A single amino acid change makes a rat neuronal sodium channel highly sensitive to pyrethroid insecticides

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Abstract Two amino acid substitutions in a housefly sodium channel, L1014F in domain IIS6 and M918T in the IIS4–S5 linker, have been identified in *kdr* and *super-kdr* pyrethroid-resistant phenotypes, respectively. Unlike their native insect counterparts, mammalian sodium channels are only weakly sensitive to pyrethroids. Do the sodium channels of mammal and pyrethroid-resistant housefly share similar structural characteristics that account for their low pyrethroid sensitivities? We report here that substitution of isoleucine for methionine at position 874 (equivalent to the *super-kdr* site 918 in the housefly) in the rat IIA α-subunit causes a 100-fold increase in sensitivity.

Key words: Sodium channel; Rat brain α-subunit; Housefly; 'Knockdown' resistance (*kdr*, *super-kdr*); Pyrethroid; Deltamethrin; *Drosophila para*-subunit; *Xenopus laevis* oocyte

1. Introduction

Voltage-activated sodium channels represent a major target for a wide range of natural or synthetic chemicals [1,2]. Physiological and biochemical studies on these proteins have revealed up to six distinct binding sites on the α-subunit, which is the main constituent of these channels [3]. Many of the molecular properties of these sites have been characterised by site-directed mutagenesis [4–9]. Far less is known about the structure–function relationships of insect sodium channels, because their cloning and functional expression have only very recently been achieved [10,11].

The pyrethroid sensitivities of wild-type insect and mammalian sodium channels differ by ~4500-fold. Pyrethroids are synthetic derivatives of toxins found in the flowers of *Chrysanthemum cinerariaefolium* [12] that cause rapid death ('knockdown') in wild-type insects. Their major target in these animals is the neuronal, voltage-gated sodium channel where they affect the voltage-dependence and kinetics of activation and inactivation. Long-term exposure of insect populations to synthetic pyrethroids has led to the development of 'knockdown' resistance (*kdr*) that has been related to structural changes in their sodium channels [13,14]. The sodium channels of *kdr* flies are ~100-fold less sensitive to pyrethroids than their wild-type counterparts [15]. *Kdr* has been identified in many important pest species, but is best characterised in the housefly where several variants, including the more resistant *super-kdr*, have been discovered [14,16,17]. Molecular analysis of the full 6.3 kb coding sequence of a neuronal sodium channel of pyrethroid-resistant houseflies has identified two amino acid substitutions, L1014F in domain IIS6 and M918T in the IIS4–S5 linker [17]. L1014F is found in both *kdr* and *super-kdr* flies, whereas M918T is present only in the *super-kdr* phenotype. Pyrethroid resistance has not been seen in the fruitfly, *Drosophila melanogaster*, but when the M918T and L1014F mutations were incorporated into a neuronal, voltage-gated sodium channel (*para*-channel) of this insect [10,11], sensitivity to pyrethroid was reduced ~1000-fold [18].

Amongst a range of physical and toxicological properties that have made pyrethroids such commercially important insecticides is their low mammalian toxicity. The *Drosophila para*-sodium channel is ~50% homologous with its counterparts in mammalian excitable tissues and the site (1014-leucine) in the wild-type housefly sodium channel that is mutated to phenylalanine in the *kdr* insect is highly conserved in wild-type insect and mammalian sodium channels. Therefore, this site could not be responsible for, or contribute to, the low sensitivity of mammalian sodium channels to pyrethroids. Vais et al. [19] have previously reported that mutation of L972 to phenylalanine (a change equivalent to the L1014F *kdr* mutation in the housefly) in the rat IIA sodium channel did not reduce its sensitivity to the pyrethroid permethrin, although there was a shift in the mid-point potential for channel activation. Intriguingly, mammalian neuronal sodium channels have an isoleucine rather than a methionine in the position (874) that corresponds to *super-kdr* site (918) in the housefly (Fig. 1). Could this account for their low sensitivity to pyrethroids? To test this possibility, we have replaced the isoleucine (I874) of the wild-type rat brain IIA sodium channel with a methionine (I874M), expressed the mutant in *Xenopus laevis* oocytes and examined its sensitivity to deltamethrin using a voltage clamp [18].

2. Materials and methods

2.1. Construction of rat brain IIA sodium channel α-I874M mutant

A plasmid containing the rat brain IIA sodium channel cDNA (pVA2580 was kindly provided by Dr C. Labarca (California Institute of Technology). The isoleucine to methionine point mutation was introduced into the rat brain α-II gene (in position 874, equivalent to 918 in the sequence for the *Drosophila para*-sodium channel) using the QuikChange site-directed mutagenesis kit (Stratagene Cloning Systems, UK). Wild-type and mutant plasmids were linearised with Cla1 and T7 transcripts synthesised using the mMESSAGE mMACHINE kit (Ambion). Transcripts were dissolved in sterile water at a final concentration of 1 μg/μl and stored at −80°C until required.

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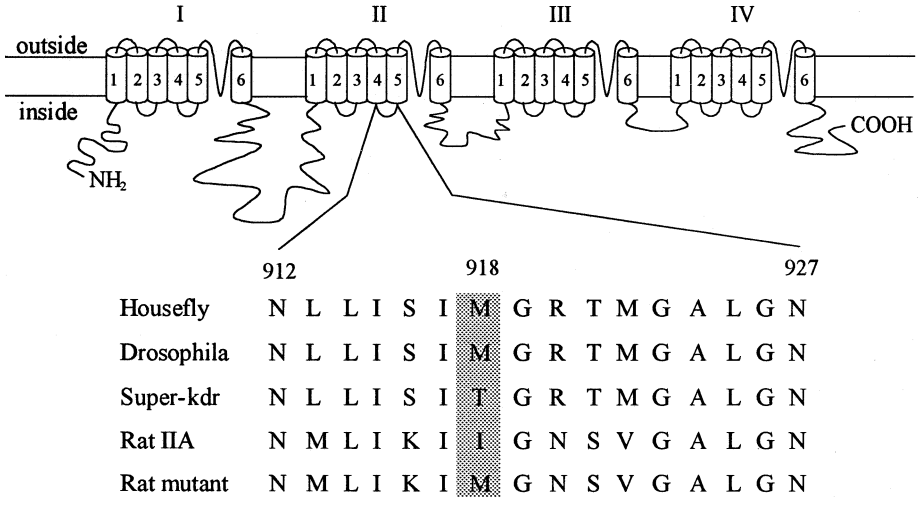

Fig. 1. Diagrammatic representation of a generalised sodium channel (e.g. rat α-subunit or its equivalent in the housefly and in *Drosophila* (*para*-subunit)) with emphasis on the amino acid sequence of the S4–S5 linker in domain II. Alignment of amino acid sequences from diverse sources in the region of the housefly *super-kdr* mutation M918T [17] suggests that mutation of the isoleucine in the 874↔918 position (highlighted with a dashed box) of the rat channel to a methionine (rat mutant) might raise the sensitivity of this channel to pyrethroids.

2.2. Oocyte preparation

X. laevis oocytes were isolated and injected according to standard procedures [20]. cRNA transcripts of either rat IIA or I874M mutant sodium channels (concentration 1 μg/μl) were mixed with β_1 transcripts and RNase-free water, the final mixture having a 1:1:3 ratio by weight. Oocytes were injected with 50 nl of correspondent transcript solution, and then incubated at 19°C for 2–5 days in ND-96 GPT solution (in mM: NaCl 96, KCl 0.2, $MgCl_2$ 1, $CaCl_2$ 1.8, Na-pyruvate 2.5, theophylline 0.5, gentamicin 50 mg/ml, HEPES 5, pH = 7.5) before recording.

2.3. Electrophysiological measurements

Voltage clamp experiments were performed using a CA-1 amplifier (Dagan Instr., Minneapolis, MN, USA). The bath solution was ND-96 (same as above, without sodium pyruvate, theophylline and gentamicin). In some experiments, the sodium concentration of this solution was reduced by equimolar replacement of NaCl with *N*-methyl-D-glucamine, in order to achieve a better voltage control in experiments on oocytes expressing large currents. Experiments were performed at room temperature (21–23°C). To improve the frequency response of the voltage clamp, we used agar bridges with Pt wires that had resistances <7 kΩ and voltage-measuring electrodes with resistances ≤ 1 MΩ when filled with 1 M KCl. The current-injecting electrodes were filled with 0.7 M KCl plus 1.7 M K-citrate and had resistances ≤ 0.5 MΩ.

2.4. Data acquisition and analysis

Data were acquired with an ITC-16 interface (Instrutech Instr., USA) using the Pulse program (HEKA Electronik, Germany), most analyses being performed with the companion program Pulsefit. Linear leak and capacitive currents were subtracted with P/5 steps from −120 mV. Data were sampled at 50 kHz and filtered at 10 kHz, unless indicated otherwise.

2.5. Chemicals

The 1*R-cis* isomer of deltamethrin, a type-II pyrethroid [21], was prepared as stock solutions ($10^{-6} \div 10^{-2}$ M) in ethanol. Experimental solutions were prepared by diluting the correspondent stock solution with ND-96 as not to exceed the amount of 0.1% ethanol in the bath. Controls undertaken to check the effect of such alcohol levels on the sodium channels were negative.

3. Results and discussion

Oocytes expressing the rat I874M and wild-type sodium channels gave inward, voltage-activated currents of more

Table 1
Effects of the I874M mutation on the voltage-dependence and kinetics of activation/inactivation and on the sensitivity to deltamethrin of the rat IIA sodium channel

Property	Wild-type	I874M mutant
Activation		
$V_{1/2}$ (mV)	−23.8 ± 0.7 (n = 11)	−24.7 ± 0.9 (n = 14)
k (mV)	5.54 ± 0.23	5.5 ± 0.12
Inactivation		
T_p = 50 ms		
$V_{1/2}$ (mV)	−50.1 ± 1.1 (n = 10)	−50.0 ± 1.0 (n = 14)
k (mV)	6.6 ± 0.18	6.7 ± 0.14
T_p = 200 ms		
$V_{1/2}$ (mV)	−54.9 ± 0.8 (n = 13)	−54.1 ± 0.5 (n = 17)
k (mV)	5.1 ± 0.1	5.2 ± 0.1
T_p = 1 s		
$V_{1/2}$ (mV)	−56.4 ± 1.1 (n = 7)	−56.7 ± 0.8 (n = 11)
k (mV)	4.9 ± 0.13	5.2 ± 0.15
τ_{decay} (ms) (−10 mV)	1.2 ± 0.07 (n = 7)	1.1 ± 0.05 (n = 14)
τ_{onset} (ms) (−40 mV)	19.0 ± 2.2 (n = 5)	17.2 ± 1.6 (n = 12)
τ_{recov} (ms) (−50 mV)	36.7 ± 0.89 (n = 5)	34.4 ± 1.9 (n = 6)
Deltamethrin		
K_{app} (μM)	95[a]	0.65 ± 0.001
τ_{tail} (−110 mV)	86 ± 2 (n = 75)	505 ± 7 (n = 194)

Activation was measured by applying series of 32 ms pulses at test potentials ranging from −60 mV and +60 mV (5 mV increments). The holding potential was −70 mV or −100 mV. Peak current values obtained at each test potential were fitted with a Boltzmann I–V characteristic: $I = G_{max}(V - V_{rev})/\{1+\exp[(V - V_{1/2})/k]\}$. Inactivation (steady-state) was measured by applying conditioning pulses of T_p duration at stepped membrane potentials ranging from −90 mV to −15 mV (in 5mV increments), prior to application of a test pulse to −10 mV. The holding potential was −70 mV or −100 mV. The peak currents at −10mV were fitted with a Boltzmann function. Onset and recovery from inactivation were measured as follows: prior to a test pulse from the holding potential of −90 mV to −10 mV, 11 conditioning pulses of increasing duration (0.5–256 ms) were applied at various membrane potentials (V_c): viz. $-110 < V_c = -50$ mV for recovery from inactivation, and $-55 < V_c = -30$ mV for onset of inactivation. Data were fitted with single exponentials giving time constants τ_{onset} and τ_{recov}, respectively. τ_{decay} represents the time constant for onset of inactivation determined by fitting a single exponential to the current decay during an activation test pulse. τ_{tail} is the time constant for tail current decay.

[a]This value for K_{app} has been extrapolated from dose–response data, assuming a Hill coefficient n = 1.

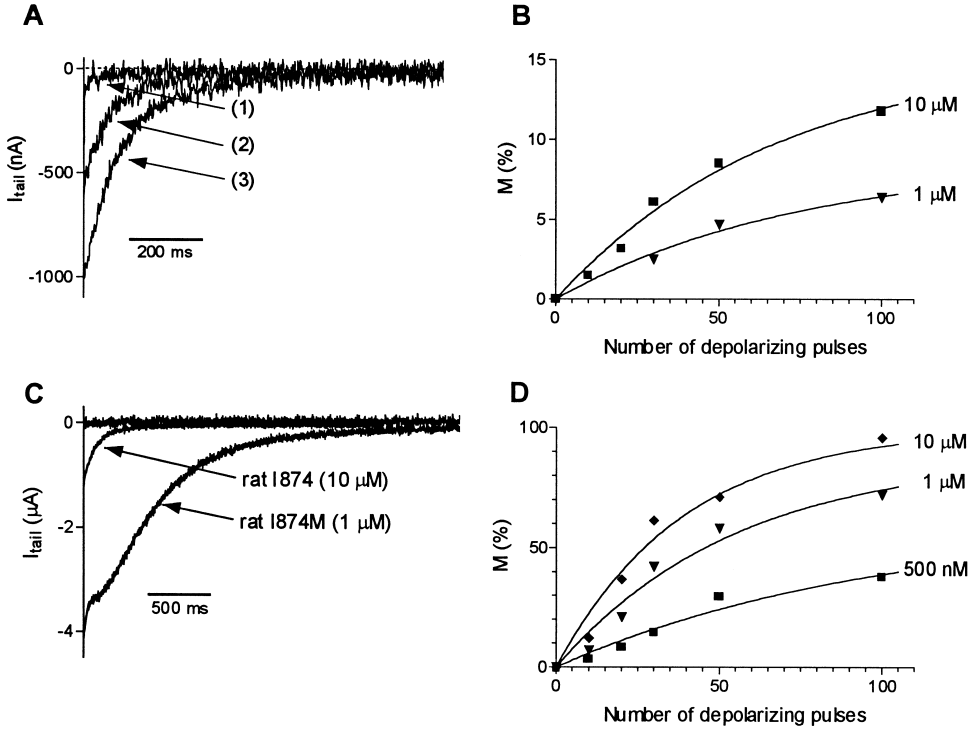

Fig. 2. Deltamethrin binds to the open states of rat wild-type and rat mutant sodium channels, but the mutant (I874M) channel is more sensitive to this pyrethroid. (A) Representative traces obtained from an oocyte expressing the wild-type channel in the presence of 1 μM (trace 2) and 10 μM (traces 1 and 3) deltamethrin. A train of 100 brief (5 ms) step depolarisations (traces 2 and 3) induced longer tail currents than a single 500 ms depolarisation (trace 1). During each 5 ms step, the oocyte was clamped at 0 mV for 5 ms and then repolarised to −100 mV for 10 ms; during the 500 ms step, the oocyte was clamped at 0 mV before being repolarised to −110 mV. Therefore, the total duration of depolarisation was the same for all three traces, i.e. 500 ms. Tail currents were recorded at −110 mV. (B) The percentage (M) (calculated using Eq. 1) of wild-type sodium channels modified by deltamethrin is plotted against the number of 5 ms depolarising steps in a train. M increased to ~12% when the number of steps was increased to 100. Data are fitted with an exponential equation, $M = M_{max}\,[1-\exp(-n/n_e)]$, where n is the number of pulses and n_e is a constant (i.e. the number of pulses inducing a modification of (1−1/e) from the maximal modification (M_{max})) for each concentration of deltamethrin (indicated beside each plot). The fitting parameters were: for 1 μM deltamethrin, $M_{max} = 8.7\%$, $n_e = 47.4$ pulses; for 10 μM deltamethrin, $M_{max} = 15.6\%$, $n_e = 51$ pulses. (C) Comparison of deltamethrin-induced tail currents recorded from oocytes injected with either rat I874 or rat I874M transcripts (the deltamethrin concentrations are in brackets). The maximal sodium conductances (G_{max}) were 64.1 μS for I918 and 61.6 μS for I874M. Note, also, the slower decay of the tail current recorded from the oocyte expressing the mutant channel (see also data in Table 1). (D) For the rat I874M transcript, M increased to 100% (compared with ~12% for the wild-type channel (B)) when the number of 5 ms depolarising steps was increased to 100. Data fitted with the same equation as in B. The fitting parameters were: for 500 nM deltamethrin, $M_{max} = 60.8\%$, $n_e = 67.6$ pulses; for 1 μM deltamethrin, $M_{max} = 88.5\%$, $n_e = 38$ pulses; for 10 μM deltamethrin, $M_{max} = 100\%$, $n_e = 27$ pulses.

than 1 μA during step depolarisations from a holding potential of −70 mV. The mid-point potential of activation, fast/slow inactivation and the voltage-dependence of onset of and recovery from fast inactivation were similar for the wild-type and mutant channels (Table 1). Both channels closed more slowly after treatment with deltamethrin (although for I874M, this change was apparent at lower deltamethrin concentrations) with the resultant appearance of 'tail currents' following the termination of step depolarisations and subsequent repolarisations to −110 mV (Fig. 2). As shown previously for *Drosophila* sodium channels [18], the tail current amplitude depended on the channel activation protocol and the deltamethrin concentration and provided a good quantitative measure of deltamethrin action. The percentage of sodium channels modified by deltamethrin was estimated using the formula:

$$M\ (\%) = \{[I_{tail}/(V_{tail}-V_{rev})]/G_{max}\}\times 100 \quad (1)$$

where I_{tail} represents the amplitude of the tail current recorded at membrane potential V_{tail} (usually −110 mV); V_{rev} is the reversal potential for the sodium current; G_{max} is the maximal sodium conductance [22]. Like the *Drosophila para*-sodium channel [18], only the open states of the rat channel were sensitive to deltamethrin. As a result, the pyrethroid had a greater effect on the tail currents obtained following a train (e.g. 100 steps) of brief (5 ms) depolarisations (separated by a 10 ms repolarisation to −100 mV between each step) than following a single long depolarising step of equivalent total duration of depolarisation (Fig. 2A). In the former case, where the 10 ms intervals between pulses allowed for full recovery from inactivation (the time constant for recovery from inactivation at −100 mV was 2 ± 0.2 ms (mean ± S.E.M.; $n = 6$)), the number of channel openings was much greater. The amplitude of the tail current increased when the number of brief depolarising pulses in a train was increased (Fig. 2B).

The relationships between tail current amplitude and deltamethrin concentration for the two rat channels are illustrated in Fig. 2C,D. They show clearly that I874M was more sensitive to deltamethrin (see also the K_{app} values given in Table 1).

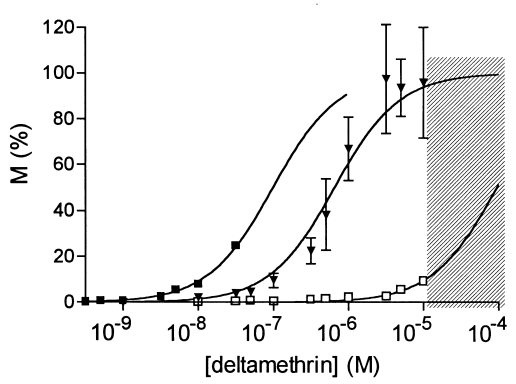

Fig. 3. The I874M mutation greatly increases the sensitivity of rat IIA sodium channel to deltamethrin. (▼) Dose–response relationship for deltamethrin action on the rat I874M sodium channel. The data were fitted with the equation: $M\ (\%) = M_{max}/[1+(K_{app}/[\text{pyrethroid}])^n]$, where K_{app} is the apparent dissociation constant, [pyrethroid] is the concentration of deltamethrin and n is the Hill coefficient. For the best fit to the experimental data, $K_{app} = 650 \pm 1$ nM (S.E.M. of fit) and $n = 1$. (□) Dose–response relationship for deltamethrin action on the rat wild-type sodium channel. The data were fitted with the same equation as those for the mutant channel, but with the additional assumption that $n = 1$. This gave an extrapolated value $K_{app} = 95$ μM. The dashed box drawn beyond 10^{-5} M indicates the probable solubility limit of deltamethrin in *Xenopus* oocyte saline [18,19]. (■) Dose–response relationship for deltamethrin action on the *Drosophila para*-sodium channel. The data are fitted with the same equation as those for the rat sodium channels. A Hill coefficient of $n = 1$ has been assumed. The extrapolated value for K_{app} is 92 nM.

Tail currents for I874M also decayed more slowly ($\tau_{decay} = 505$ ms) than those for the wild-type channel ($\tau_{decay} = 86$ ms) (Fig. 2C and Table 1). A comparison of the data in Fig. 2B,D supports the claim that the I874M mutation affects the sensitivity of the rat sodium channel to deltamethrin. In both cases, values for τ_{tail} were independent of deltamethrin concentration, a result that is consistent with a 1:1 drug–receptor reaction [23] and with data obtained from other studies on mammalian sodium channels [24].

Small differences in sensitivity to pyrethroids between TTX-sensitive and TTX-resistant sodium channels in neurones of rat dorsal root ganglia have recently been reported [25]. In our studies, the TTX sensitivity of I874M was similar to that of the wild-type channel, i.e. apparent dissociation constants for TTX were 3.1 ± 0.1 nM (mean ± S.E.M.; $n = 5$) and 3.9 ± 0.3 nM ($n = 4$), respectively. This finding is consistent with the location of the TTX binding site in the outer vestibule of the pore [5] rather than in the domain IIS4–S5 linker where residue 874 is located.

Although rat I874M is >100-fold more sensitive to deltamethrin (and to permethrin; data not shown) than the wild-type rat sodium channel, it is ~10 times less sensitive than the *D. melanogaster* wild-type sodium channel [10,11] (Fig. 3). Nevertheless, the fact that a single point mutation (I874M) induces a large increase in sensitivity to pyrethroids suggests that low pyrethroid toxicity in mammals may be accounted for, at least in part, by structural differences between mammalian and insect sodium channels rather than by metabolic factors [1], although further studies are required to test this idea. Although binding of pyrethroids is probably influenced by the amino acid at position 874↔918, it is likely that other residues are also influential and, thereby, contribute to the different sensitivities of wild-type insect and rat sodium channels to pyrethroids. Identification of these sites should lead to the design of a rat neuronal sodium channel that is as sensitive to pyrethroids as its counterparts in native insects.

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