

# Functional analysis of a rat sodium channel carrying a mutation for insect knock-down resistance (*kdr*) to pyrethroids

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**Abstract** Pyrethroid insensitivity in resistant (*kdr*) insects has been correlated with a leucine to phenylalanine replacement in the S6 transmembrane segment of domain II of the axonal sodium channel  $\alpha$ (*para*)-subunit. An  $\alpha$ -subunit of rat brain type II sodium channel containing this mutation has been expressed and its sensitivity to permethrin compared with that of the wild-type channel. The steady-state activation curve of the mutant was shifted 14 mV in the depolarizing direction. We propose that an equivalent shift of the sodium current activation curve in *kdr* insects could account for their low sensitivity to permethrin toxicity.

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**Key words:** Sodium channel; *Xenopus* oocyte; Rat brain  $\alpha$ -subunit; Housefly *para*-subunit; Knock-down resistance (*kdr*); Permethrin

## 1. Introduction

Voltage-activated sodium channels are responsible for the generation of the inward current for neuronal action potential generation and propagation. The mammalian sodium channel contains a large (1800–2000 amino acids)  $\alpha$ -subunit. In addition, some sodium channel proteins contain one or two smaller subunits, the  $\beta$ -subunits [1]. Following its expression in *Xenopus* oocytes, the  $\alpha$ -subunit qualitatively exhibits all of the functional properties associated with sodium channel proteins in vivo [2,3]. However, its co-expression with  $\beta_1$ -subunit produces channels that are qualitatively and quantitatively more similar to those that occur in vivo [4]. In *Drosophila*, two genes *DSC1* [5] and *para* [6] show a high level of structural homology to the  $\alpha$ -subunits of vertebrate sodium channels. Although both of the *Drosophila* genes are expressed in the nervous system [7], only the *para* gene has a known physiological role [8]. A third *Drosophila* gene, *tipE* [9], was recently reported to stimulate functional expression of the *Drosophila para*-subunit in *Xenopus* oocytes [10].

From a toxicological perspective, voltage-activated sodium channels represent a major target for a wide range of naturally occurring or synthetically derived chemicals [11]. In insects, such chemicals are selected and developed especially for their potential application as powerful insecticides [12]. For example, the pyrethroids [13], which are synthetic derivatives of the natural pyrethrin esters of chrysanthemic acid [14], have been used extensively to control insect pests, especially because they combine high insecticidal potency with low mammalian toxicity. Pyrethroids modify sodium channels by slow-

ing their activation and inactivation kinetics, resulting in long-lived and repeated channel openings [15]. Unfortunately, the widespread use of these insecticides has led to the development of resistance in many pest insect species, a development that represents an increasing threat to their continued effective use [16]. Knockdown resistance or *kdr*, which is characterised by a reduced sensitivity of the nervous system towards not only pyrethroids but also DDT, was first recognised in the housefly *Musca domestica* [17] and has subsequently been found in several other insect pest species [18]. Variant forms of *kdr* have also been identified in the housefly, such as the more potent *super-kdr* that confers up to 500-fold resistance to cyano group-containing (type II) pyrethroids [19]. Although the molecular basis of pyrethroid insensitivity in the *kdr* and *super-kdr* insect strains is not known, it is thought to be related to modifications of the sodium channel [20].

Recently, the full sequence of a housefly sodium channel subunit (*para*-type) has been described and point mutations in *kdr* and *super-kdr* housefly strains have been identified. A single leucine to phenylalanine replacement in the S6 transmembrane segment of domain II of the *para* subunit is associated with the *kdr* phenotype, while an additional methionine to threonine change in the cytoplasmic S4–S5 inter-segmental loop within the same domain has been identified in several *super-kdr* strains [21]. The same leucine to phenylalanine mutation has also been observed in a cockroach *kdr* strain [22].

Although mammalian sodium channels are less sensitive to pyrethroids than insect wild-type sodium channels, they experience similar qualitative changes in activation and inactivation kinetics when exposed to high concentrations of a pyrethroid [15]. Is it possible, therefore, to confer resistance to pyrethroids on a mammalian sodium channel through the incorporation of these point mutations? Like the *para*-subunit of the housefly, the  $\alpha$ -subunit of the rat brain type II sodium channel contains a leucine residue at the corresponding position of domain II-S6 (leucine<sup>972</sup>).

We report here on the functional expression of a recombinant rat  $\alpha$ -*kdr* mutant that was constructed by site-directed mutagenesis of the rat wild-type  $\alpha$ -subunit. Expression of the wild-type  $\alpha$ -subunit alone resulted in sodium channels that responded to  $10^{-5}$  M permethrin with a 5 mV shift in the voltage dependence of activation towards a more negative membrane potential. When the mutant channel was expressed in *Xenopus* oocytes, its voltage dependence of activation in the absence of pyrethroid was found to be shifted by 14 mV towards a more positive potential compared with the wild-type channel. However,  $10^{-5}$  M pyrethroid still caused a 5 mV shift in the voltage dependence of activation towards a more negative membrane potential. The change in the voltage dependence of activation seen in the mutant would be expected to counteract the effects of a pyrethroid, because

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the latter involves a shift of the voltage dependence of activation towards a more negative potential. If the axonal sodium channels of *kdr* insects behave in similar fashion to the rat  $\alpha$ -*kdr* mutant sodium channel, this could provide, at least in part, a biophysical explanation for *kdr* resistance which would not necessarily involve a reduction in the affinity of the insect sodium channel for pyrethroids.

## 2. Materials and methods

### 2.1. Construction of rat brain sodium channel $\alpha$ -*kdr* mutant

Plasmid constructs containing the rat-brain  $\alpha$ -II and rat  $\beta_1$  genes were kindly provided by Dr. C. Labarca (California Institute of Technology) and Dr. T. Tanada (University of Washington) respectively. The leucine to phenylalanine point mutation was introduced into the rat-brain  $\alpha$ -II gene (Leu<sup>972</sup>, equivalent to Leu<sup>1014</sup> in housefly *para* sequence) construct by site-directed mutagenesis (CTT to TTT) using the Chameleon mutagenesis kit (Stratagene Ltd., UK) (primer for mutagenesis: GGTGATTGGGAACCTTGTGCTCCTGAACC). T<sub>7</sub> transcripts containing the rat wild-type  $\alpha$ -subunit,  $\alpha$ -*kdr* and  $\beta_1$  subunit sequences were synthesised using the mMESSAGE mMACHINE in vitro transcription kit (Ambion Inc.). Transcription reactions were carried out according to the supplier's recommended protocols, with the  $\alpha$ -subunit reactions supplemented by the addition of 1.5 mM GTP. The transcripts were dissolved in sterile water at a final concentration of 1  $\mu$ g  $\mu$ l<sup>-1</sup>.

### 2.2. Chemicals

The 1R-*cis* isomer of permethrin, a Type I pyrethroid [23], was prepared as a stock solution of 10<sup>-3</sup> M in ethanol. Experimental solutions (usually 10<sup>-5</sup> M) were prepared by diluting the stock solution with frog Ringer (see below). Controls undertaken to determine the effects of the alcohol content of the experimental solutions on the sodium channels were negative.

### 2.3. Oocyte preparation

Adult female *Xenopus laevis* were anaesthetized by immersion in iced water for 1 h followed by surgical removal of several ovarian lobes. Stage V and VI oocytes were mechanically separated from the connective tissue, selected by general inspection, and incubated at 19°C in Barth's saline containing (in mM): NaCl, 96; KCl, 2; CaCl<sub>2</sub>, 1.8; MgCl<sub>2</sub>, 1; sodium pyruvate, 2.5; HEPES, 5; and antibiotics (per liter: penicillin, 10 000 IU; streptomycin, 10 mg; neomycin, 0.144 g); pH 7.5. 24 h later, batches of 40–50 oocytes were injected with 50 nl of a 1:1 mixture of RNA transcripts for the  $\beta_1$ -subunit gene and for either the rat wild-type  $\alpha$ -subunit gene or its  $\alpha$ -*kdr* mutant counterpart.

### 2.4. Electrophysiological measurements

The methodology described by Methfessel et al. [24] was employed. The follicle cell layer was removed from an oocyte after enzymic treatment with collagenase (1 mg ml<sup>-1</sup> of collagenase Type 1 (Sigma), for 30 min) and the oocyte was then immersed in frog Ringer (in mM): NaCl, 115; KCl, 2.5; CaCl<sub>2</sub>, 1.8; HEPES, 10; pH 7.5. Experiments were usually done at 6°C to maximize the effect of the pyrethroid [25]. The voltage-clamp studies were performed with an Axoclamp-2A amplifier (Axon Instruments, USA). The 'charging-compensator' technique was used to reduce the effect of the series resistance of the bath [26]. To acquire and to display the electrophysiological data, the current output was passed through an 8-pole low-pass Bessel filter set at 7.5 kHz. PClamp software (Axon Instr.) was used to apply various voltage-step protocols, to correct for leakage currents (P/4 method), and to acquire the data. Corrections for capacitive transients were done by subtracting from each record a similar record acquired with an identical protocol, but at a holding potential ( $V_H$ ) of -40 mV. The sodium currents are inactivated at this  $V_H$ . Capacitive artifacts still remaining after correction, which never extended over more than 200  $\mu$ s, were blanked in all records presented herein (Figs. 1–3). Intracellular current and voltage electrodes were pulled from thin-wall borosilicate glass (CG-150TF, Clark). After filling their tips with agarose-KCl solution (1% agarose in 3 M KCl) [26]), the electrodes were back-filled with 3 M KCl. Electrodes typically had resistance values of 0.4–0.8 M $\Omega$ .

### 2.5. Data analysis

Current-voltage (*I*-*V*) relationships were fitted using an iterative non-linear regression protocol to the following transform of a Boltzmann function:

$$I_{Na} = G_{max}(V - V_{rev}) / \{1 + \exp[(V - V_{0.5})/k]\} \quad (1)$$

where  $I_{Na}$  is the peak sodium current elicited by the voltage pulse,  $V$  is the test potential,  $V_{rev}$  is the reversal potential,  $G_{max}$  is the maximal conductance,  $V_{0.5}$  is the voltage for half-maximal current activation, and  $k$  is the slope factor (in mV). Conductance values at each test potential were then calculated by dividing each peak current value by its correspondent driving force (i.e.  $G = I/(V - V_{rev})$ ). The ratios  $G/G_{max}$  plotted versus the correspondent test potentials were fitted to the following Boltzmann equation:

$$G/G_{max} = 1 / \{1 + \exp[(V_{0.5} - V)/k]\} \quad (2)$$

## 3. Results

Control injections of RNA for the rat  $\alpha$ -subunit alone led to the expression of functional sodium channels, but channels with faster inactivation kinetics were obtained following co-injection of RNAs for  $\alpha$ - and  $\beta_1$ -subunits, as has been shown previously [4]. This report concentrates on the latter.

Voltage-activated sodium currents were recorded as early as day 2 post-co-injection of the two RNAs (encoding rat  $\alpha$ - and  $\beta_1$ -subunits). Fig. 1A presents a typical family of sodium currents obtained during application of the voltage protocol given in the inset to the figure ( $V_H$  was -100 mV). The current records were corrected for capacitive transients by subtracting a family of currents recorded with the same protocol as in Fig. 1A, but at a  $V_H$  of -40 mV (the sodium currents were inactivated at this  $V_H$ ). Identical results were obtained using tetrodotoxin to eliminate the sodium currents, but this pharmacological approach was more time-consuming. Fig. 1B shows the effect of 10<sup>-5</sup> M permethrin on the family of sodium currents. The pyrethroid altered the current-voltage (*I*-*V*) relationship (peak sodium current (*I*) versus *V*). Fig. 1C presents plots of the relative conductance ( $G/G_{max}$ ; see Section 2) versus membrane potential, in control conditions and following permethrin application. When these data were fitted with the Boltzmann Eq. 2, it appeared that the pyrethroid shifted the potential at which 50% of the sodium channels were open (mid-point potential,  $V_{0.5}$ ) from  $-35 \pm 2$  mV (SD,  $n = 6$  experiments) to  $-40 \pm 0.6$  mV (SD,  $n = 5$ ). Due to the low sensitivity of the rat  $\alpha$ -subunit to pyrethroids and the poor solubility of permethrin in water, it was not possible to obtain a dose-response relationship for this pyrethroid.

When the rat  $\alpha$ -*kdr* subunit was co-expressed with the rat  $\beta_1$  subunit, sodium currents of 1–3  $\mu$ A were consistently recorded at  $V_H = -100$  mV during the application of voltage steps between -60 mV and +30 mV (Fig. 2A: correction of the capacitive transients was as for Fig. 1A). Typically, sodium currents could be observed only during depolarisations to -30 mV or greater. Fig. 2B illustrates conductance-voltage curves for the  $\alpha$ -*kdr* mutant (Fig. 2B, ●) and for the rat wild-type  $\alpha$ -subunit (Fig. 2B, ○). A fit of the data with the Boltzmann Eq. 2 disclosed a depolarising shift of 14 mV for sodium channels containing the  $\alpha$ -*kdr* mutant compared with the expressed wild-type sodium channel, i.e. the mid-point potential for the  $\alpha$ -*kdr* mutant  $G/G_{max} - V$  curve was  $-21 \pm 0.8$  mV (SD,  $n = 8$  experiments; Fig. 2B) compared with  $-35 \pm 2$  mV for the wild-type.

The effect of permethrin on channels containing the  $\alpha$ -*kdr*

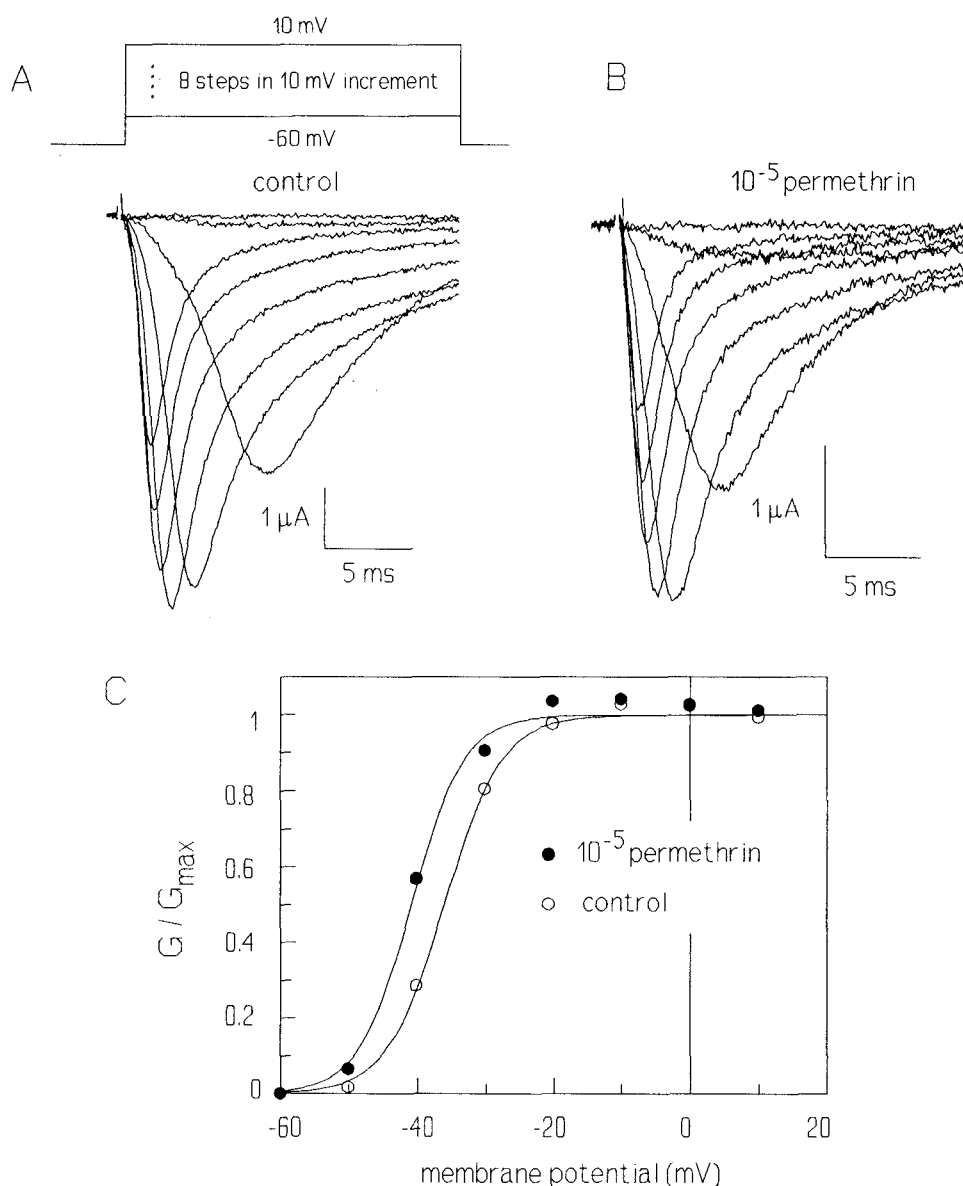


Fig. 1. Sodium currents recorded from *Xenopus* oocytes co-injected with cRNA transcripts for rat wild-type  $\alpha$ -subunit plus  $\beta_1$ -subunit. The currents were recorded (A) under control conditions (frog Ringer solution, see Section 2) and (B) during continuous exposure to  $10^{-5}$  M permethrin. The oocyte bath was thermostatically maintained at 6°C. The voltage protocol was identical for (A) and (B). Voltage steps, schematically presented at the top of (A), were applied from a  $V_H$  of  $-90$  mV. The blanking interval (see Section 2) was 200  $\mu$ s. C: Relative peak sodium conductance ( $G/G_{max}$ ) vs. membrane potential in control conditions ( $\circ$ ); same as in A) and in the presence of  $10^{-5}$  M permethrin ( $\bullet$ ); same as in B). Data points were fitted with the Boltzmann Eq. 2 (continuous lines), defined by the following parameters: ( $\circ$ ), mid-point potential  $V_{0.5} = -35.9$  mV ( $\pm 0.3$  mV, SE of the fit) and slope  $k = 4.2$  mV ( $\pm 0.2$ , SE of the fit); ( $\bullet$ ),  $V_{0.5} = -40.8$  mV ( $\pm 0.5$  mV, SE of the fit) and  $k = 3.9$  mV ( $\pm 0.5$  mV, SE of the fit).

mutant subunit was investigated using two additional voltage protocols. The first was used to determine whether permethrin induced a change in sodium channel activation and inactivation kinetics. It was also used to test the effect of the insecticide on tail currents (Fig. 3A). The latter were examined by repolarising the oocyte membrane to  $-50$  mV after a voltage step to detect possible delayed openings of sodium channels [27]. Fig. 3A shows that permethrin slowed down both activation and inactivation, and also reduced the peak sodium current recorded at  $-10$  mV. These changes are consistent with the single channel data of Chinn and Narahashi [28] that were recorded from native sodium channels in mouse neuroblastoma cells. Fig. 3B shows that the current elicited

by depolarising the membrane to  $-40$  mV was increased 3-fold upon permethrin treatment. Both sets of protocols failed to detect any change in the tail currents. In this respect, the data are similar to those obtained from channels containing the rat wild-type  $\alpha$ -subunit (data not shown).  $10^{-5}$  M permethrin shifted the mid-point potential of the steady-state activation curve of the rat  $\alpha$ -*kdr* mutant subunit by  $5 \pm 0.6$  mV (SD,  $n = 3$  exp.), a change equal to that obtained for the rat wild-type  $\alpha$ -subunit.

To check whether the  $\beta_1$ -subunit modulates the effect of permethrin on the steady-state activation of the  $\alpha$ -*kdr* mutant, we compared the effect of the pyrethroid on oocytes expressing the  $\alpha$ -*kdr* mutant alone with those co-expressing the  $\alpha$ -*kdr*

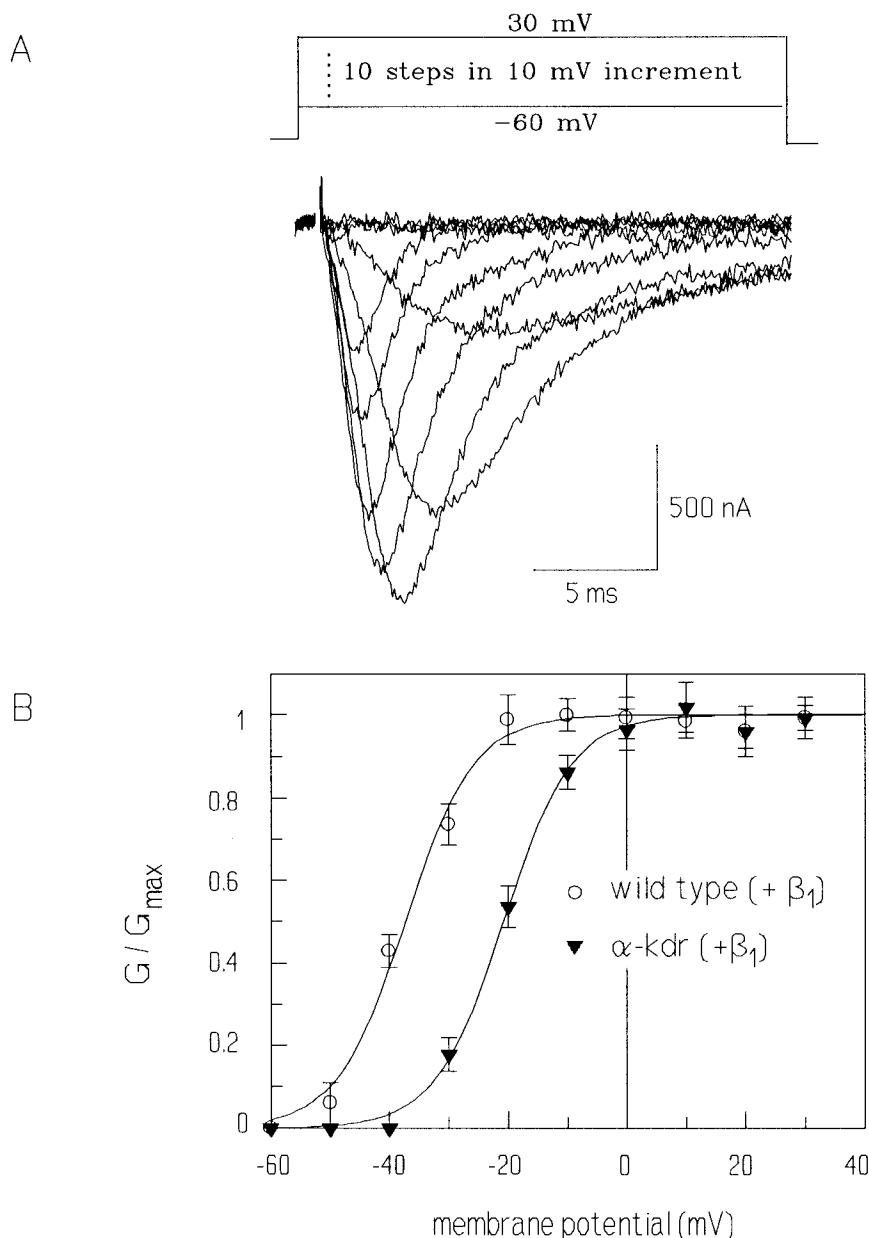


Fig. 2. A: Sodium currents recorded in the absence of permethrin from *Xenopus* oocytes co-injected with cRNA transcripts for rat  $\alpha$ -kdr mutant subunit plus wild-type  $\beta_1$ -subunit. The oocyte bath temperature was 6°C. The voltage protocol is schematically presented at the top of (A). Voltage steps were applied from  $V_H$  of -100 mV. The blanking interval was 200  $\mu$ s. B: Steady-state activation of sodium channels (relative peak sodium conductance values ( $G/G_{\max}$ ) plotted versus membrane potential). (○-○-) Derived from currents recorded from an oocyte co-injected with cRNA transcripts for rat  $\alpha$ -subunit plus  $\beta_1$ -subunit upon application of the voltage protocol illustrated in Fig. 1A, but from a  $V_H$  of -100 mV. (-▼-) Derived from currents from an oocyte co-injected with cRNA transcripts for the rat  $\alpha$ -kdr mutant subunit plus wild-type  $\beta_1$ -subunit upon application of the voltage protocol illustrated in Fig. 1A, but from a  $V_H$  of -100 mV. The two plots in (B) were fitted with the Boltzmann Eq. 2 (continuous lines), defined by the following parameters: (○-○-),  $V_{0.5} = -37.3$  mV ( $\pm 0.6$  mV, SE of the fit) and  $k = 5.8$  mV ( $\pm 0.5$  mV, SE of the fit); (-▼-),  $V_{0.5} = -20.8$  mV ( $\pm 0.4$  mV, SE of the fit) and  $k = 5.7$  mV ( $\pm 0.4$  mV, SE of the fit).

mutant with the  $\beta_1$ -subunit. Fig. 4 shows that the  $\beta_1$ -subunit had no modulatory action in this respect.

#### 4. Discussion

This study constitutes the first report on the likely functional consequences of the *kdr* L→F mutation in domain II-S6 for the operation of a voltage-activated sodium channel. We have used the readily available rat  $\alpha$ -subunit as a template to express this mutation. It is important to note that data

presented in Figs. 3 and 4 clearly show that the rat  $\alpha$ -kdr mutant and its wild-type counterpart were equally affected by  $10^{-5}$  M permethrin. The 14 mV shift of the steady-state activation of the rat  $\alpha$ -kdr mutant channel towards a more positive membrane potential (Fig. 2) would counteract the relatively small negative shift induced by permethrin (Fig. 1C Fig. 4). This provides a possible explanation for the apparent lack of toxicity of permethrin in *kdr*-resistant insects. One would predict that *kdr*-resistant insects would be less excitable than their wild-type counterparts because the shift in the

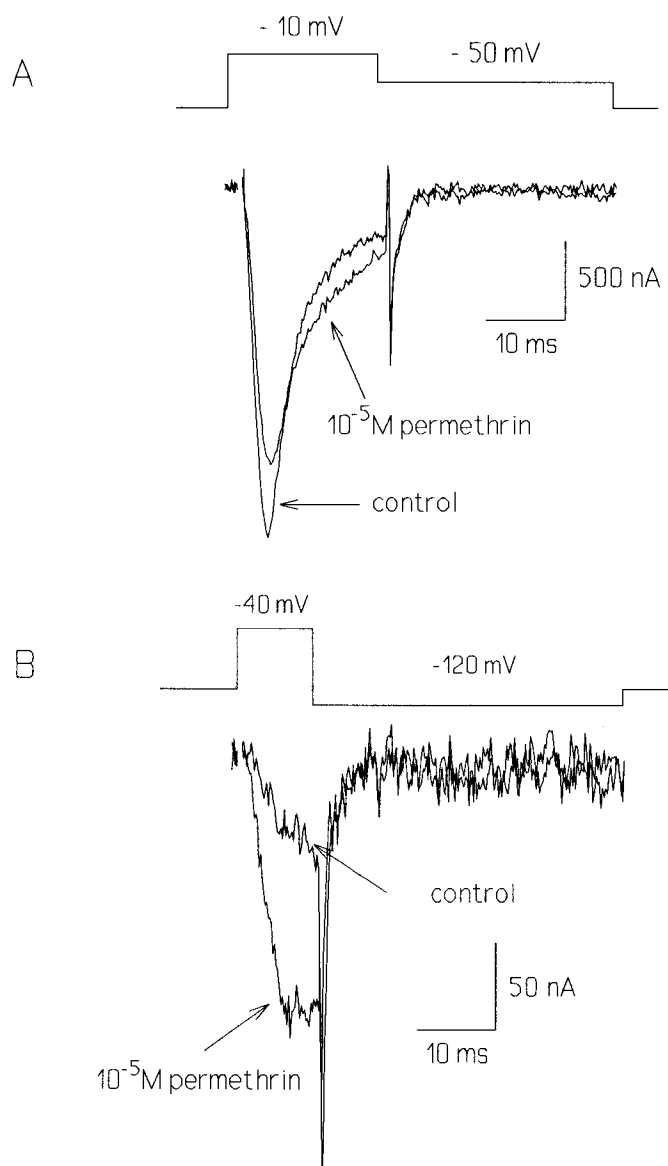


Fig. 3. Permethrin effect on sodium currents expressed in two (A,B) *Xenopus* oocytes co-injected with cRNA transcripts for rat  $\alpha$ -*kdr* mutant subunit (plus wild-type  $\beta_1$ -subunit).  $V_H$  was  $-100$  mV. The blanking interval was  $200$   $\mu$ s. The oocyte bath was at  $6^\circ\text{C}$ . A: Sodium currents elicited in response to the voltage steps depicted at top of (A). One record was obtained under control conditions, the other during continuous application of  $10^{-5}$  M permethrin. Although permethrin altered the activation and inactivation of the sodium current, no tail currents appeared during its application to the oocyte. B: Permethrin increased the amplitude of the sodium current obtained in response to a  $10$  ms step depolarisation to  $-40$  mV.

steady-state activation to a more positive membrane potential would result in an increase in nerve action potential threshold. Interestingly, an even greater ( $25$  mV) shift in the voltage-dependent activation of the rat  $\alpha$ -subunit sodium channel is caused by a leucine to phenylalanine mutation in the S4 segment (the voltage sensor) of domain II of this subunit (mutation introduced as a possible PCR error) [29]. When the biophysical properties of sodium channels in central neurons of pyrethroid-susceptible and pyrethroid-resistant (*kdr*) tobacco budworm *Heliothis virescens* were compared, a similar shift in the steady-state activation was found [30]. Although we have shown that the  $\beta_1$ -subunit does not influence the effects of permethrin on the rat sodium channel (Fig. 4), it will be important in future studies to determine whether the  $\beta_2$ -subunit is modulatory in this respect, because of the marked quanti-

tative differences in responses to pyrethroids shown by recombinant  $\alpha$ -subunit channels [31] and native sodium channels of vertebrate brain [15].

At the outset of this work we had expected from data on insects [12,13] that the inactivation kinetics of the rat  $\alpha$ -*kdr* mutant sodium channel would be less affected by pyrethroids than those of the wild-type channel. However, *kdr* resistance to pyrethroids cannot be explained on this basis because the inactivation kinetics of the mutant  $\alpha$ -*kdr* and wild-type rat sodium channels are apparently affected similarly by these toxins. Nevertheless, more detailed studies on inactivation kinetics are required to establish this unequivocally. Also, until more information on the effects of pyrethroids on the inactivation kinetics of the rat sodium channels is available it might be unwise to extrapolate too readily from rat to insect.

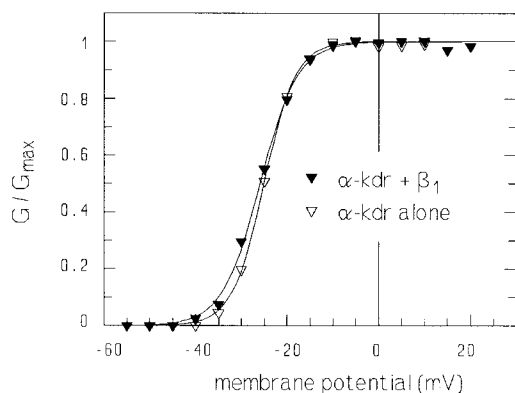


Fig. 4. Steady-state activation (relative peak sodium conductance values ( $G/G_{\max}$ ) plotted against membrane potential) derived from currents recorded from oocytes injected with cRNA transcripts for rat  $\alpha$ -kdr mutant, expressed either alone ( $\nabla$ ) or in conjunction with rat wild-type  $\beta_1$ -subunit ( $\blacktriangledown$ ). Oocytes were continuously perfused with saline containing  $10^{-5}$  M permethrin. Sodium currents were recorded upon application of a voltage protocol similar to that illustrated in Fig. 2A, but consisting in 16 steps in 5 mV increment. Data points were fitted with the Boltzmann Eq. 2 (continuous lines), defined by the following parameters: ( $\nabla$ ),  $V_{0.5} = -25.1$  mV ( $\pm 0.3$  mV, SE of the fit) and  $k = 4.2$  mV ( $\pm 0.5$  mV, SE of the fit); ( $\blacktriangledown$ ),  $V_{0.5} = -25.9$  mV ( $\pm 0.2$  mV, SE of the fit) and  $k = 5.1$  mV ( $\pm 0.4$  mV, SE of the fit).

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