

Novel effects of dendrotoxin homologues on subtypes of mammalian Kv1 potassium channels expressed in *Xenopus* oocytes

Brian Robertson^{a,*}, David Owen^b, John Stow^c, Carolyn Butler^c, Claire Newland^d

^aDepartment of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AZ, UK

^bElectrophysiology Laboratory, Wyeth Research (U.K.) Ltd., Taplow, Maidenhead SL6 0PG, UK

^cChemistry, Wyeth Research (U.K.) Ltd., Taplow, Maidenhead SL6 0PH, UK

^dNeurosciences Group, Institute of Molecular Medicine, Oxford OX3 9DU, UK

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Abstract We have examined the effects of two DTX homologues, toxin I and toxin K, on Kv1.1, Kv1.2 and Kv1.6 channels expressed in *Xenopus* oocytes. Toxin I blocked all three channels; in contrast, toxin K was selective for Kv1.1. Both toxins slowed channel activation and inactivation kinetics with 10 nM toxin I approximately doubling activation and inactivation time constants of Kv1.1. For the first time, we have demonstrated the selectivity of a DTX homologue for a single cloned Kv1 channel and suggest that these toxins may sterically hinder the conformational changes that occur during channel gating.

Key words: Potassium channel; Dendrotoxin; Oocyte; Toxin; Ion channel; Rat brain

1. Introduction

The symbiosis of molecular biology and electrophysiology has provided unparalleled access to the physiological and pharmacological properties of voltage-gated K channels important in the mammalian CNS [1]. Basic physiological and pharmacological studies of the properties of single K channel α subunits will be essential to a complete understanding of their role in modulating neuronal activity. Here we examine for the first time the actions of two dendrotoxin homologues, toxins I and K, on three cloned K channels from the mammalian brain. These toxins bind to synaptosomal membranes with high affinity and increase neurotransmitter release in both peripheral and central preparations through block of specific voltage-activated conductances [2]. We show that in addition to differences in the specificity of block of cloned K channel subtypes, channel activation and inactivation gating may also be slowed. A preliminary account of some of this data has been published [3].

2. Materials and methods

2.1. Expression in oocytes

cRNAs coding for rat (r) or human (h) Kv1.1, Kv1.2 or Kv1.6 were kindly provided by Professor Olaf Pongs (Institut für Neuronale Signalverarbeitung, Hamburg, Germany). Oocytes were obtained from mature female *Xenopus laevis* under anaesthesia (0.1% solution of 3-aminobenzoic acid ethyl ester in water, Sigma). Theca and follicular cell layers were removed by incubation in 1.5–2 mg/ml collagenase (Boehringer Mannheim) in a Ca^{2+} free medium (in mM: 82.5 NaCl, 2 KCl, 1 MgCl_2 , 5 HEPES, pH 7.6) for a total of 3 h. Stage V and V oocytes were injected with 0.5–2 ng of cRNA dissolved in RNase free water (total volume 50 nl), using a Drummond Nanoject microinjec-

tor system (Broomall, PA, USA) within 24 h of collagenase treatment. Injected oocytes were maintained at 18°C in Barth's solution (in mM: 88 NaCl, 10 HEPES, 1 KCl, 0.82 MgCl_2 , 0.33 $\text{Ca}(\text{NO}_3)_2$, 0.41 CaCl_2 , 2.4 NaHCO_3 , 0.5 theophylline, pH 7.4, plus 0.1 mg/ml gentamycin sulphate, 0.01 mg/ml streptomycin sulphate and 0.01 mg/ml penicillin-G).

2.2. Electrophysiological recording and analysis

Currents were recorded with a two-microelectrode voltage-clamp (TEC-10CD, NPI, Germany) 1–5 days after injection and at 20–24°C. Microelectrodes were filled with filtered 3 M KCl, and had resistances of 0.3–1 M Ω . During recording oocytes were perfused with Ringer's solution (in mM: 115 NaCl; 2.5 KCl; 10 HEPES; 1.8 CaCl_2 , pH 7.2). Data acquisition and analysis was performed with an EPC9 and Pulse+Pulsefit software (version 7.4, Heka, Germany) running on a Macintosh Ilii. Leak and capacity artefacts were subtracted on-line using a p-on-4 procedure. Toxins were stored as frozen stocks and diluted before each experiment. Toxin containing solutions were bath applied at a rate of 1–3 ml/min. Toxin I was applied first to each oocyte, and washed away until full recovery to control values was achieved. Toxin K was applied subsequently, and a cumulative dose-response relationship was constructed. To quantify the effects of toxins, the response (denoted Q_{step}) was measured as the integral of the total outward ionic current during the voltage step, i.e. charge transferred during the step (–80 to +10 mV). This method more accurately describes the overall effects of the toxins, since it includes effects of agents on channel kinetics [4]. Data are presented as mean $\pm$ S.E.M. of measurements made from n number of oocytes.

Activation and inactivation time constants were obtained using a Simplex optimization algorithm in the Pulsefit (Heka, Lambrecht, Germany) analysis program. Activation time constants (τ_n) were obtained using the Hodgkin-Huxley formalism: $I(t + \text{delay}) = I_0 n(t)^3$ where $n(t) = 1 - \exp(-t/\tau_n)$, I_0 is maximal current amplitude and t is time during the voltage step. Inactivation time constants from long voltage steps were fitted according to the equation: $I(t) = I_p + a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$; where I_p is the current plateau amplitude, and τ_1 and τ_2 are the two inactivation time constants with amplitudes a_1 and a_2 respectively.

2.3. Toxin isolation and purification

Toxins I and K were isolated from crude venom of the black Mamba snake *Dendroaspis polylepis polylepis*, which contains at least 28 different peptides [5]. The toxins of interest here were purified from crude venom after ultrafiltering using a 5000 nominal molecular weight cutoff filter to remove low molecular weight material. The ultrafiltrate was then further purified by preparative cation exchange HPLC followed by preparative reverse-phase HPLC. The purity of the toxins was found to be greater than 99.5% by capillary electrophoresis. The molecular weight was confirmed by electrospray mass spectrometry as matching the calculated mass from the published amino acid sequence (e.g. [6]). Fig. 1 illustrates typical electropherograms showing the constituents of the crude venom, and purified toxin I and K fractions.

3. Results

3.1. Selectivity and voltage dependence of block by toxins I and K

Toxin I blocked all three types of Kv1 channel, with 10 nM

*Corresponding author. Fax: (44) (171) 225 0960.
E-mail: brian.robertson@ic.ac.uk

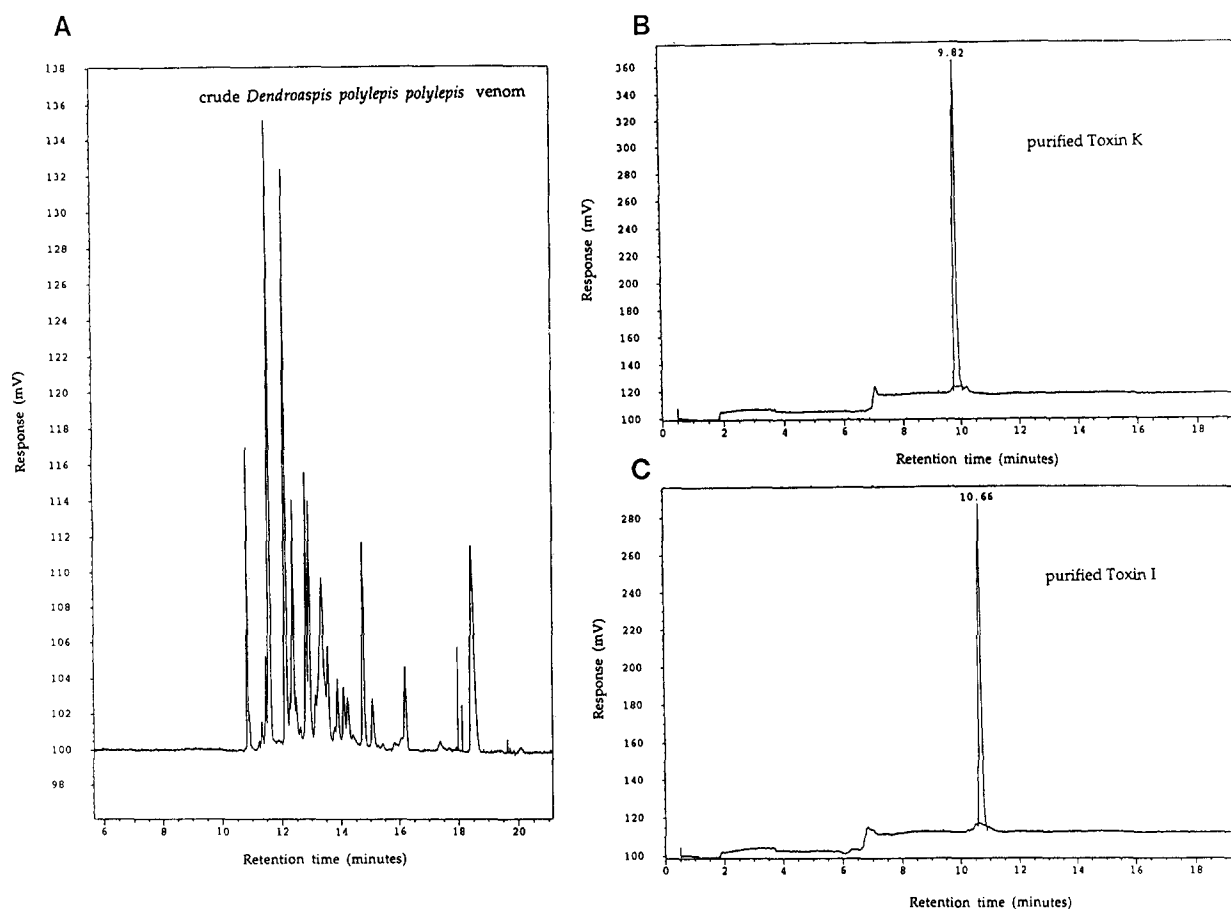


Fig. 1. Purification of toxin fractions from crude venom. Electropherograms, showing response (in mV) versus retention time of (a) crude *Dendroaspis polylepsis polylepsis* venom, (b) purified toxin I and (c) purified toxin K from *Dendroaspis polylepsis polylepsis* venom.

toxin I blocking control Q_{step} at +10 mV by the following amounts: rKv1.1: $43 \pm 6\%$ ($n = 10$); rKv1.2: $22 \pm 3\%$ ($n = 7$); hKv1.2: $36 \pm 6\%$ ($n = 7$) and rKv1.6: $26 \pm 4\%$ ($n = 6$). Block was rapid in onset and usually fully reversible. Toxin K was not only more potent than its close homologue toxin I in blocking Kv1.1 channels (Fig. 2a), but was also

much more selective, effectively only blocking Kv1.1 channels with an $IC_{50} = 2.5$ nM (Fig. 2b). Block by toxin K was rapid in onset, but seldom showed complete reversal despite extensive (>30 min) washout.

Block by toxins I and K was voltage-independent over the range -30 to $+30$ mV (Fig. 3). 10 nM toxin I blocked rKv1.1

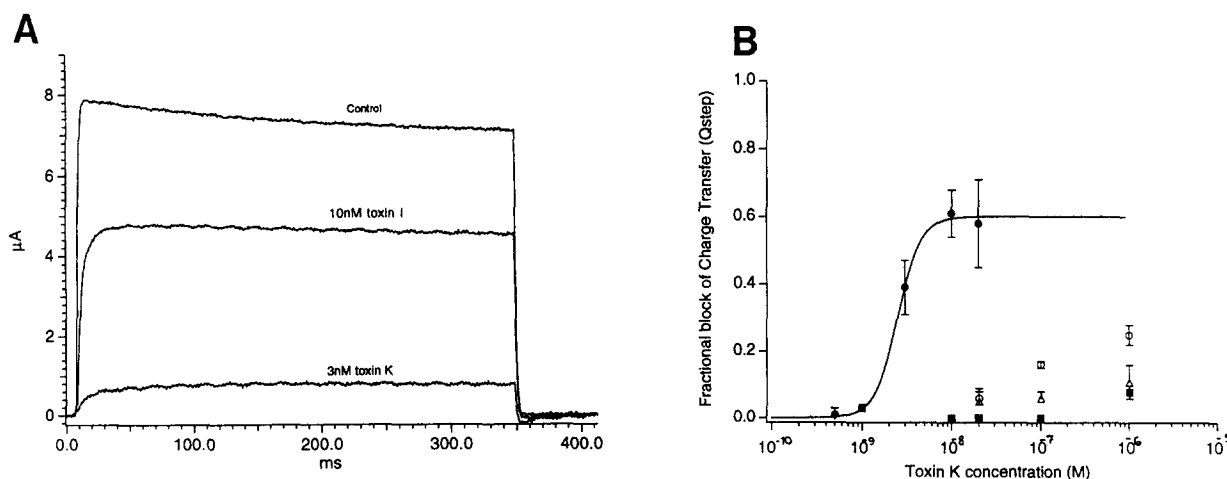


Fig. 2. Concentration dependence of block by toxins I and K. (a) Control rKv1.1 current (top) was reduced by 10 nM toxin I (middle trace) and 3 nM toxin K (bottom trace). Steps from -80 to $+10$ mV. (b) Concentration-response relationship for block of rKv1.1 (●), rKv1.2 (○), hKv1.2 (■) and rKv1.6 (△) by toxin K. Block expressed as a fraction of control Q_{step} . The data for rKv1.1 (●) were fitted with a standard logistic function, with an IC_{50} of 2.5 nM. Each point is the mean $\pm$ S.E.M. of 3–14 oocytes.

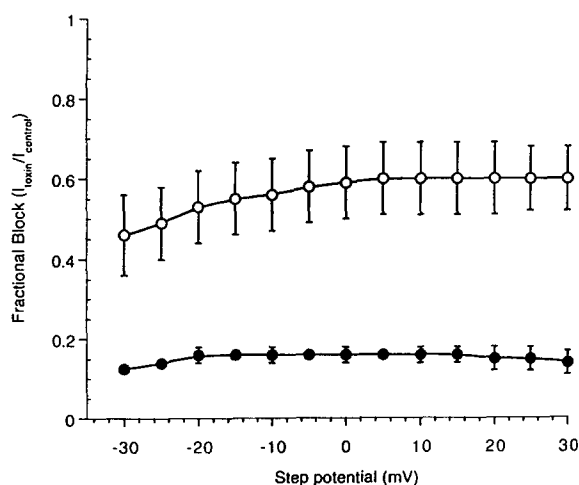


Fig. 3. Voltage dependence of block of rKv1.1 by toxins I and K. Relative peak current ($I_{\text{toxin}}/I_{\text{control}}$) in the presence of 10 nM toxin I (○) or 3 nM toxin K (●). Each point is mean $\pm$ S.E.M. for 4–5 oocytes.

peak current by $56 \pm 8\%$ ($n = 5$) at -30 mV, and by $42 \pm 7\%$ at $+30$ mV ($n = 5$). Similarly, 3 nM toxin K blocked rKv1.1 by $87.5 \pm 0.8\%$ ($n = 4$) at -30 mV and $86 \pm 3\%$ at $+30$ mV ($n = 4$). Values for inhibition at these voltages were not significantly different as determined by a Wilcoxon signed rank test.

3.2. Slowing of the rate of activation by toxin I

In addition to blocking certain Kv1 channels, both toxins altered gating behaviour, slowing activation kinetics. These actions were investigated in detail for toxin I on rKv1.1 channels. 10 nM toxin I slows the time to peak of rKv1.1 (Fig. 4a). The activation time constants in the presence and absence of toxin I were determined by fitting the currents with an n^3 Hodgkin-Huxley equation (see section 2); Fig. 4a illustrates that this formula accurately describes the current traces. In the absence of toxin the activation time constant, τ_n , decreased as the step potential became more positive, ranging

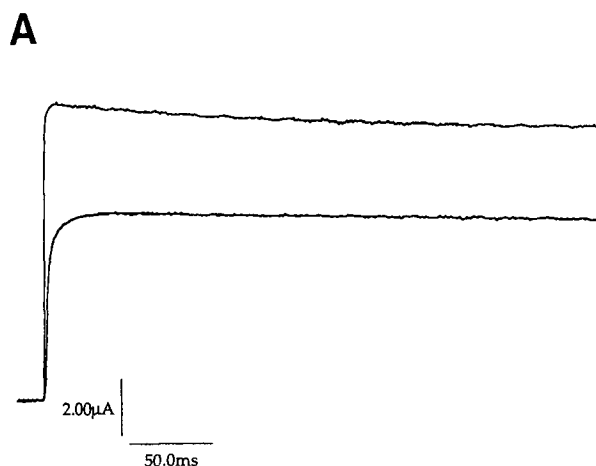


Fig. 4. Effect of toxin I on the rate of activation of rKv1.1. (a) Toxin I slowed the activation kinetics of rKv1.1. The top trace is control, and the lower trace is in the presence of 10 nM toxin I. Both traces are superimposed with Hodgkin-Huxley fits to peak current (smooth curves, see section 2), with activation time constants (τ_n) of 0.7 ms in control and 1.5 ms in toxin I. The voltage steps were from -80 to $+30$ mV. (b) Dependence of activation time constant (τ_n) on step potential. Values for τ_n were derived from fits such as that shown in (a). Each point is the mean $\pm$ S.E.M. from 5 oocytes. The activation time constant was increased approximately 2.3-fold in the presence of 10 nM toxin I at all voltages tested (-30 to $+30$ mV).

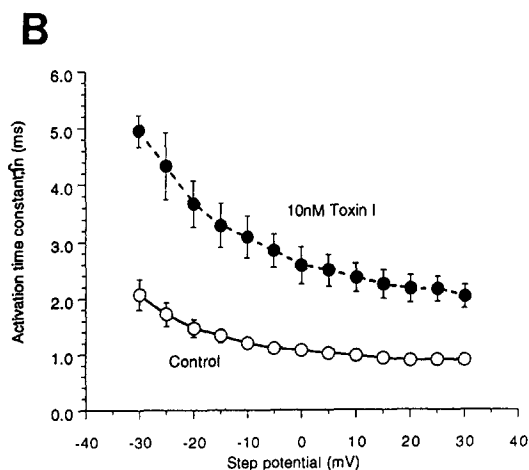
from 2.1 ± 0.3 ms at -30 mV ($n = 5$) to 0.9 ± 0.1 ms at $+30$ mV ($n = 5$). At all potentials, 10 nM toxin I lengthened the activation time constant, increasing it approximately 2.3-fold over the entire voltage range tested (see Fig. 4b). Thus, 10 nM toxin I increased the activation time constant to 5.0 ± 0.3 ms ($n = 5$) at -30 mV, and 2.0 ± 0.2 ms ($n = 5$) at $+30$ mV. Toxin K also slowed activation kinetics; for example, for the oocyte shown in Fig. 4a, 3 nM toxin K slowed τ_n from 1 ms in control to 3.1 ms (at $+10$ mV). Overall, activation time constant (at $+10$ mV) was 1.0 ± 0.1 ms ($n = 9$) in control, and increased to 2.1 ± 0.3 ms ($n = 6$) in 3 nM toxin K.

3.3. Slowing of inactivation by toxin I

rKv1.1 channels showed little inactivation during short depolarizing steps (see e.g. Fig. 2a) but slow inactivation was revealed when voltage steps were several seconds long (Fig. 5a). Fig. 5b shows expanded and scaled traces (with the residual plateau current removed), showing more clearly that there are two components to the slow inactivation, both of which are slowed by toxin I (and by toxin K, not shown). In control solution the time constants were: $\tau_1 = 244 \pm 18$ ms ($n = 4$) and $\tau_2 = 8.3 \pm 1.2$ s ($n = 4$). In 10 nM toxin I these time constants were prolonged to 584 ± 70 ms ($n = 5$) and 13.4 ± 1.1 s ($n = 5$) respectively.

4. Discussion

We have examined the actions of black mamba toxins I and K on three key cloned potassium channels from mammalian brain. There are two important new results from the present study. Firstly, while all three clones Kv1.1, 1.2 and 1.6 were blocked by toxin I, the closely related homologue toxin K was selective for Kv1.1. Thus toxin K will be useful in separating out the contributions from Kv1.1 subunits from other mammalian *Shaker*-family subunits in native cells, such as central neurones. Recent work [7] has shown that only one of the four α subunits in a tetrameric Kv1.1 potassium channel is necessary to confer sensitivity to α -dendrotoxin (a close homologue of toxin K); here there is a linear relationship between the free energy of α -dendrotoxin binding and the



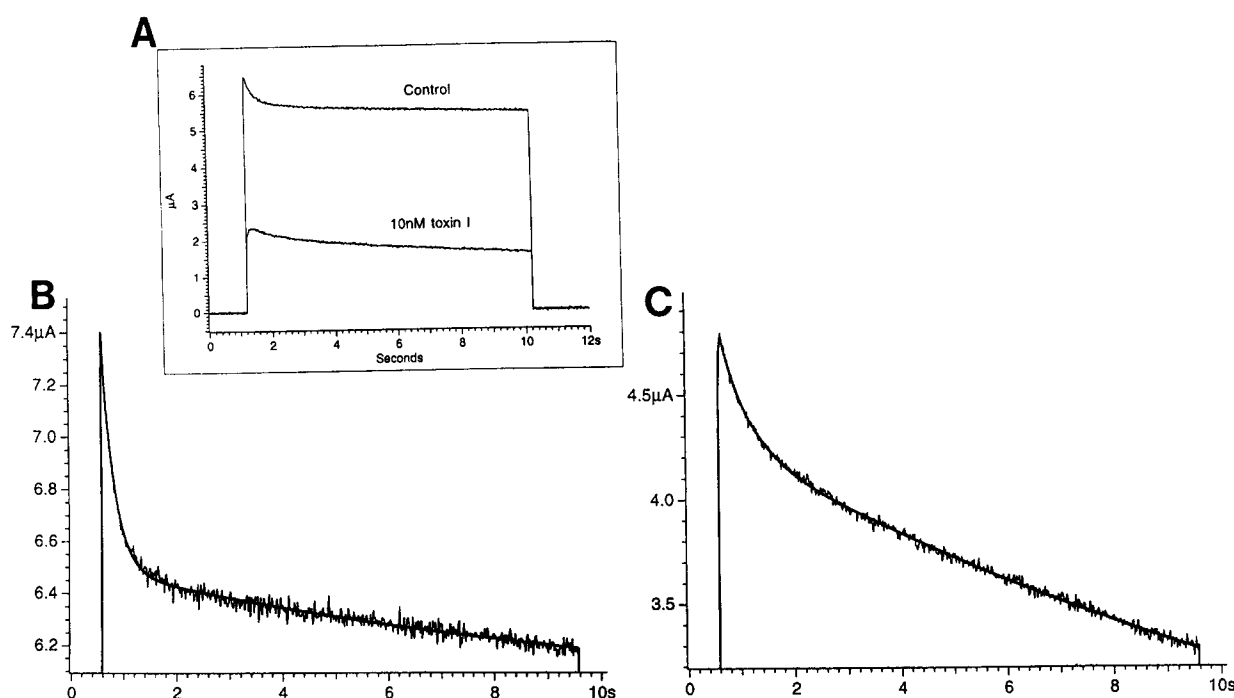


Fig. 5. Inactivation of rKv1.1 channels is slowed by 10 nM toxin I. (a) Long voltage steps (9 s) show slow inactivation of control current (upper trace). This inactivation is further slowed in the presence of 10 nM toxin I (lower trace). (b and c) Double exponential fits (smooth curves, see section 2) to expanded and scaled current traces, with plateau current (I_p) removed. (b) Control time constants are: $\tau_1 = 247$ ms and $\tau_2 = 11.1$ s; in toxin I (c) these increase to: $\tau_1 = 512$ ms and $\tau_2 = 16.2$ s respectively. All voltage steps from -80 to $+10$ mV.

number of Kv1.1 binding subunits. Related studies with the smaller, more compact charybdotoxin molecule have also revealed that the sensitivity of heteromultimeric potassium channels to block depends on the number of toxin sensitive and toxin-insensitive α -subunits present [8,9]. Our results may also help to identify which amino acids on the toxin and on the channel are necessary to confer high affinity block and selectivity. Previous approaches using both mutated channels [10] and toxins [11] have revealed that several regions on both the toxin and channel α -subunit are required for full activity.

We have also demonstrated that toxin I slows the activation and inactivation kinetics of Kv1.1 channels. Werkman et al. [12] showed a slowing of the rising phase of Kv1.2 channels by dendrotoxin, but suggested that this was due to a voltage-dependent dissociation of the toxin from the channel. However, we observed that the slowing of activation produced by toxin I was not voltage-dependent over the range -30 to $+30$ mV. An alternative explanation is that the toxin slows the conformational changes associated with gating. Modelling studies of the structure and dynamics of voltage-gated K channels suggest that there are major conformational changes as these channels open and close, with certain transmembrane segments, such as S4, perhaps moving a considerable distance out of the membrane [13]. Indeed recent experiments have indicated that the S4 voltage-sensing transmembrane segment of Na⁺ channels may indeed move outward into the extracellular space following depolarization [14]. We suggest that in addition to directly occluding the channel pore, these toxins sterically hinder some of the conformational changes that occur during potassium channel opening and closing. A similar mechanism would explain the results of Schaaf [15] who observed that dendrotoxin was able to slow *sodium* channel inactivation completely independently of block. The dendro-

toxins employed here are quite large molecules (~ 2.9 nm) compared to voltage-gated K channels; perhaps the sheer size of the toxin above the K channel may cause sufficient steric hindrance to slow the conformational changes necessary for channel opening and closing. Toxin induced changes in K channel gating should be taken into account when interpreting results from commonly used pharmacological methods. For instance one must exercise caution when subtracting out toxin-sensitive K channels from total K currents in a neurone, since one will get a false representation of the current if gating is altered. Furthermore, where flux studies are used to examine toxin-sensitive currents, one must be aware that changes in the dynamics of the channel by toxin could lead to erroneous estimates of potency depending on sampling interval used.

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