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Block of Voltage-gated Potassium Channels by Pacific Ciguatoxin-1 Contributes to Increased Neuronal Excitability in Rat Sensory Neurons

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ABSTRACT

The present study investigated the actions of the polyether marine toxin Pacific ciguatoxin-1 (P-CTX-1) on neuronal excitability in rat dorsal root ganglion (DRG) neurons using patch-clamp recording techniques. Under current-clamp conditions, bath application of 2-20 nM P-CTX-1 caused a rapid, concentration-dependent depolarisation of the resting membrane potential in neurons expressing tetrodotoxin (TTX)-sensitive voltage-gated sodium (Na_v) channels. This action was completely suppressed by the addition of 200 nM TTX to the external solution, indicating this effect was mediated through TTX-sensitive Na_v channels. In addition P-CTX-1 also prolonged action potential and afterhyperpolarisation (AHP) duration. In a sub-population of neurons, P-CTX-1 also produced tonic action potential firing, an effect that was not accompanied by significant oscillation of the resting membrane potential. Conversely, in neurons expressing TTX-resistant Na_v currents, P-CTX-1 failed to alter any parameter of neuronal excitability examined in this study. Under voltage-clamp conditions in rat DRG neurons, P-CTX-1 inhibited both delayed-rectifier and 'A-type' potassium currents in a dose-dependent manner, actions that occurred in the absence of alterations to the voltage-dependence of activation. These actions appear to underlie the prolongation of the action potential and AHP and contribute to repetitive firing. These data indicate that a block of potassium channels contributes to the increase in neuronal excitability, associated with a modulation of Na_v channel gating, observed clinically in response to ciguatera poisoning.

Key Words: ciguatoxin; potassium channel, dorsal root ganglia, neuronal excitability, patch clamp.

INTRODUCTION

Ciguatera poisoning is a form of ichthyosarcotoxism that occurs as a consequence of eating certain tropical and sub-tropical reef fish species that have bioaccumulated the marine neurotoxin, ciguatoxin. Each year, ciguatera poisoning affects more than 25,000 people worldwide and is the most frequent foodborne illness related to fin fish consumption. Typically the poisoning is characterised by gastrointestinal, neurological and to a lesser extent, cardiovascular disturbances. The gastrointestinal symptoms, such as vomiting, diarrhoea, nausea and abdominal pain, tend to occur within 24 hours of ingestion of a toxic fish, and last 1-3 days (Gillespie *et al.*, 1986). The neurological symptoms usually take longer to develop, often between 2-5 days, but may last for

weeks to months or even longer in some cases. They involve the peripheral nervous system causing weakness and diaphoresis but predominantly result in sensory neuropathies including paraesthesia of the extremities, pruritus, myalgia, arthralgia, dental pain, and a paradoxical temperature reversal.

Ciguatoxins are heat-stable, highly oxygenated, lipid-soluble cyclic polyethers with molecular weights of approximately 1000-1200 Da that underlie ciguatera in the Pacific, Indian Ocean and Caribbean regions (Lewis, 2001; Hamilton *et al.*, 2002a; Hamilton *et al.*, 2002b; Pottier *et al.*, 2003). In general, Pacific and Indian Ocean ciguatoxins cause predominantly neurological symptoms, while Caribbean ciguatoxins are associated with more gastrointestinal symptoms (Lewis, 2001; Hamilton *et al.*, 2002b). Of all the different strains, Pacific CTX-1 (P-CTX-1) is the most potent, with an LD_{50} in mice of 0.25 μg/kg i.p. (Murata *et al.*, 1990; Lewis *et al.*, 1991). These toxins originally derive from precursors found in epiphytic benthic marine dinoflagellates of the order *Dinoflagellatae*, in particular *Gambierdiscus spp*. These dinoflagellates produce less polar and less potent ciguatoxins (formerly known as gambiertoxins) that are biotransformed into more polar ciguatoxins in the liver of fish by oxidative metabolism and spiroisomerisation. The neurological symptoms observed in clinical cases of ciguatera poisoning are believed to be the consequence of the direct interaction of ciguatoxin with voltage-gated sodium (Na_v) channels (reviewed by (Lewis *et al.*, 2000)). Previous radioligand binding experiments have shown that ciguatoxin competes with another cyclic polyether toxin, brevetoxin (PbTX), from the marine dinoflagellate *Gymnodinium breve* (formerly *Ptychodiscus brevis*), for neurotoxin receptor site 5 on Na_v channels. This interaction causes membrane depolarisation and stimulates sodium influx in neuroblastoma cells (Bidard *et al.*, 1984). Pharmacological studies have shown that ciguatoxins activate Na_v channels in a variety of preparations to cause depolarisation, spontaneous action potentials and an elevation of $[Na^+]_i$, with resulting oedema of Schwann cells and axons (Bidard *et al.*, 1984; Allsop *et al.*, 1986; Benoit *et al.*, 1986; Lewis and Endean, 1986; Molgó *et al.*, 1993; Benoit *et al.*, 1996; Mattei *et al.*, 1999). This results in a stimulation of transmitter release from rat brain synaptosomes and enhanced spontaneous and evoked transmitter release from motor nerve terminals (Molgó *et al.*, 1990; Brock *et al.*, 1995; Hamblin *et al.*, 1995). Under voltage-clamp conditions these actions appear to be the result of hyperpolarising shifts in the voltage dependence of activation of TTX-sensitive Na_v channels. Furthermore P-CTX-1 induces a TTX-sensitive leakage current in DRG neurons (Strachan *et al.*, 1999) most likely from the spontaneous opening of a sub-population of Na_v channels at resting and hyperpolarised membrane potentials. In contrast, the only significant action of P-CTX-1 on TTX-resistant Na_v channels in DRG neurons was an

increase in the rate of recovery from Na_v channel inactivation (Strachan *et al.*, 1999).

Dinoflagellate-derived ciguatoxins (formerly gambiertoxins) are not especially toxic to mammals because they are less oxidised than ciguatoxins and are only present in small quantities in fish muscle. Nevertheless, it has been suggested that high concentrations of P-CTX-4B, the less polar precursor of P-CTX-1, blocks nodal voltage-gated potassium (K_v) channels in frog myelinated nerve fibres resulting in a decrease in the amplitude, and an increase in the duration of action potentials (Benoit and Legrand, 1994). This is due to the finding that tetraethylammonium failed to further prolong action potential duration following exposure to 24 nM P-CTX-4B. However, to date no studies have investigated the actions of ciguatoxins commonly implicated in human poisoning on neuronal excitability in mammalian sensory neurons, particularly actions on voltage-gated ion channels other than Na_v channels.

The aim of the present study was to examine the effects of P-CTX-1 on action and resting potentials in mammalian sensory neurons. Dorsal root ganglion (DRG) neurons were chosen to investigate the actions of P-CTX-1 on action potential electrogenesis as their cell bodies and/or afferent fibres are presumably the origin of the characteristic sensory neurological disturbances reported clinically for ciguatera. Given that P-CTX-4B has been shown to block K_v channels, we determined if the P-CTX-1-induced alterations to neuronal excitability involved the modulation of K_v channels in addition to effects on Na_v channel gating reported previously. Specifically, we investigated if P-CTX-1 modulated delayed-rectifier (K_{DR}) or transient 'A-type' (K_A) potassium channels, given their importance in controlling in neuronal excitability (Hille, 2001). These experiments were carried out using current- and voltage-clamp methods employing the whole-cell patch clamp technique in acutely dissociated newborn rat DRG neurons.

MATERIALS AND METHODS

Isolation and purification of P-CTX-1

Pacific ciguatoxin-1 (P-CTX-1) was isolated from the viscera of moray eels (*Lycondontis javanicus*) that were collected from a region of Tarawa (1.3°N, 173°E) in the Republic of Kiribati (central Pacific Ocean) where ciguatera is endemic. The isolation and purification techniques required to extract P-CTX-1 have been previously described. Briefly, the purification technique

involved heating the viscera to 70°C and extracting the lipid-soluble components using acetone. This product was then subjected to silica gel vacuum liquid chromatography followed by five chromatographic steps. Samples of isolated ciguatoxins were reapplied to HPLC columns and eluted with different polarity solvents to confirm homogeneity, and an array detector was used to determine the UV profile and establish purity. The sample was >95% pure and contained no detectable P-CTX-4B, which is not present in carnivorous fish where the moray eels were captured. P-CTX-1 stock was dissolved in 50% aqueous methanol and stored in glass at –20°C. Concentrations of P-CTX-1 ranging from 2-20 nM were made by dilution with external solution and applied directly to the patch-clamp recording chamber. Control experiments were performed with 50% aqueous methanol at a maximum concentration of 2.2 mg/ml to assess the effects on neuronal excitability.

DRG Isolation and preparation

All electrophysiological experiments were carried out on acutely dissociated rat DRG neurons as previously described. Briefly, DRG neurons were isolated from 2-12 day-old Wistar rat of either sex and the connective sheath weakened by incubation in 25 mg/ml of trypsin (Type XI). After enzyme treatment the DRGs were washed twice with sterile Dulbecco's modified Eagles medium (DMEM) (Gibco, Grand Island, NY, USA), containing 10% newborn calf serum (Gibco) and 80 mg/ml gentamycin. After the final wash the ganglia were re-suspended in 2 ml of the remaining DMEM culture media and mechanically triturated through a heat-flamed Pasteur pipette. The neurons were then evenly distributed onto 12 mm glass coverslips (Assistent, West Germany) that were pre-coated with poly-L-lysine. DRG neurons were incubated overnight in 1 ml of DMEM at 37ºC (10% CO_2, 90% O_2 and 100% relative humidity) to allow isolated neurons to adhere to the coverslips. All animal experimentation was approved by the joint Animal Care & Ethics Committee of the University of Technology, Sydney and the Royal North Shore Hospital, Sydney, Australia.

Electrophysiology

Electrophysiological experiments employed the patch-clamp recording technique in whole-cell configuration to measure current and voltage changes from single DRG neurones. Neurons were bathed in iso-osmotic external solution using a continuous gravity-fed perfusion system (Barrington, IL, USA), that maintained a flow rate of 0.3-0.6 ml/min. Patch pipettes were pulled from borosilicate glass capillary tubing (Corning 7052 Glass, Warner Corp.) and had

resistances of 0.8-2 MΩ when filled with internal pipette solution. Experiments were performed at 22°C.

Membrane voltage and current were recorded using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA) and current and voltage pulse protocols were generated using pClamp software system (Axon Instruments). Signals were filtered using an internal 1-kHz low-pass, 5-pole Bessel filter (-3dB) and digitised at 15-25 kHz, depending on protocol length. Leakage and capacitive currents were subtracted electronically with *P-P*/4 procedures and series resistance compensation was >80% for all neurons. The voltage-clamp data recorded in this study were rejected if there were large leak currents or currents showed signs of inadequate space clamping such as an abrupt activation of currents upon relatively small depolarising pulses. Current-clamp data were rejected if the initial resting membrane potential was more depolarised than -45 mV.

Effects on action and resting membrane potentials - The effects of P-CTX-1 on action and resting potentials were investigated using current-clamp recordings from DRG neurons. The extracellular solution for current-clamp recordings (solution 1) contained (in mM): 120 NaCl, 3 KCl, 10 D-glucose, 1.8 $CaCl_2$, 1.8 MgCl, 10 HEPES-acid and was adjusted to pH 7.4 using 1 M NaOH. The internal patch pipette solution contained (in mM): 110 KCl, 10 NaCl, 5 HEPES-acid and was buffered to pH 7.0 with 1 M KOH. Action potentials were elicited by 1-2 ms supramaximal currents delivered at 0.1 Hz.

The predominant Na_v channel present in each neuron was determined prior to current-clamp recordings using a modified steady-state Na_v channel inactivation protocol under voltage-clamp conditions. This takes advantage of the separation of steady-state inactivation curves for TTX-sensitive and TTX-resistant Na_v channels (Roy and Narahashi, 1992). Larger diameter neurons from older animals tended to express fast TTX-sensitive Na_v channels, whilst smaller neurons tended to express predominantly slow TTX-resistant Na_v channels (Roy and Narahashi, 1992). In those experiments that assessed the actions of P-CTX-1 on neurons expressing TTX-resistant Na_v channels, 200 nM TTX was applied in the external solution to eliminate any residual TTX-sensitive I_{Na}. Only those neurons that exhibited less than 10% TTX-resistant I_{Na}, were used to determine the actions of P-CTX-1 in neurons expressing predominantly TTX-sensitive Na_v channels. Once the identity of the major type of sodium current (I_{Na}) was established, the patch-clamp amplifier was switched over to current-clamp mode and changes in the membrane

voltage were measured. In those experiments investigating the action of P-CTX-1 on K_v channels the current clamp external solution (solution 1) was then switched to the voltage clamp solution for recording I_K (see solution 2 below)

Effects on K^+ channels - Rat DRG neurons express a variety of K_v channels. The present study investigated the effects of P-CTX-1 specifically on delayed-rectifier potassium (K_{DR}) channels and transient 'A-type' potassium (K_A) channels, given their importance in the controlling action potential duration and firing frequency (Hille, 2001). The extracellular solution for voltage-clamp recordings (solution 2) of delayed-rectifier potassium currents ($I_{K(DR)}$) and transient 'A-type' potassium currents ($I_{K(A)}$) contained (in mM): tetramethylammonium (TMA) chloride 120, KC1 5, $CaCl_2$ 1.8 $MgCl_2$ 1, D-glucose 25 and HEPES-acid 5, with the pH adjusted to 7.4 using 1 M TMA-OH. The internal patch pipette solution contained (in mM): KF 80, TMA-chloride 50, D-glucose 5, EDTA 5 and HEPES-acid 5, with the pH adjusted to 7.0 using 1 M KOH.

When recording I_K, 200 nM tetrodotoxin (TTX) was added to the external solution to block tetrodotoxin-sensitive I_{Na}. Neurons displaying TTX-resistant I_{Na} were not used for experiments investigating actions on I_K. Since I_{Na} were blocked by TTX, any involvement of Na^+-dependent I_K was eliminated. In addition, the internal solution contained 80 mM KF and the external solution contained 30 nM charybdotoxin and 1 mM $CdCl_2$, eliminating any Ca^{2+}-dependent components of I_K. To record $I_{K(DR)}$, 5 mM 4-aminopyridine (4-AP) was added to the external solution to eliminate any possible contamination by $I_{K(A)}$. This required readjustment of the pH to 7.4 using 1 M HCl. For recordings of $I_{K(A)}$, 25 mM tetraethylammonium (TEA) chloride was added to the external solution to block $I_{K(DR)}$. The osmolarity of all external and internal solutions was adjusted to 300 mOsmol/l with sucrose using a vapour pressure osmometer (Gonotec Osmomat, Berlin, Germany) to reduce osmotic stress on the DRG neurons. The liquid junction potential was determined using the JPCalc® software program and all data were compensated for this value.

Delayed-rectifier potassium currents were generated using a single-pulse protocol from a holding potential of –80 mV to +10 mV for 100 ms every 5 seconds. In the presence of 5 mM 4-AP, this test pulse evokes only $I_{K(DR)}$ in isolation. The voltage-dependence of K_{DR} activation was determined using a 100-ms voltage step protocol from a holding potential of –80 mV to voltages between –80 to +40 mV in 10-mV steps every 5 seconds. Fast transient 'A-type' potassium currents ($I_{K(A)}$) were isolated by subtraction in the presence of 25 mM TEA-Cl in the external solution to eliminate any residual $I_{K(DR)}$. Currents were elicited by a test pulse (V_{test}) to +20 mV for 500 ms.

$I_{K(A)}$ recorded following an initial 1.5-s prepulse ($V_{prepulse}$) to –40 mV, to inactivate $I_{K(A)}$, were subtracted from those recorded from a holding potential of –80 mV. The effect of P-CTX-1 on the voltage-dependence of activation of K_A was determined using a similar subtraction protocol except the test pulse was varied from –80 to +30 mV in 10-mV steps every 10 seconds.

Reagents used

All chemicals used were analytical grade and, unless otherwise stated, were obtained from Sigma Chemical Co., St. Louis, MO, USA. Tetrodotoxin (Calbiochem, San Diego, CA), supplied as a citrate buffer, was made up to 100 μM stock solution with sterile water and stored at –20°C for up to 6 months. The stock was then diluted with external solution to a final concentration of 200 nM on the day of an experiment.

Data analysis.

Numerical data are presented as the mean ± S.E. (*n*, number of observations). Statistical differences in current-clamp experiments were determined using a one-way ANOVA followed by a Tukey's HSD post-hoc test, at $P < 0.05$. For voltage-clamp experiments on K_v channels, statistical differences were determined using a Student's *t*-test, at $P < 0.05$. Mathematical curve fitting was accomplished using GraphPad Prism version 4.00 for Macintosh (GraphPad Software, San Diego CA, USA). All curve-fitting routines were performed using non-linear regression analysis employing a least squares method.

The I/V data were normalised to maximum control current and fitted with the following equation:

$$I_K = g_{max}\left(1 - \frac{1}{1 + \exp\left[\left(V - V_{1/2}\right)/s\right]}\right)\left(V - V_{rev}\right) \quad (1)$$

where I_K is the amplitude of the I_K at a given test potential, V, g_{max} is the maximal K^+ conductance, $V_{1/2}$ is the voltage at half-maximal activation, s is the slope factor and V_{rev} is the reversal potential.

RESULTS

Actions of P-CTX-1 on neuronal excitability

An increase in neuronal excitability resulting in membrane depolarisation and repetitive action potential discharges has previously been observed with ciguatoxins at frog motor terminals and myelinated axons as well as rat parasympathetic neurons and neuroblastoma cells. To investigate membrane excitability in response to P-CTX-1 in mammalian sensory neurons, DRG neurons were held under current-clamp conditions and action potentials generated from a single depolarising pulse. A variety of action and resting potential parameters were then measured, including the resting membrane potential, action potential amplitude and duration, afterhyperpolarisation amplitude and duration, and spontaneous action potential firing frequency.

A typical response of 10 nM P-CTX-1 on neuronal excitability in a DRG neuron expressing predominantly TTX-sensitive Na_v channels is shown in Fig. 1A-C. This shows the depolarisation of the resting membrane potential, and increases in action and afterhyperpolarisation duration. These changes occurred in the absence of significant alterations in the amplitude of the spike or afterhyperpolarisations, although there was a small decrease their amplitude. The application of P-CTX-1 from 2-20 nM induced an almost immediate, concentration-dependent membrane depolarisation in neurons expressing TTX-sensitive Na_v channel subtypes (Fig. 1C and D). The average resting membrane potential (E_m) for DRG neurons expressing TTX-sensitive Na_v channel subtypes was significantly depolarised from –54.7 ± 1.1 mV (n = 22) in controls to –42.9 ± 1.6 mV (n = 13; $P < 0.0001$) and –36.2 ± 2.9 mV (n = 5; $P < 0.0001$) after a 10-min perfusion in 10 nM and 20 nM P-CTX-1, respectively (Fig. 1D). The effects of P-CTX-1 on the resting membrane potential were completely suppressed by the addition of 200 nM TTX to the external solution (n = 5, data not shown), indicating this effect was mediated through TTX-sensitive Na_v channels. Furthermore, Figure 1C also shows that P-CTX-1 caused a concentration-dependent increase in action potential duration, measured at both 0 mV and 50% maximum spike amplitude. At 50% of maximum action potential amplitude, the average duration increased 3.4-fold from 1.3 ± 0.1 ms (n = 22) in control recordings to 4.4 ± 0.2 ms (n = 13; P = 0.02) in the presence of 10 nM P-CTX-1. The duration of the afterhyperpolarisation, measured at 50% decay, was also significantly increased at all concentrations of P-CTX-1 tested, while the amplitude of the afterhyperpolarisation tended to be reduced after P-CTX-1 (Figure 1B and C). This data is summarised in Table 1.

During depolarisation of the resting membrane potential, 10 nM P-CTX-1 induced a short

5-10 minute episode of stimulus-evoked repetitive firing of action potentials. This occurred in response to a single depolarising stimulus but only in neurons expressing predominantly TTX-sensitive Na_v channels (Fig. 2A-B). Moreover these neurons then underwent spontaneous tonic action potential firing (Fig 2C-D). However, this evoked and tonic repetitive action potential firing only occurred in 25% of neurons exposed to P-CTX-1. The frequency of these repetitive discharges averaged 56 ± 6 Hz (n = 4) and always occurred after the membrane had depolarised to between approximately –45 to –50 mV. This spontaneous firing ceased when the resting membrane potential became depolarised beyond –40 mV. However, manual hyperpolarisation of the membrane potential resulted in the re-emergence of spontaneous action potential firing in those TTX-sensitive neurons that had initially displayed repetitive firing in response to a single stimulus Fig. 2F). Interestingly, these reappearing spontaneous action potential discharges were of a much higher frequency of 100 ± 5 Hz (n = 4). It was also noted that in most cells the resting membrane depolarisation was accompanied by small oscillations of the membrane potential (Fig. 2D). These oscillations were observed at potentials between –49 and –58 mV and had an amplitude of 0.5-5 mV (mean 1.8 ± 0.1 mV, n = 5 cells) and frequencies of 23-40 Hz (mean 33 ± 2 Hz, n = 5 cells). Spontaneous action potential firing following addition of P-CTX-1 was not observed in the presence of 200 nM TTX (n = 4, data not shown).

To investigate the effect of P-CTX-1 on the threshold of action potential firing, a current-step protocol of increasing amplitude was applied. Under control conditions, the threshold for generation of action potentials occurred at a mean current injection of 10 ± 1 nA (n = 29). However, following a 10-min perfusion with 10 nM P-CTX-1 on neurons not showing spontaneous firing an average current injection of 20 ± 1 nA (n = 13) was required to induce action potential firing. Neurons undergoing repetitive firing of action potentials did not require an increase in current injection to stimulate the high frequency repetitive discharges. In contrast to its actions in neurons expressing predominantly TTX-sensitive Na_v channels, P-CTX-1 at concentrations up to 10 nM did not induce significant membrane depolarisation, or alter any aspect of action potential electrogenesis in neurons expressing predominantly TTX-resistant Na_v channels (n = 8, data not shown). In addition, experiments that assessed the action of the vehicle showed that at a concentration up to 2.2 mg/ml of methanol, which represents the amount of methanol in solutions containing 20 nM P-CTX-1, had no significant effect on the resting or action potential parameters.

Actions of P-CTX-1 on delayed-rectifier potassium (K_{DR}) channels

Fast activating delayed-rectifier potassium channels function to limit the duration of action potentials by remaining open for as long as depolarisation occurs, thereby promoting the onset of repolarisation (Hille, 2001). To assess the actions of P-CTX-1 on the kinetics of $I_{K(DR)}$, initial experiments measured the amplitude and time course of $I_{K(DR)}$ currents following perfusion with P-CTX-1. Currents were recorded under control conditions prior to a 10-min perfusion with P-CTX-1 and the change in maintained $I_{K(DR)}$ amplitude determined. Figure 3C-D shows typical $I_{K(DR)}$ recorded before, and 15 min following, the addition of 5 and 20 nM P-CTX-1. Mean maintained $I_{K(DR)}$ amplitude, measured at the end of the depolarising test pulse, was significantly reduced by 24 ± 3 % (n = 5; P < 0.005) and 64 ± 6% (n = 4; P < 0.05) by 5 and 20 nM P-CTX-1, respectively. The magnitude of the block of $I_{K(DR)}$ at 20 nM P-CTX-1 is comparable to inhibition by 10-25 mM TEA (Figure 3H). Only a partial 26 ± 9% (n = 5) reversal of the effects of 5 nM P-CTX-1 were noted following washout in toxin-free external solution (Fig. 3C). The effects of 20 nM P-CTX-1 were not reversible, even after prolonged washout in toxin-free solution. The time taken to reach peak $I_{K(DR)}$ amplitude was also not significantly altered following addition of 5 or 20 nM P-CTX-1. The time to peak was unchanged in the presence of 5 nM P-CTX-1 and only slightly decreased by 0.1 ± 0.9 ms following perfusion with 20 nM P-CTX-1 (n = 4, P > 0.9). This indicates that toxin does not alter the kinetics of channel activation.

The voltage dependence of K_{DR} channel activation was also analysed to determine whether the block by P-CTX-1 was caused by a depolarising shift in the voltage-dependence of activation. Maintained $I_{K(DR)}$ amplitude, measured at the end of the 100-ms depolarising test pulse, was measured and plotted against membrane potential. Under control conditions, $I_{K(DR)}$ activated in response to depolarising test pulses at potentials greater than -60 mV (Fig. 3G). Following a 10-min perfusion with either 5 of 20 nM P-CTX-1, there was no significant alteration in the threshold of K_{DR} channel activation (Fig. 3G). The only observed change was a significant reduction in peak $I_{K(DR)}$ amplitude at all test potentials indicating the absence of any voltage-dependent block of K_{DR} channels by P-CTX-1 (Fig. 3G).

Actions of P-CTX-1 on A-type potassium (K_A) channels

One of the main functions of the transient 'A-type' potassium channels (K_A) is to modulate

action potential firing frequency and slow the rate of depolarisation by altering the duration of the afterhyperpolarisation (Hille, 2001). To determine whether the ability of P-CTX-1 to initiate tonic action potentials involves a block of K_A channels the amplitude and timecourse of A-type potassium currents ($I_{K(A)}$) was determined. Figure 4C-D shows typical $I_{K(A)}$ recorded before, and 10 min following, the addition of 5 and 20 nM P-CTX-1. In the presence of 5 nM P-CTX-1, peak $I_{K(A)}$ were reduced by 18 ± 8 % in the presence of 5 nM P-CTX-1 (n = 5, P < 0.05). At a concentration of 20 nM, P-CTX-1 inhibited $I_{K(A)}$ by 47 ± 8% (n = 5, P < 0.02). The magnitude of the block of $I_{K(DR)}$ at 20 nM P-CTX-1 is comparable to inhibition by 1-5 mM 4-AP (Figure 4H). Again only a partial 27 ± 8% (n = 5) reversal of the effects of 5 nM P-CTX-1 were noted following washout in toxin-free external solution (Fig. 4C). The effects of 20 nM P-CTX-1 were not reversible, even after prolonged washout in toxin-free solution. The time taken to attain peak $I_{K(A)}$ amplitude and the timecourse of decay was assessed to determine if P-CTX-1 significantly altered the kinetics of channel activation or inactivation. No significant alteration in the time to peak $I_{K(A)}$ or decay were noted following addition of 5 or 20 nM P-CTX-1. The time to peak was only slightly increased by 3 ± 2 ms from 14 ± 2 ms in controls to 17 ± 3 ms in the presence of 5 nM P-CTX-1 (n = 5, P > 0.1) Similarly the time to peak was only slightly increased by 2 ± 1 ms following perfusion with 20 nM P-CTX-1 from 9 ± 2 ms in controls to 11 ± 2 ms in toxin (n = 5, P > 0.5). This indicates that toxin does not alter the kinetics of channel activation.

The voltage-dependence of K_A channel activation was also analysed to determine whether P-CTX-1 altered the voltage-dependence of activation. Peak $I_{K(A)}$ was measured and plotted against membrane potential on an $I_{K(A)}/V$ curve, as shown in Fig 4G. Under control conditions, $I_{K(A)}$ activated in response to the depolarising test pulse at potentials greater than -60 mV (Fig. 4G). Following a 10-min perfusion with either 5 or 20 nM P-CTX-1, there was no significant alteration in the threshold of K_A channel activation. The only observed change was a significant reduction in peak $I_{K(A)}$ amplitude which occurred at all test potentials again indicating the absence of any voltage-dependent block of K_A channels by P-CTX-1 (Fig. 4G).

DISCUSSION

The present study shows that, under current-clamp conditions, purified P-CTX-1 induces a concentration-dependent membrane depolarisation in rat sensory neurones expressing predominantly TTX-sensitive Na_v channels. This membrane depolarisation, combined with an

ability to prolong spike and afterhyperpolarisation duration, and the previously observed action to cause a hyperpolarising shift in the activation of Na_v channels, all act to promote tonic firing of action potentials in DRG neurons. These results clearly show for the first time that P-CTX-1 significantly alters neuronal excitability in mammalian sensory neurones that underlie the paraesthesiae and dysesthesiae observed clinically in ciguatera poisoning. DRG neurons, or their axons, have been previously identified to be sites of ectopic impulse generation leading to these types of spontaneous or altered sensations (Wall and Devor, 1983; Rizzo *et al.*, 1996). We also report that nanomolar concentrations of P-CTX-1 inhibit K_v channels in DRG neurons, an action that underlies changes in spike and afterhyperpolarisation duration and contributes to tonic firing of action potentials.

The capacity of ciguatoxins to induce membrane depolarisation in many excitable cells is commonly thought to be due to the ability of ciguatoxins to increase Na^+ influx through Na_v channels. In the present study we have confirmed that P-CTX-1 also causes membrane depolarisation in rat somatosensory neurons. This action appears to be mediated via Na_v channels given that the resting membrane depolarisation was blocked by the addition of TTX. In support, we have previously shown that P-CTX-1 precipitates a large increase in the leakage current in whole-cell voltage-clamp experiments on rat DRG neurons, an action that was reversed upon the addition of TTX. This indicates that the rise in leakage current is mediated via TTX-sensitive Na_v channels. This P-CTX-1-induced leakage current is most likely due to the spontaneous opening of a sub-population of Na_v channels at resting and hyperpolarised membrane potentials that remain in a permanent open state (Hogg *et al.*, 1998) and no doubt underlies the marked membrane depolarisation observed in the present study.

The ability of ciguatoxins to cause repetitive action potential firing in many cell types is commonly thought to be due to their ability to shift the voltage dependence of Na_v channel activation to potentials closer to the resting membrane potential (Bidard *et al.*, 1984; Seino *et al.*, 1988; Molgó *et al.*, 1990; Brock *et al.*, 1995; Hamblin *et al.*, 1995; Strachan *et al.*, 1999; Hogg *et al.*, 2002). This action, in combination with ongoing membrane depolarisation and in some neurons a marked oscillation in the membrane potential (Hogg *et al.*, 1998; Hogg *et al.*, 2002), triggers spontaneous action potential firing. Unlike previous studies in rat parasympathetic neurons where membrane oscillations were in the range of 10-25 mV (Hogg *et al.*, 2002), oscillations observed in the present study were subthreshold and only between 0.5-5 mV in amplitude. These oscillations may contribute to repetitive firing of action potentials but is unlikely to be important unless the

membrane potential is close to the threshold of Na_v channel activation. It is also interesting to note that in the present study spontaneous repetitive firing only occurred in 25% of neurons undergoing membrane depolarisation and occurred in the absence of significant membrane potential oscillations. This differential sensitivity of individual DRG neurons to P-CTX-1 is similar to that reported for P-CTX-4B isolated from the dinoflagellate *Gambierdiscus toxicus* in frog myelinated nerve (Benoit and Legrand, 1994) and guinea-pig sympathetic ganglia (Hamblin *et al.*, 1995). In the case of DRG neurons, it may represent differences in the action of P-CTX-1 on distinct subtypes of Na_v channels that are differentially expressed in these neurons (see (Goldin, 2001) for details of nomenclature). Given that P-CTX-1 failed to alter neuronal excitability in current-clamp recordings from small diameter DRG neurons expressing TTX-resistant Na_v channels, it would appear that the $Na_v1.8$ (PN3/SNS) and $Na_v1.9$ (NaN/SNS2) channel subtypes (Sangameswaran *et al.*, 1996; Dib-Hajj *et al.*, 1998) are not involved to any appreciable extent. Nevertheless, one or several of the TTX-sensitive Na_v subtypes such as $Na_v1.1$ (rat I), $Na_v1.6$ (NaCh6/PN4) and $Na_v1.7$ (PN1) that have been shown to be highly expressed in larger diameter DRG neurons (Beckh, 1990; Toledo-Aral *et al.*, 1997; Caldwell *et al.*, 2000), or even $Na_v1.2$ (rat II) which is expressed at low levels (Felts *et al.*, 1997), may be involved.

An unexpected action of P-CTX-1 was that it caused a significant increase in action potential and afterhyperpolarisation duration. This is in contrast to previous findings in frog myelinated nerves, whereby action potential duration was not significantly modified by ciguatoxin at concentrations up to 1.25 nM. However a prolongation of action potential duration has been previously reported with P-CTX-4B and C-CTX-1 on single frog myelinated axons. This increase in spike duration suggests that P-CTX-1 may either slow the inactivation of Na_v channels or may block K_v channels. Given that a previous study has shown that P-CTX-1 does not alter the kinetics of I_{Na} inactivation in DRG neurons (Strachan *et al.*, 1999), we determined if P-CTX-1 modulated the gating or kinetics of K_v channels.

Voltage-gated potassium channels are regulators of neuronal excitability, opening in response to depolarising membrane potentials allowing outward flux of K^+ to repolarise the neuron (Hille, 2001). The modulation of potassium channel gating by classical organic K_v blockers such as TEA and 4-AP can profoundly affect the resting membrane potential, spike amplitude and duration, and the afterhyperpolarisation amplitude and duration in DRG neurons (Amir *et al.*, 2002). The dominant outward K_v current in DRG neurons is a delayed-rectifier (K_{DR}) whose kinetic and pharmacological properties are consistent with expression of $K_v1.1/K_v1.2/Kv\beta2.1$ channels

(Fedulova *et al.*, 1998; Rasband *et al.*, 2001). Previous studies have shown that block of K_{DR} channels in DRG neurons using TEA, depolarized the cell membrane, prolonged action potential duration, reduced the amplitude of afterhyperpolarisations and lowered the threshold for action potential firing (Safronov *et al.*, 1996). The present study has shown that nanomolar concentrations of purified P-CTX-1 also block $I_{K(DR)}$ and produces similar changes in neuronal excitability to those described above. Thus the block of K_{DR} channels would most likely contribute to membrane depolarisation previously identified to be due to the permanent activation of a sub-population of Na_v channels (Hogg *et al.*, 1998; Strachan *et al.*, 1999). In addition it would also contribute to a lowering of action potential threshold in concert with the hyperpolarising shift in the voltage dependence of TTX-sensitive Na_v channel activation (Strachan *et al.*, 1999).

Although the majority of K_v channels present in DRG neurones are of the delayed-rectifier K_v subtype, transient 'A-type' K_v channels also play a vital role. Their function is to dampen developing interspike depolarisations to space successive action potentials (Hille, 2001). Electrophysiological and immunochemical studies suggest that, in large diameter DRG neurons, K_A channels are heterotetrameric combinations most likely expressing either $K_v1.4$ or $K_v4.2$ (Baldwin *et al.*, 1991; Fedulova *et al.*, 1998; Rasband *et al.*, 2001). The $I_{K(A)}$ seen in DRG neurons has similar properties to the slowly-inactivating $I_{K(A)}$ found in nodose and hippocampal neurons which have previously been shown to play an important role in limiting firing frequency (McFarlane and Cooper, 1991; Klee *et al.*, 1995). Inhibition of this current can induce profound repetitive firing of action potentials (Stansfeld *et al.*, 1986; Storm, 1988). Agents that block $I_{K(A)}$ such as 4-AP (Safronov *et al.*, 1996) have also been shown to induce repetitive firing in rat dorsal root fibres (Baker *et al.*, 1985) and DRG neurons (Amir *et al.*, 2002). Importantly, at concentrations that profoundly affect spike electrogenesis and Na_v channel gating, P-CTX-1 produced a significant inhibition of $I_{K(A)}$. This promotes a faster firing frequency by speeding the rate of interspike membrane potential depolarisation .

Interestingly the spike amplitude was unaffected by concentrations of P-CTX-1 up to 20 nM, despite a block of K_{DR} channels. Previous studies in rat DRG neurons have shown that blockers of K_{DR} channels cause an increase in spike amplitude (Amir *et al.*, 2002). This apparent inconsistency can be reconciled by the fact that in voltage clamp experiments P-CTX-1 has previously been shown to reduce TTX-sensitive I_{Na} in these rat DRG neurons (Strachan *et al.*, 1999), an action that would decrease the action potential amplitude. These competing actions on distinct voltage-gated ion channels involved in action potential electrogenesis would therefore tend

to oppose one another, leaving spike amplitude unaltered.

A variety of small-, large- and possibly intermediate-conductance calcium-activated potassium (K_{Ca}) channels are also present in DRG neurons (Gold *et al.*, 1996; Scholz *et al.*, 1998; Abdulla and Smith, 2001; Boettger *et al.*, 2002). Here they function to increase the afterhyperpolarisation and prevent repetitive firing in small diameter DRG neurons (Scholz *et al.*, 1998). Indeed block of the large-conductance $I_{K(Ca)}$ by iberiotoxin has been shown to prolong action potential duration, reduce the amplitude of the afterhyperpolarisation and cause repetitive firing during long depolarisations (Scholz *et al.*, 1998). Given that P-CTX-1 causes a maintained depolarisation of the resting membrane potential (Table 1), K_{Ca} channels may be involved in shaping the action potential discharge patterns seen during P-CTX-1 exposure (see Fig. 2F). Unfortunately the effects of P-CTX-1 on $I_{K(Ca)}$ could not be determined in the present study for two reasons (i) $I_{K(Ca)}$ could not be isolated from $I_{K(DR)}$ using the standard K_{DR} channel blocker TEA since it is an effective blocker of K_{Ca} channels in DRG neurons, with an EC_{50} of 0.4 mM (Scholz *et al.*, 1998), and (ii) $I_{K(Ca)}$ could not be isolated using current subtraction procedures since P-CTX-1 also blocks $I_{K(DR)}$ (Fig. 3). Future investigations should address the possibility of P-CTX-1 modulating $I_{K(Ca)}$ using single channel patch clamp techniques.

In conclusion, this is the first study to find that P-CTX-1 causes a significant block of K_v channels with IC_{50} values around 20 nM. Given that K_{DR} and K_A channels play multiple roles in the excitability of DRG neurons block of these channels by P-CTX-1 influences the shape of the action potential, its firing threshold and the resting membrane potential. The block of K_{DR} and K_A channels act in concert with permanent activation of a sub-population of Na_v channels to depolarise the resting membrane potential and a hyperpolarising shift in the threshold of TTX-sensitive Na_v channel activation (Strachan *et al.*, 1999), as the underlying determinants for the spontaneous action potential firing induced by P-CTX-1 in sensory neurons. These actions on neuronal excitability provide us with further understanding of the predominant paraesthesiae, dysesthesiae and other neurological symptoms associated with ciguatera poisoning in the Pacific region.

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Table 1

Effects of P-CTX-1 on neuronal excitability in DRG neurones

P-CTX-1 Concentration	E_m (mV)	AP Amplitude (mV)	AP $Duration_0$ (ms)	AP $Duration_{50}$ (ms)	AHP Amplitude (mV)	AHP $Duration_{50}$ (ms)
Control (n = 22)	**-54.7 ± 1.1**	**115.5 ± 4.5**	**1.5 ± 0.1**	**1.3 ± 0.1**	**11.2 ± 0.6**	**7.2 ± 1.8**
2 nM (n = 10)	**-51.4 ± 1.7**	**117.3 ± 10.5**	**3.8 ± 0.4***	**3.6 ± 0.3***	**12.4 ± 1.4**	**11.9 ± 0.9***
5 nM (n = 9)	**-47.2 ± 1.7***	**113.2 ± 11.1**	**4.3 ± 0.3***	**4.2 ± 0.3***	**11.7 ± 1.5**	**12.7 ± 1.4***
10 nM (n = 13)	**-47.2 ± 1.7***					

E_m, membrane potential; AP, action potential; AHP; afterhyperpolarisation; $Duration_0$, duration at 0 mV; $Duration_{50}$, duration at 50% of maximal amplitude. * $P<0.05$ using Tukey's HSD post-hoc test

FIGURES

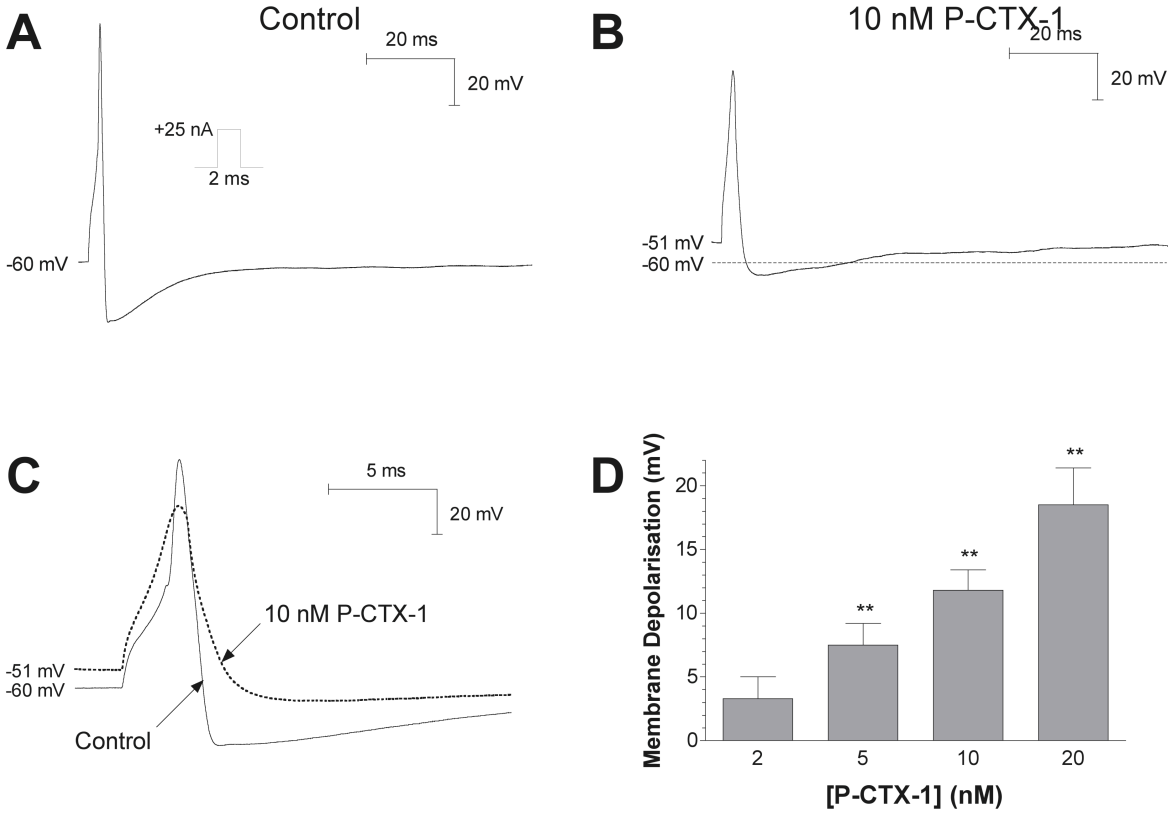

Fig. 1. Effect of P-CTX-1 on action and resting potentials in DRG neurons expressing TTX-sensitive Na_v channels. Typical action potentials generated by a single current pulse (see *inset*), before (A) and following a 10-min perfusion with 10 nM P-CTX-1 (B). The dotted line in panel B represents the control resting membrane potential and highlights the depolarisation induced by 10 nM P-CTX-1. (C) Superimposed voltage traces from panels A and B showing reduced spike and afterhyperpolarisation amplitude, prolongation of action potential and afterhyperpolarisation duration, and membrane depolarisation. (D) Concentration-dependent membrane depolarisation occurred in response to a 5-10 min perfusion with P-CTX-1 ($n \geq 5$). Statistical significance indicated at the ** $P < 0.01$ level.

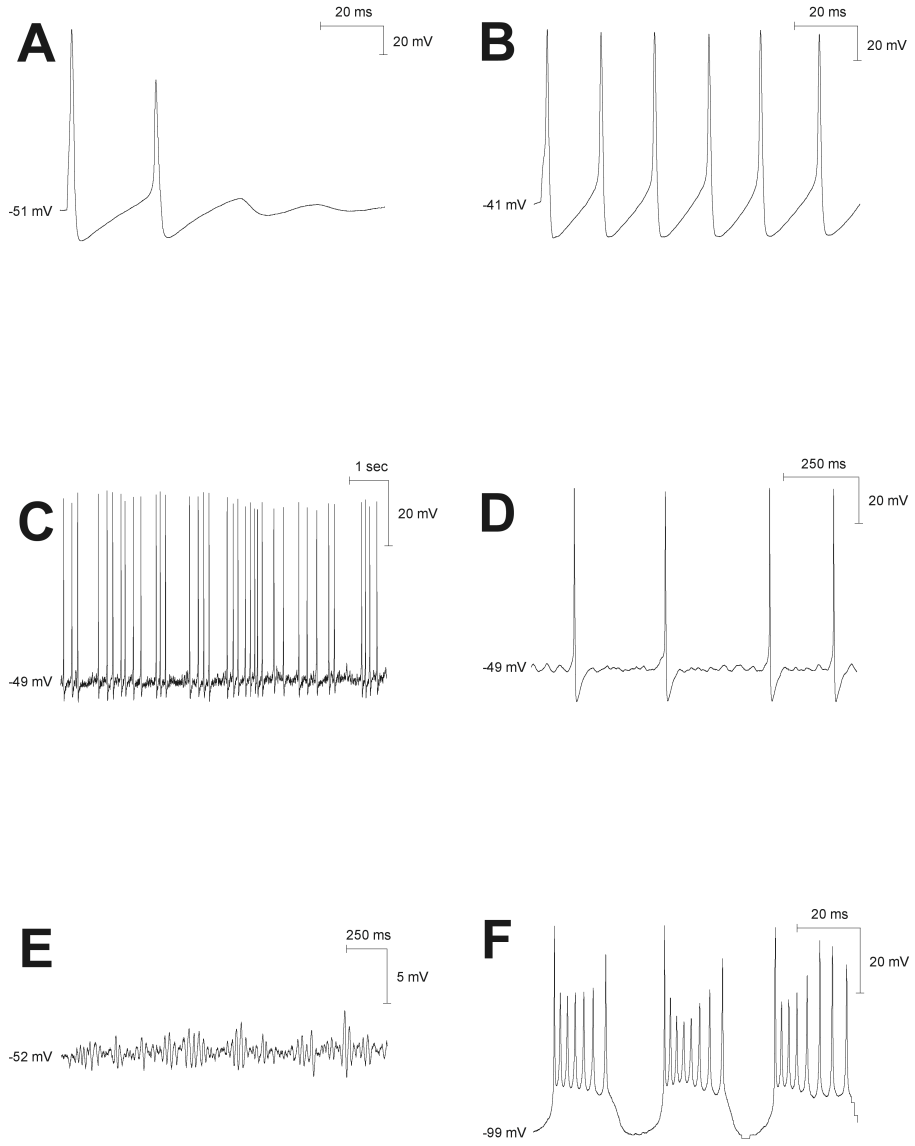

Fig. 2. Actions of P-CTX-1 to cause repetitive action potential firing in DRG neurons expressing TTX-sensitive Na_v channels. (A-B) Typical progression of stimulus-locked repetitive action potential firing from limited repetitive firing (A), to trains of action potentials (B), in the presence of 10 nM P-CTX-1. (C-D) Typical tonic action potential firing in the presence of 10 nM P-CTX-1, where spontaneous firing was not locked to a depolarising stimulus. Note the slow time scale in panel C. (E) Typical example of the slight oscillation in the resting membrane potential in the presence of 10 nM P-CTX-1. (F) Return of repetitive firing following manual hyperpolarisation of the resting membrane potential.

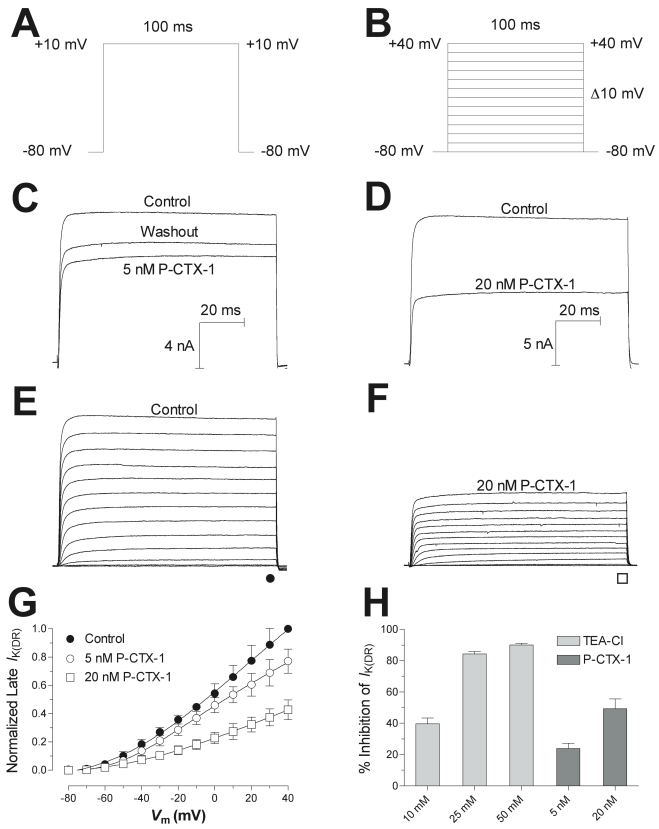

Fig. 3. Actions of P-CTX-1 on delayed-rectifier potassium currents ($I_{K(DR)}$) in rat DRG neurons. (A-B) Depolarising voltage command protocols used to elicit outward $I_{K(DR)}$. $I_{K(DR)}$ were elicited using 100-ms depolarising test pulses (V_{test}) to +10 mV applied from a holding potential of -80 mV every 5 seconds (A). Families of $I_{K(DR)}$ were elicited by 100-ms depolarising V_{test} from -80 mV to +40 mV in 10-mV steps (B). (C-D) Representative superimposed current traces before, and following, a 20-min perfusion with 5 nM (C) and 20 nM (D) P-CTX-1. Currents were elicited using the voltage command protocol shown in panel A. Note the partial reversal of current amplitude following perfusion with toxin-free external solution in panel C. (E-G) Effect of P-CTX-1 on $I_{K(DR)}/V$ relationships. Typical families of control currents (E) were recorded prior to a 20-min perfusion with 20 nM P-CTX-1 (F). Calibration for panels E and F is the same as panel D. (G) Normalised maintained $I_{K(DR)}/V$ relationship recorded at the end of the depolarising test pulse before (●), and following, perfusion with 5 nM (○, n = 5) and 20 nM (□, n = 4) P-CTX-1. Families of currents were elicited using the voltage command protocol shown in panel B. Data were fitted with equation 1 in the materials and methods. (H) Comparison of the block of $I_{K(DR)}$ by TEA-Cl and P-CTX-1. Data represents the mean ± SEM of $n \geq 4$. All $I_{K(DR)}$ data were recorded in the presence of 5 mM 4-AP to block any residual $I_{K(A)}$.

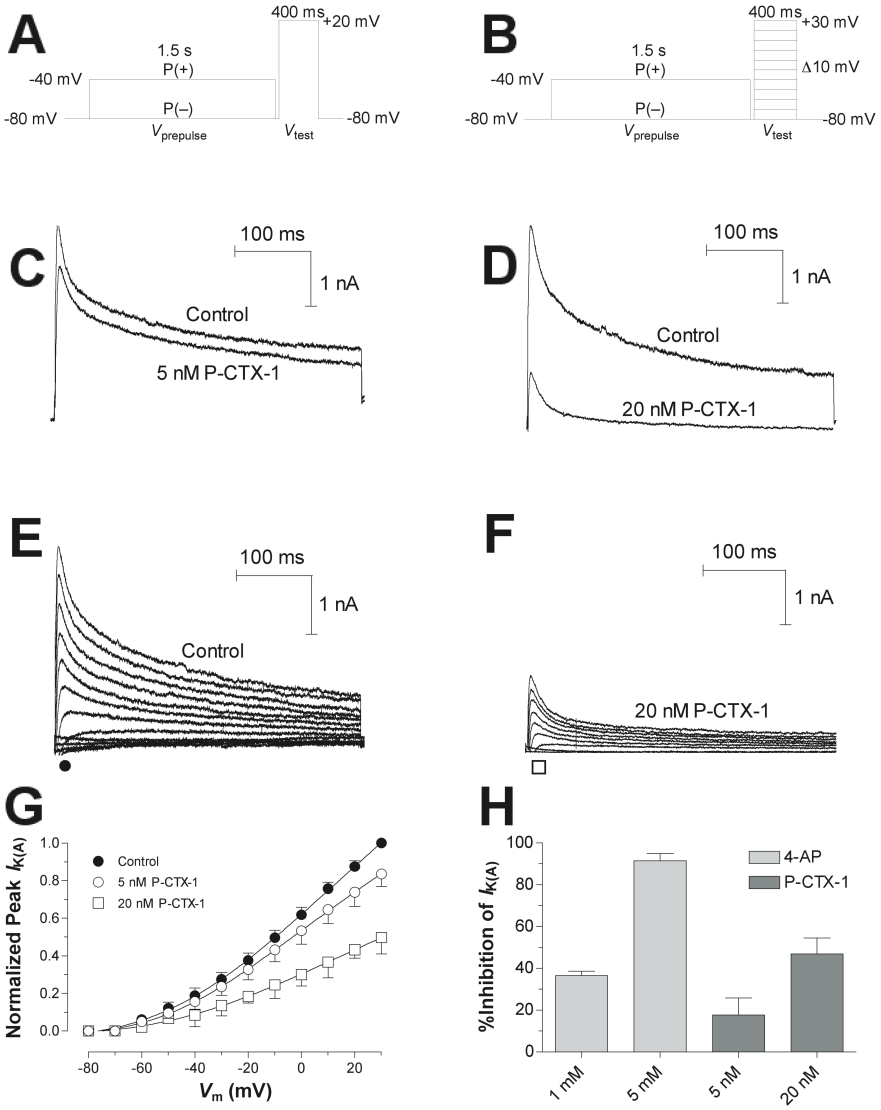

Fig. 4. Actions of P-CTX-1 on A-type potassium currents ($I_{K(A)}$) in rat DRG neurons. (A-B) Depolarising voltage command protocols used to elicit outward $I_{K(A)}$. (A) $I_{K(A)}$ were evoked by 400-ms depolarising test pulses (V_{test}) to +20 mV applied from a holding potential of –80 mV ($P_{(–)}$) or applied subsequent to a 1.5-second hyperpolarising prepulse ($V_{prepulses}$) to –40 mV ($P_{(+)}$). $I_{K(A)}$ was obtained by subtracting the current produced by $P_{(+)}$ from the current produced by $P_{(–)}$. (B) Families of $I_{K(DR)}$ were also isolated by subtraction except that V_{test} was varied from -80 mV to +30 mV in 10-mV steps. (C-D) Representative superimposed current traces before, and following, a 20-min perfusion with 5 nM (C) and 20 nM (D) P-CTX-1. Currents were elicited using the voltage command protocol shown in panel A. (E-G) Effect of P-CTX-1 on $I_{K(A)}/V$ relationships. Typical families of control currents (E) were recorded prior to a 20-min perfusion with 20 nM P-CTX-1 (F). (G) Normalised peak $I_{K(A)}/V$ relationship recorded before (●), and following, perfusion with 5 nM

(○, n = 5) and 20 nM (□, n = 4) P-CTX-1. Families of currents were elicited using the voltage command protocol shown in panel B. Data were fitted with equation 1 in the materials and methods. (H) Comparison of the block of $I_{K(A)}$ by 4-AP and P-CTX-1. Data represents the mean ± SEM of $n \geq 4$. All $I_{K(A)}$ data were recorded in the presence of 25 mM TEA-Cl to eliminate any residual $I_{K(DR)}$.