The poison Dart frog's batrachotoxin modulates $Na_v1.8$

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Abstract Batrachotoxin is a potent modulator of voltage-gated sodium channels, leading to irreversible depolarisation of nerves and muscles, fibrillation, arrhythmias and eventually cardiac failure. Since its discovery, field researchers also reported numbness after their skin came into contact with this toxin. Intrigued by this phenomenon, we determined the effect of batrachotoxin on the voltage-gated sodium channel $Na_v1.8$, which is considered to be a key player in nociception. As a result, we discovered that batrachotoxin profoundly modulates this channel: the inactivation process is severely altered, the voltage-dependence of activation is shifted towards more hyperpolarised potentials resulting in the opening of $Na_v1.8$ at more negative membrane potentials and the ion selectivity is modified.

Keywords: Pain; Voltage-gated Na^+ channel; $Na_v1.8$; Batrachotoxin

1. Introduction

Poison Dart frogs (*Phyllobates* spp.) are often strikingly coloured anurans, which excrete several alkaloid toxins from their skin [1] (Fig. 1). Among these excretions is one of the most potent toxins known in nature, batrachotoxin (BTX) [2]. Close relatives like homobatrachotoxin occur in the skin and feathers of three passerine bird species of the genus *Pitohui* and the *Ifrita kowaldi* bird endemic to New Guinea (Fig. 1) [3,4]. These toxins are potent modulators of voltage-gated sodium channels (VGSCs), which orchestrate the generation of action potentials and are key elements in the signal transduction process of membranes of neurons and most excitable cells [5–7]. VGSC modulation by BTX causes an irreversible depolarisation of nerves and muscles, fibrillation, arrhythmias and eventually cardiac failure [8]. Aside from the aforementioned effects, BTX causes a feeling of numbness upon contact with human tissue, an effect that remains unexplained [9].

Several reports on native cells and cloned channels have been published showing that BTX drastically modifies the gating of VGSCs: this agonist shifts the voltage dependence of activation to more hyperpolarised voltages. In addition, both fast and slow inactivation processes of the channel are inhibited. Aside from these effects, the ion selectivity is reduced. BTX-modified VGSCs display increased permeability for NH_4^+, K^+ and Cs^+ ions [10–17].

In the literature, there is evidence that a specific VGSC plays an important role in nociception, namely $Na_v1.8$ (Scn10A). $Na_v1.8$ is expressed in peripheral neurons such as the small diameter nociceptor neurons of dorsal root ganglia (DRG) that originate in afferent nociceptive fibres such as the C-fibres and Aδ fibres [18–22]. Behavioural studies on $Na_v1.8$ null mutant mice have shown that $Na_v1.8$ plays an important role in the detection of noxious stimuli [23]. Until now, this challenging tetrodotoxin (TTX)-resistant VGSC still largely remains *terra incognita* since there are only a few, not very potent or selective modulators of this VGSC described [24–26]. For instance, widely used local anaesthetics and class I anti-arrhythmics, like lidocaine and tetracaine, block $Na_v1.8$ [27,28]. The synthetic vincamine derivative, vinpocetine, produces a concentration- and state-dependent inhibition of $Na_v1.8$ channels expressed in a DRG-derived cell line [26]. Recently, John and co-workers reported that the novel anticonvulsant drug lamotrigine and a range of structural analogues block both $Na_v1.8$ and other TTX-resistant VGSCs [29]. A comparative study of the action of tolperison on different VGSCs has revealed that this organic drug preferentially blocks $Na_v1.8$ at high micromolar concentrations [30].

Here, we wanted to elucidate the analgesic effect (numbness) of BTX and, therefore, studied its effect on $Na_v1.8$ co-expressed with the β_1 subunit ($Na_v1.8/\beta_1$) in *Xenopus laevis* oocytes. In addition, since there are only a few modulators known for this VGSC, it is an interesting target to screen for novel ligands.

2. Materials and methods

For this study, capped cRNAs were synthesised from the linearised plasmids, $Na_v1.8$/pSP64T and β_1/pSP64T, and injected into oocytes harvested from anaesthetised female *Xenopus laevis* frogs according to established procedures [31–33]. Oocytes were injected with 50 nl of cRNA at a concentration of 1 ng nl^{-1}. The solution used for incubating the oocytes contained (in mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$ and 5 HEPES (pH 7.4), supplemented with 50 mg l^{-1} gentamycin sulfate and 180 mg l^{-1} theophyllin.

Two-electrode voltage-clamp (TEVC) recordings were performed at room temperature (18–22 °C) using a GeneClamp 500 amplifier controlled by a pClamp data acquisition system (Axon instruments, USA). Whole-cell currents from oocytes were recorded 4 days after injection. Currents were sampled at 10 kHz and filtered at 2 kHz using a four-pole low-pass Bessel filter. Leak subtraction was performed using a P/4

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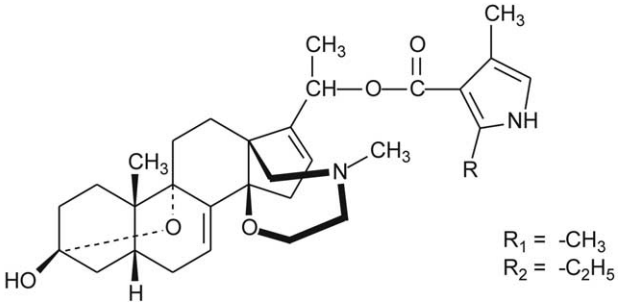

Fig. 1. Left panel: *Phyllobates terribilis*; right panel: *Pitohui dichrous*; lower panel: structure of batrachotoxin (R_1) or homobatrachotoxin (R_2).

protocol. The voltage protocols are shown in the figures. All of the measurements were performed in control conditions and in the presence of BTX at concentrations ranging from 500 nM to 10 μM. The displayed data in Fig. 2 for toxin conditions were recorded after a 900 pulse train experiment at a frequency of 1 Hz.

Fig. 2. (A) Representative current traces in response to the voltage protocol (inset). Displayed data in the presence of BTX were recorded after a 900 pulse train experiment, each lasting 2 s using a frequency of 1 Hz. From a holding potential of −90 mV, a 50 ms pulse was given to +30 mV (part I) corresponding to the maximum Na^+ influx of the unaffected $Na_v1.8/\beta_1$. Next, a 50 ms pulse to −10 mV (part II) evoked a transient deactivation response. After 50 ms at +30 mV in the presence of BTX, the open channel probability is very high caused by the inhibition of the inactivation process. As a consequence, the jump to −10 mV causes an initial increase of inward Na^+ current due to the increased driving force (this is not instantaneous due to the sluggishness of TEVC when measuring oocytes). Next, $Na_v1.8/\beta_1$ starts to deactivate at −10 mV which is rather slow and which causes the current decay over the next 25 ms. The ensuing jump to −120 mV (Part III) reveals a faster and complete deactivation. In part IV, a pulse is given to −20 mV in order to reveal a shift in the current–voltage relationship (*I–V* curve). In control conditions, $Na_v1.8/\beta_1$ is not yet activated at −20 mV so there is no current visible. In the presence of BTX, Na^+ ions are passing through even at −20 mV. (B) Corresponding current–voltage relationship (left panel) before (■) and after addition of 5 μM (●) and 10 μM (▲) BTX. Current traces were evoked using 50 ms step depolarisations of 10 mV to a voltage range between −70 and 70 mV from a holding potential of −90 mV. The arrow indicates the shift in E_{rev}. (C) Superimposed graphics of the corresponding activation and steady-state inactivation curves. Current traces for the inactivation protocol were evoked by 50 ms depolarisations from −120 mV to 0 mV followed by a 50 ms pulse to +30 mV, from a holding potential of −90 mV. As indicated by the arrows, BTX clearly shifts the activation curve (horizontal arrow) to more negative potentials and inhibits the inactivation process (vertical arrow).

Curve manipulations were performed using pClamp8 (Axon Instruments) and Origin software (Microcal, USA). Voltage-dependence of activation was determined from the *I–V* curves and the reversal potential was measured experimentally for each oocyte. The voltage dependence of relative current (activation and fast inactivation) was fit by a Boltzmann function.

BTX was kindly provided by John Daly (N.I.H., USA). The molecular weight was checked on a LCQ Deca XP iontrap mass spectrometer (Finnigan Corp., USA).

3. Results and discussion

BTX radically and irreversibly affects $Na_v1.8/\beta_1$. Fig. 2A shows representative current traces in response to voltage steps. Parts I and IV clearly show that $Na_v1.8/\beta_1$ does not inactivate in the presence of 10 μM BTX, illustrating that the inactivation process is severely altered. Fig. 2B shows the corresponding current–voltage (*I–V*) relation and Fig. 2C displays the steady-state activation and inactivation curves. The voltage-dependence of activation is shifted towards more hyperpolarised potentials, resulting in the opening of $Na_v1.8/$

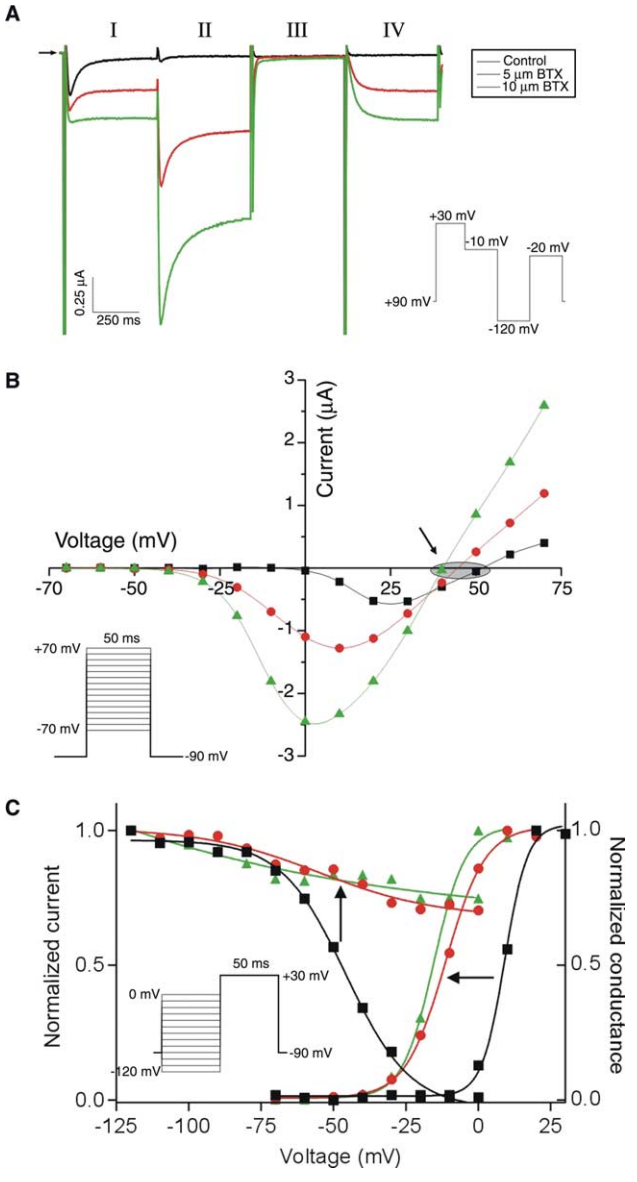

Table 1
$Na_v1.8$ gating behaviour in control conditions and in the presence of BTX

	E_{rev} (mV)	$V_{1/2}$ (mV)	k	AUC (%)
Control	57.3 ± 2.9	2.9 ± 5.1	5.1 ± 0.4	100
+5 μM BTX	52.4 ± 4.7	-9.1 ± 7.7	9.6 ± 1.1	218 ± 28
+ 10 μM BTX	46.2 ± 2.9	-11.7 ± 4.9	10.4 ± 1.3	427 ± 105

Pooled data from 4–6 oocytes. AUC, area under the curve and k, slope factor.

β_1 at more negative membrane potentials (Fig. 2C). The voltage of half-maximal activation ($V_{1/2}$) shifted towards more hyperpolarised voltages with ±12.0 mV when 5 μM BTX was applied ($n = 6$) and with ±14.8 mV in the presence of 10 μM BTX ($n = 4$) (Table 1). As can be seen in part IV of Fig. 2A, $Na_v1.8/\beta_1$ activates at −20 mV under the influence of BTX, whereas in control conditions the channels are still closed. As a result of the total loss of inactivation and the leftward shift of the activation curve, the Na^+ ion influx increases dramatically. A clear marker to demonstrate this is the area under the I–V curve (AUC) (Fig. 2B). Compared to the control situation, the AUC increases with ±427% when 10 μM BTX is present ($n = 4$, Table 1). BTX also changes the ion selectivity as indicated by the shift in the reversal potentials (E_{rev}) (Fig. 2B). An average hyperpolarising shift of 5.0 mV is present when 5 μM BTX is added ($n = 6$) and a shift of 11.1 mV was noted in the presence of 10 μM BTX ($n = 4$) (Table 1). So both the gating and selectivity of $Na_v1.8$ are affected by BTX, with the effect on inactivation being the most drastic. Lower concentrations were also tested and we observed that 1 μM of BTX, measured after a 900 pulse train experiment at a frequency of 1 Hz, already starts to affect $Na_v1.8/\beta_1$. At this concentration, a small initial block of the current is seen, which is soon followed by an influx of Na^+ ions caused by the inhibition of the inactivation. In addition, the activation is clearly shifted to more hyperpolarised voltages. In order to simulate neurons in living organisms, which have a higher firing rate than 1 Hz (e.g., 20–50 Hz), we also tested higher frequency pulse trains. At 10 Hz, we noticed that lower concentrations (500 nM) cause effects that are related to higher concentrations at 1 Hz (data not shown). Hence, we believe that in vivo the effects of BTX may be as catastrophic at even lower concentrations.

Now, how can we tie these results to an analgesic effect? A general idea about an ideal painkiller is a compound that blocks $Na_v1.8$ in a use-dependent way (e.g., lidocaine). Here, we discuss how a compound like BTX, which does not simply block but profoundly modulates $Na_v1.8$, can also cause an analgesic effect. It is known from disease-associated mutations that alterations in either a channel's selectivity or gating alter the electrical excitability of the cell. In practice, functional studies of mutant channels have shown that the primary defect is usually an alteration in gating and that for sodium channelopathies the gating defects tend to increase the likelihood of mutant Na^+ channels being open. Such gating defect will depolarise the cells, which may either cause hyperexcitability (as in myotonia) by triggering spurious action potentials or chronically depolarise the cell to a refractory unexcitable state (as in paralysis). Computer simulations in a model cell demonstrated that 2% of non-inactivating Na^+ channels cause a myotonic response, however, a slightly larger proportion of non-inactivating channels (3% or more) results in paralysis [34]. BTX causes a dose-dependent increase in the amplitude of TTX-resistant Na^+ currents ($Na_v1.8/\beta_1$), accompanied by a left-ward shift in the voltage-dependence of activation. Furthermore, BTX causes an irreversible dramatic decrease in inactivation and deactivation rate of $Na_v1.8/\beta_1$ (see Fig. 2, panel II). The resulting persistent inward Na^+ current will lead to a sustained depolarisation of the cell membrane in vivo. As a consequence, the remaining VGSCs that were not affected by BTX will be trapped in the inactivated state, resulting in the loss of electrical excitability of nociceptor neurons.

To confirm this analgesic effect of BTX, a preliminary small scale clinical experiment was conducted in our laboratory. In a blind study, three volunteers applied an ointment containing BTX and a placebo on their hands. This was repeated once with two different concentrations (5 and 25 μg per 100 g of ointment), which are far below the estimated lethal blood concentrations (approximately 5–200 μg per adult). An initial tingling sensation followed by a feeling of numbness was clearly noticed when the ointment with BTX was applied. The effect lasted for over 30 min, was reversible and did not cause any side-effects.

In conclusion, BTX, the first profound modulator of $Na_v1.8$, causes nociceptive neurons to go into arrest, resulting in the inhibition of action potential trains. We believe that this is the main reason for the feeling of numbness when human tissue comes into contact with BTX. We think that this trademark makes BTX eligible for exploitation in topical applications in order to inhibit the propagation of pain stimuli.

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