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Kirchhof, Paulus; Tal, Tzachy; Fabritz, Larissa; Klimas, Jan; Nesher, Nir; Schulte, Jan S; Ehling, Petra; Kanyshkova, Tatayana; Budde, Thomas; Nikol, Sigrid; Fortmueller, Lisa; Stallmeyer, Birgit; Müller, Frank U; Schulze-Bahr, Eric; Schmitz, Wilhelm; Zlotkin, Eliahu; Kirchhefer, Uwe

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First report on an inotropic peptide activating TTX-sensitive, "neuronal" sodium currents in the heart

Paulus Kirchhof MD[1,2,a], Tzachy Tal PhD[3,4,a], Larissa Fabritz MD[1,2], Jan Klimas PhD[5,6], Nir Nesher PhD[4], Jan S. Schulte MD[6], Petra Ehling PhD[7,8], Tatayana Kanyshkova[7], Thomas Budde PhD[7], Sigrid Nikol MD[1,9], Lisa Fortmueller PhD[1], Birgit Stallmeyer PhD[10], Frank U. Müller MD[6], Eric Schulze-Bahr MD[10], Wilhelm Schmitz MD[6], Eliahu Zlotkin PhD[4,b,+], Uwe Kirchhefer MD[6,b]

1. Department of Cardiovascular Medicine, Hospital of the University of Muenster, Muenster, Germany
2. University of Birmingham Center for Cardiovascular Sciences, School of Clinical and Experimental Medicine, and SWBH NHS Trust, Birmingham, United Kingdom
3. Technion Israel Institute of Technology, Haifa, Israel
4. Department of Animal and Cell Biology, Institute of Life Sciences, The Hebrew University of Jerusalem, Israel
5. Department of Pharmacology and Toxicology, Faculty of Pharmacy, Comenius University in Bratislava, Bratislava, Slovak Republic
6. Department of Pharmacology, Hospital of the University of Muenster, Germany
7. Institute of Physiology, Division of Neuropathophysiology, and Department of Neurology, University of Muenster, Germany
8. Institute of Physiology I, University of Muenster, Germany
9. Asklepios Hospital Hamburg St. Georg, Germany
10. Institute for Genetics of Heart Disease (IfGH), Department of Cardiovascular Medicine, Hospital of the University of Muenster, Germany.

a shared first authorship
b shared senior authorship
+ deceased prior to submission

Short title: BjIP activates TTX-sensitive Na currents

Address for correspondence:
Prof. Dr. Paulus Kirchhof
University of Birmingham Centre for Cardiovascular Sciences
School of Clinical and Experimental Medicine and SWBH NHS trust
IBR, 1st Floor
Birmingham B15 2TT
England, UK
Phone: +44 121 4147042
Email: p.kirchhof@bham.ac.uk

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Abstract

Background. New therapeutic approaches to improve cardiac contractility without severe risk would improve the management of acute heart failure. Increasing systolic sodium influx can increase cardiac contractility, but most sodium channel activators have proarrhythmic effects that limit their clinical use. Here, we report the cardiac effects of a novel positive inotropic peptide isolated from the toxin of the Black Judean scorpion that activates "neuronal" TTX-sensitive sodium channels.

Methods and results. All venoms and peptides were isolated from Black Judean Scorpions (Buthotus Hottentotta) caught in the Judean Desert. The full scorpion venom increased left ventricular function in sedated mice in vivo, prolonged ventricular repolarization, and provoked ventricular arrhythmias. An inotropic peptide (BjIP) isolated from the full venom by chromatography increased cardiac contractility, but did neither provoke ventricular arrhythmias nor prolong cardiac repolarization. BjIP increased intracellular calcium in ventricular cardiomyocytes, and prolonged inactivation of the cardiac sodium current. Low concentrations of tetrodotoxin (200nM) abolished the effect of BjIP on calcium transients and sodium current. BjIP did not alter the function of $Nav_{1.5}$, but selectively activated the brain-type sodium channels $Nav_{1.6}$ or $Nav_{1.3}$ in cellular electrophysiological recordings obtained from rodent thalamic slices. $Nav_{1.3}$ (SCN3A) mRNA was detected in human and mouse heart tissue.

Conclusion. Our pilot experiments suggest that selective activation of TTX-sensitive neuronal sodium channels can safely increase cardiac contractility. As such, the peptide described here may become a lead compound for a new class of positive inotropic agents.

Key words. acute heart failure, sodium channel, inotropic agents, arrhythmia, pharmacotherapy

Introduction

Heart failure kills in equal proportions either by a critical loss of contractile cardiac function or by sudden due to ventricular arrhythmias [1-3]. Currently available inotropic agents (sympathomimetic agents, calcium sensitizers, or phosphodiesterase inhibitors) improve hemodynamic function at the price of potentially fatal proarrhythmic side effects and/or the deleterious consequences of chronic β-adrenoreceptor stimulation. Hence, inotropic therapy is confined to patients with severe, acutely decompensated heart failure treated on intensive care units [1, 2]. An inotropic therapy without these side effects would clearly be desirable in the management of acute heart failure.

Cardiac contraction is initiated by calcium release from the sarcoplasmic reticulum in cardiomyocytes. The trigger for calcium influx into the cell is an influx of sodium into the cardiomyocyte initiated through opening of voltage-gated sodium channels. Thereafter, opening of voltage-gated calcium channels, exchange of sodium and calcium via the sodium-calcium exchanger, and calcium-activated calcium release from the sarcoplasmic reticulum accentuate this initial stimulus. Voltage-gated sodium channels, an evolutionary well-preserved group of sarcolemmal transmembrane proteins, regulate voltage-dependent sodium influx and electrical excitability in neuronal and cardiac tissue [4]. Activators of cardiac sodium channels are known to exert positive inotropic effects, but also cause arrhythmias [5]. Cardiac sodium channels can be divided into TTX resistant (μM range) and TTX-sensitive (nM range) channel subtypes [4]. The main isoform of the TTX-resistant sodium channels $Nav_{1.5}$ is expressed predominantly in cardiomyocytes [4]. Selective increase in the opening kinetics of $Nav_{1.5}$ causes atrial fibrillation and heart failure [6, 7], and genetic variants in the $Nav_{1.5}$ channel are associated with defibrillator shocks in heart failure patients [8]. This may explain why activators of $Nav_{1.5}$ are not used in patients with heart failure, despite their

positive inotropic effects [9]. TTX-sensitive sodium channels, in contrast, are mainly expressed in mammalian brain and in skeletal muscle [4, 10], but also in T-tubules of cardiomyocytes close to calcium-releasing organelles [10, 11]. Selective activation of TTX-sensitive sodium channels could hence provide a leverage to improve cardiac contraction without interfering with the electrophysiological function of cardiomyocytes. This has, however, never been formally tested. Here, we report about a peptide isolated from the venom of the Black Judean Scorpion, BjIP, which confers positive inotropy without proarrhythmic effects. This beneficial combination of effects appears to be mediated by selective activation of neuronal TTX-sensitive sodium channels (most likely $Nav_{1.3}$ and/or $Nav_{1.6}$).

Methods

All methods are explained in detail in the on-line supplement.

Venom harvesting and isolation of the inotropic peptide BjIP. The full venom was harvested from *Hottentotta judaicus* scorpions. All venom and peptide fractions were harvested and prepared in Israel, and shipped to Muenster for functional and molecular analyses.

Molecular characterization of BjIP. The inotropic peptide was characterized by Matrix-Assisted Laser-Desorption Ionization-Time-Of-Flight Mass Spectrometry (MALDI-TOF MS). The N-terminal amino acid sequence (33 amino acids) was determined using Edman degradation. Total RNA isolated from the Black scorpion's venom gland was used for cDNA synthesis by the aid of SMART approach (Clontech, Park Tamar, Israel).

Real time quantitative PCR (qPCR) of Na^+ channels in heart tissue. Total RNA was prepared from freshly dissected tissue by extraction with Trizol® reagent (RNeasy Lipid Tissue, Qiagen, Düsseldorf, Germany). Further details in supplemental methods.

Dose selection for the full venom and the inotropic peptide. The limited access to BjIP required careful dose selection throughout the project. Preliminary experiments showed a positive inotropic effect of the full venom at 4µg/ml. Considering the expected weight contribution of BjIP to the protein content of the full venom (10%), we chose the concentrations given below for the initial functional assessment of the full venom and of BjIP (Table). Following the trabecular experiments demonstrating a positive inotropic effect of the BjIP at 1/10 of the initial concentration of BjIP, we reduced the concentration of BjIP for further cellular mechanistic experiments.

Murine echocardiography. Cardiac function was measured during anesthesia on HP5500 and Vevo770 ultrasound microscopy systems (Visualsonics, Canada, 50-70 MHz transducers) in adult three months old CD-1 wild type mice [12]. In brief, sedated animals were studied while spontaneously breathing. After establishing a stable physiological situation, left ventricular function was assessed in real-time by M mode echocardiography at baseline and after intraperitoneal injection of the full venom (40µg) or the inotropic peptide (BjIP, 2µg). Cardiac contraction was monitored for 20 minutes after injection.

Electrophysiological measurements in the isolated heart. Hearts of three months old wild type mice were isolated and retrogradely perfused (oxygenated modified Krebs-

Henseleit solution, 37°C temperature, pH 7.4) on a modified Langendorff apparatus. Monophasic action potentials were recorded from the right and left ventricles of beating hearts [12, 13]. The atrioventricular node was ablated to generate ventricular bradycardia. After a stabilization period of 10 minutes, spontaneous rhythm was observed, and ventricular pacing was performed at fix rates to assess ventricular action potential durations. The protocol was repeated after adding BjV or BjIP to the perfusate (Table).

Recording of sodium current (I_{Na}) in murine cardiomyocytes. Ventricular cardiomyocytes were isolated and prepared for patch clamp experiments as described [14]. Cells were exposed to BjIP (4 µg/ml) and TTX (200 nM) by a gravity driven fast application system (Warner, Holliston, USA). The inactivation time constant τ was derived from single exponential fits to the I_{Na} decay phase (Fitmaster, HEKA, Lambrecht, Germany). The total sodium influx was estimated by I_{Na} integration as the area under curve.

Cardiomyocytes shortening and calcium transient measurements. Cell shortening was measured in electrically driven (0.5 Hz) mouse ventricular cardiomyocytes. Cells were isolated and mounted in tissue chambers, containing a modified Tyrode's solution, for recording of cell shortening [15]. After a preequilibration period of 30 min, BjIP solution (0.4 µg/ml) was superfused to the cardiomyocytes for 5 min. As a control, 200 nM TTX was applied, followed by BjIP. Cell shortening, time to peak tension, and relaxation time (90%) were continuously monitored during the measurement. For calcium transient measurements, indo1 fluorescence ratio (Ca^{2+}) and cell shortening (Crescent Electronic Video Edge Motion Detector) were recorded simultaneously from isolated murine cardiomyocytes at room temperature during field stimulation at 0.5 Hz [16].

Single-cell electrophysiology in acute living brain slices. Brains were removed from decapitated rats and placed in an ice-cold, oxygenated solution. Acute living brain slices (280 µm thickness) containing the dorsal part of the lateral geniculate nucleus of the thalamus (dLGN) were cut on a vibratome (Leica VT1200, Wetzlar, Germany) and kept submerged in solution, and recording pipettes inserted into neuronal cells. Slices were continuously superfused with a bath solution. After establishment of the whole cell configuration, cells were superfused with a low Na^+-solution. Signals were amplified (EPC-10, HEKA) and digitally analyzed using Pulse. µ-Conotoxin PIIIA (µCtx, Alomone, Jerusalem, Israel), ProTX-II (ProTX, Alomone) which selectively inhibit distinct isoforms of voltage-dependent Na^+ channels, or TTX (Tocris, Bristol, UK) were added directly into the bath. Recordings were obtained at baseline and during BjIP superfusion in identical cells.

All functional experiments were approved by the Bezirksregierung in Münster, Germany, and conformed to the local and international regulation for experiments in animals.

Statistical analysis. We compared categorical data using Fishers exact test. Numerical data were compared by two sided paired t tests (e.g. measurements before and after perfusion of BjIP or BJCV), Wilcoxon signed-rank tests. For multiple measurements data were analyzed by repeated measures analysis of variance followed by a multiple comparison procedure (Bonferroni t-test) if the overall test was significant. For thalamic recordings a one-sided (expected increase for BjIP; expected decrease for commercial blockers) test was used. Data were considered significantly different at p values <0.05. When data are displayed as boxplots, boxes and box limits indicate the data range mean, and standard

error. Whiskers indicate the minimum and maximum of the respective data. Individual measurements are shown in the boxplots as points.

Results

BjIP has inotropic effects without prolonging cardiac repolarization or provoking arrhythmias. The complete venom of *Hottentotta judaicus* (BjV), when injected intra-peritoneally into anesthetized albino CD1 mice (0.7-1.0 mg/mouse n=5) did neither kill nor affect locomotion. BjV (0.2 mg/mouse i.p.) increased ventricular contractility by 20-25% and caused ventricular arrhythmias (Fig1 a,b). When perfused in isolated, beating mouse hearts, BjV provoked ventricular arrhythmias and prolonged the ventricular action potential (Fig 1c-f). Reversed-phase HPLC chromatography identified an inotropic peptide. Intraperitoneal application of 20 µg BjIP increased contraction of the murine heart *in vivo* to a similar extent as the full venom (Fig 1a,b). In contrast to the full venom, BjIP did neither provoke ventricular arrhythmias when administered intraperitoneally in vivo (Figure 1a) nor during perfusion in the isolated heart (Fig 1c,d). Furthermore, BjIP did not prolong action potential duration in the isolated, beating heart (4 µg /ml BjIP, Fig 1e-g).

The primary sequence and predicted tertiary structure of BjIP closely resemble scorpion venom long chain neurotoxins affecting the voltage-gated sodium channels. BjIP was sequenced and its tertiary structure modeled (Fig 2): The full sequence includes 63 amino acids of mature polypeptide, 19 amino acids of the signal peptide (red underline), 111 base pairs of untranslated nucleotides, and a polyadenylation site (black underline) – followed by 30 base pairs of poly A. Based on the molecular mass of the peptide, the last two amino acids of the mature peptide are cleaved (blue underline in Fig

2b). The modeled structure of BjIP resembles that of β-type insect toxins (Fig 2c,d), consistent with the transient contraction of *Sarcophaga falculata* blowfly larvae upon exposure to 20-50 ng BjIP /100 mg body weight (ED_{50} for flaccid paralysis 30-40ng/100mg body).

The positive inotropic effects of BjIP are TTX-sensitive. BjIP superfusion enhanced contractility in right ventricular mouse trabecles (Fig. 3a) and increased contractility and intracellular calcium transients in paced mouse ventricular cardiomyocytes (Fig 3b-e). Furthermore, BjIP delayed inactivation of the cardiac sodium current without affecting peak current amplitude (Fig 3f-i). Nanomolar concentrations of TTX antagonized the effect of BjIP on contractility (Fig 3a), calcium transients (Fig 3d,e), and I_{Na} (Fig 3f-i).

BjIP activates neuronal sodium channels. Fast inward currents in thalamocortical neurons[17] indicated a sufficient voltage control and space clamp to assess Na currents (Fig. 4a). Current amplitudes increased by application of BjIP (0.44 µg/ml; +16.1±8.4 %, n=7; Fig. 4a,c). The inactivation time constant of I_{Na} was best fitted by a single exponential function at room temperature (Fig. 4b, e.g. τ = 4.9±0.8 ms for the step to -30 mV, n=7). No changes in current decay were detected in the presence of BjIP τ = 5.0±0.5 ms (n=7; Fig. 4b). µCtx [18] reduced I_{Na} by -19.4±3.9 % (n=4; Fig. 4f). In the presence of µCtx, BjIP increased I_{Na} amplitudes by 13.2±6.0 % compared to control (n=4; Fig. 4f). ProTx (0.5 µM [19]) reduced I_{Na} amplitudes by -27.5±6.6 % (n=5; Fig. 4d, g). In the presence of ProTx, BjIP had no effect on current amplitudes (n=5; Fig. 4d, g). Additional application of TTX (1 µM) nearly completely inhibited I_{Na} in TC neurons (Fig. 4d).

Expression of TTX-sensitive Na channel genes in murine brain and heart and in human heart tissue. From the ten known Na_V1 channel genes, mRNA of SCN1A ($Na_V1.1$), SCN2A ($Na_V1.2$), SCN3A ($Na_V1.3$), SCN8A ($Na_V1.6$), SCN11A ($Na_V1.9$) and SCN7A (Na_X) was detected in thalamic tissue (Fig. 4h). mRNA of the other Na^+ channels were below detection limit in thalamus. qPCR was used for heart tissue. Relevant expression of SCN3A mRNA, but not of SCN8A mRNA was detected in human and murine ventricular tissue, and slightly higher SCN3A mRNA levels were found in specialized conduction tissue (Purkinje fibers and AV node, Fig. 5). These findings together with the subtype specificity of μCtx and ProTx make $Na_V1.3$ channels the most likely candidates for positive modulation by BjIP.

Discussion

Main findings. Within the limitations of studying a biological toxin that had to be harvested and purified for each experiment, our findings suggest that selective activation of "neuronal", TTX-sensitive sodium channels in the heart (most likely $Nav_{1.3}$ channels) by a peptide isolated from the venom of Buthotus Hottentotta may be a novel therapeutic principle to increase cardiac contractility without proarrhythmic side effects. Further experiments are needed to validate our encouraging preliminary findings.

Currently available positive inotropic agents modulate unspecific kinases in cardiomyocytes, such as protein kinase C, but also calcineurin or calcium-dependent calmodulin kinase II (CaMKII) [20, 21]. These kinases increase cAMP levels in the myocardium and thereby increase calcium release from the sarcoplasmic reticulum, but also alter the function of the main cardiac sodium channel $Nav_{1.5}$ by phosphorylation [21], and thereby increase cardiac contractility. Activators of the main cardiac sodium channel $Nav_{1.5}$ increase contractility [5, 22]. Activation of $Nav_{1.5}$, however, also prolongs repolarization [5, 22] and provokes ventricular arrhythmias [23].

TTX-sensitive sodium channels contribute to sodium influx and increased contractility in the heart: Approximately 25% of mammalian cardiac sodium channels are TTX-sensitive, and preferentially localized close to the T tubules [11]. Our own qPCR measurements confirm that SCN3A/Nav1.3 mRNA is present in human and mouse ventricular tissue (Figure 5), while SCN8a/Nav1.6 mRNA is not found in the heart, consistent with a recent report in rats (Figure 6 in [24]). Insect toxins can have inotropic effects in large mammals [25], and nanomolar concentrations of TTX exert a negative inotropic effect (-20%) in beating guinea-pig hearts

[10]. It is noteworthy that a similar phenomenon, although less noticeable, can be seen in Fig. 3d,e, where TTX (200 nM) reduces contractility slightly below control values.

Similar to the $Nav_{1.5}$ activators and to apelin [26], the full venom of the *Hottentotta judaicus* has inotropic effects, but also prolongs the ventricular action potential and provokes ventricular arrhythmias (Figure 1). BjIP, in contrast, increased contractility in vivo and in cardiomyocytes without proarrhythmic effects (Figure 1). Furthermore, the effect of BjIP was reversed by TTX (Figure 3), suggesting an action on TTX-sensitive cardiac sodium channels, i.e. $Nav_{1.1}$, $Nav_{1.2}$, $Nav_{1.3}$, $Nav_{1.4}$, $Nav_{1.6}$ or $Nav_{1.7}$ [4, 27], or alternatively spliced $Nav_{1.5}$ sodium channels [28]. Although we could not measure full I-V-relations due to limited amounts of BjIP, we have sufficient data so show that BjIP delays I_{Na} inactivation without altering I_{Na} amplitude. Our electrophysiological experiments in thalamic slices suggest that BjIP activates $Nav_{1.3}$ and/or $Nav_{1.6}$ (Fig. 4). Consistent with published reports [4, 11, 27, 29-31], we find expression of $Nav_{1.3}$ channel mRNA in ventricular myocardium. Thus, it seems likely that the inotropic effect of BjIP is mediated by activation of $Nav_{1.3}$, possibly initiating a localized increase in sodium close in the T tubules of cardiomyocytes [11] close to the sarcoplasmic reticulum, where even a small sodium influx can increase local calcium concentrations and facilitate calcium release from the sarcoplasmic reticulum [32]. A similar alteration in local calcium homeostasis was proposed in multiple sclerosis where axonal injury is caused by increased $Nav_{1.6}$ channels in demyelinated axons and alters sodium-calcium exchange [33]. Alternatively, sodium influx close to the T tubules may synchronize excitation-contraction coupling, and thereby improve inotropy [10, 11]. BjIP may also increase diastolic sodium load in the cell by providing additional substrate to the sodium-calcium exchanger through activation of $Nav_{1.3}$ channels, as has been shown for the $Nav_{1.5}$ opener veratridine [34]. These possibilities deserve further experimental examination. Of note, $Nav_{1.6}$, but not $Nav_{1.3}$ mRNA

expression is increased in response to pressure overload, rendering activation of $Nav_{1.6}$ channels relevant in hypertrophied and failing hearts [30]. Thus, activation of $Nav_{1.3}$ and/or $Nav_{1.6}$ seems a promising new cardiac inotropic principle.

Limitations. We performed the experiments over a period of several years using different preparations of the peptide, limited by the available amount of the purified peptide obtained from freely living scorpions collected in the Negev Desert. Our attempts to generate recombinant BjIP have so far not allowed us to obtain an adequately folded peptide in sufficient amounts [35]. Clearly, recombinant BjIP would be desirable to perform experiments in large animal models and in failing hearts, and to provide unambiguous evidence that the observed effects were mediated by BjIP and not by contaminations. These limitations notwithstanding, our data suggest that selective activation of the neuronal, TTX-sensitive sodium channels, most likely including $Nav_{1.3}$, is a promising target for the development of novel inotropic substances.

Further research directions. Our observations invite further studies, e.g. examining the following issues: 1. Providing dose-response curves for the effect of BjIP on different Na channels; 2. Determining whether $Nav_{1.3}$ is indeed the main molecular target mediating the effect of BjIP; 3. Investigating the effect of BjIP in failing hearts, where Na channel expression, calcium handling, metabolic state, and Na channel function may be altered; 4. Determining the effect of BjIP in ischemia-reperfusion models ; 5. Assessing whether BjIP may have a specific antiarrhythmic effect that could explain that cardiac APD and ventricular arrhythmias are not affected by BjIP.

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Figure 1. *BjIP increases cardiac contraction without affecting cardiac rhythm.*

a: Representative M mode echocardiographic tracings at baseline, after injection of the full venom of Buthotus Hottentotta (BjV, 200µg), and after injection of the inotropic peptide (BjIP, 20µg). White lines indicate left ventricular systolic and diastolic dimensions. The tracing after BjV injection shows ventricular arrhythmias.

b: Mean fractional shortening at baseline and after injection of BjV (200µg, grey) and BjIP (20µg, black).

c-f: *Effects of BjV (20µg/ml) and BjIP (4µg/ml) on cardiac electrophysiology in the beating heart* as assessed in contracting, Langendorff-perfused (37°C, modified Tyrode' s solution) mouse hearts with simultaneous recording of right and left ventricular monophasic action potentials. Ventricular arrhythmias were assessed after AV nodal block.

c: Example of a spontaneous polymorphic ventricular tachycardia during BjV perfusion.

d: Number of hearts with (black bars) and without (white stacked bars) spontaneous arrhythmias at baseline, during BjV perfusion, and during BjIP perfusion.

e: Representative monophasic action potential recordings at baseline (top), during perfusion with the full venom BjV (middle), and during perfusion with BjIP (bottom). BJV prolongs the ventricular action potential, BjIP does not.

f: Mean action potential durations at 70% repolarization and 140 ms paced cycle length at baseline (left), during perfusion with the full venom BjV (middle, grey), and during perfusion with BjIP (right, black). BjV prolongs ventricular action potential duration.

g: Mean action potential durations at baseline (dotted lines) and during BjIP perfusion (solid lines) at different repolarization levels (APD50, APD70, APD90) and different paced cycle lengths (80 – 140 ms, x axis) in isolated, beating mouse hearts (n=7 hearts, n=18-21

action potentials per mean data point). All values are given as mean and standard deviation.

Figure 2: *Isolation, sequence and modeled structure of BjIP.*

a: Fractionation of the BjV (75 mg) by reversed phase HPLC. Preparative column of RP-C18 (Vydac, USA) equilibrated by DDW + 0.1% TFA and eluted by a linear gradient of acetonitrile + 0.1% TFA with a changed steepness (not shown). Fraction 21 contains BjIP.

b: Amino acid sequence of BjIP (fraction 21) as deduced from the cDNA clone. Total RNA isolated from Black scorpion venom gland was analyzed by SMART (Clontech, USA). The full sequence contained 61 amino acids of mature polypeptide (predicted weight 6609 Dalton), 19 amino acids of the signal peptide (red underline), 111 base pairs of untranslated nucleotide after the stop codon, and a polyadenylation site (black underline), followed by a long segment of 30 base pairs of poly A. Matrix-Assisted Laser-Desorption Ionization–Time-Of-Flight (MALDI-TOFF) mass spectrometry of BjIP confirmed a 6609 Dalton component (duplicate measurement from two independent venom batches).

c: Superimposition of BjIP and five β toxins of known 3D structure (pdb codes 1B7D, 1I6F, 1I6G, 1NRA, 1NRB) on the Swiss-Model modeling server, displayed in color RMS PyMOL: Dark blue thinner lines represent strong homology, red thick lines diversity.

d: Structure of BjIP (PyMOL). The reduced length of BjIPs alpha helix is due to the absence of two adjacent lysines.

Figure 3: *BjIP increases cardiomyocyte contractility, increases intracellular calcium transient amplitude, and prolongs opening of TTX sensitive sodium channels*

a: BjIP increases force of contraction by 13% in mouse right ventricular muscle strips. 200 nM TTX abolish the inotropic effect of BjIP.

b: Representative simultaneous recording of calcium transients and cell shortening at baseline and during superfusion with BjIP (0.4 µg/ml).

c: Corresponding mean calcium transient amplitude and cell shortening (n=12/3). BjIP increases calcium transient and cell shortening amplitudes.

d: Representative cell shortening at baseline, with BjIP, and with BjIP+ 200 nM TTX.

e: Mean cell shortening is increased by BjIP. This effect is reversed by 200 nM TTX (n=7).

f: Representative recordings of I_{Na} from the same adult ventricular mouse cardiomyocyte at baseline, with 4 µg/ml BjIP and with BjIP and 200 nM TTX.

g : BjIP does not affect the peak current amplitude of I_{Na}

h: BjIP prolongs inactivation of I_{Na} as illustrated by increased inactivation time constant τ.

i: Increased total Na^+ influx estimated as area under the curve (AUC) (f-i: n=2-6; *$p<0.05$ vs. base). The BjIP effects on I_{Na} are abolished by 200 nM TTX.

Figure 4: *BjIP effect on I_{Na} in thalamic neurons.*

a: Current-voltage relationship of I_{Na} under control conditions (open squares) and in the presence of BjIP (closed squares) in a thalamocortical neuron. Continuous lines indicate Boltzmann fits corrected for driving force to the data points. The cell was held at -60 mV before stepping to -90 mV for 2 s and eliciting I_{Na} by voltage steps of increasing amplitudes (-60 to -30 mV, 10 mV increment, 30 ms duration).

b: Inactivation kinetics of I_{Na} were best fitted by a single exponential function (see inset) and not different between control and BjIP.

c: Original traces of I_{Na} recorded from thalamic neurons of acute living rat brain slices under control conditions (base, black trace) and in the presence of BjIP (0.44 µg/ml, dark grey trace).

d: Current traces under control conditions (base, black trace), during application of ProTx-II (ProTx, 0.5 µM, light grey trace), in combination with BjIP (ProTx + BjIP, dark grey trace) and with tetrodotoxin (TTX, 1 µM, dashed black line) added to the extracellular solution. I_{Na} was elicited by voltage steps from -90 mV (2 s) to -30 mV (30 ms, see inset).

e-g: Box plots of current amplitudes under different recording conditions (as indicated; * $p<0.05$, ** $p<0.01$). µCtx is a blocker $Na_V1.2$, $Na_V1.4$ and $Na_V1.7$. The most likely subtypes activated by BjIP are underlined. pro-Tx is an inhibitor of $Na_V1.2$, $Na_V1.3$, $Na_V1.5$, $Na_V1.6$, $Na_V1.7$ and $Na_V1.8$.

h: mRNA expression of SCN1A ($Na_V1.1$), SCN2A ($Na_V1.2$), SCN3A ($Na_V1.3$), SCN4A ($Na_V1.4$), SCN5A ($Na_V1.5$), SCN8A ($Na_V1.6$), SCN9A ($Na_V1.7$), SCN10A ($Na_V1.8$), SCN11A ($Na_V1.9$), SCN7A (Nax) in thalamic brain tissue. DNA marker bands are located in the left margin. “x” indicate channels that are inhibited by µCtx or ProTx.

Figure 5: *Expression of SCN3A, but not SCN8A mRNA in murine and human heart tissue.*

a: PCR showing detection of SCN3A and SCN5A mRNA in human heart and blood and in mouse heart tissue. SCN8A mRNA is not detectable in the heart, but is readily detected in human leucocytes.

b: Mean expression levels of SCN3A (black), SCN5A (grey), and SCN8A (non detectable) mRNA in mouse ventricular tissue derived from qPCR.

c: Mean expression levels derived from qPCR of SCN3A (black), SCN5A (grey, reference, set to 1), and SCN8A (not detected) mRNA in human heart tissue taken from different regions, i.e. atria, ventricles, sinus node, AV node, and Purkinje fibers.
ΔCt-means were used to express mRNA expression normalized to SCN5A expression applying 2-ΔΔCt-method.

Table: Concentrations of the full venom (BjV) and of the inotropic peptide (BjIP, 6609 Da estimated weight) used for functional assessment. All numbers rounded to 0.1µg/ml, where applicable

		Full venom (BjV)	**Ionotropic peptide (BjIP)**
in vivo	Toxicity (i.p.)	200 µg	20 µg (3 µmol)
	inotropy, arrhythmias (echocardiography, i.p.)	40 µg	2 µg (300 nmol)
perfused preparations	arrhythmias, AP, perfused heart	20 µg/ml	4 µg/ml (600 nM)
	Na current, superfused cardiomyocytes	-	4 µg/ml (600 nM)
	isolated trabecles	-	0.4 µg/ml (60 nM)
	Ca transients, superfused cardiomyocytes	4 µg/ml	0.4 µg/ml (60 nM)
	thalamic slices	-	0.4 µg/ml (60 nM)