Jellyfish and other cnidarian envenomations cause pain by affecting TRPV1 channels

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Received 3 August 2006; revised 5 September 2006; accepted 13 September 2006
Available online 22 September 2006

Edited by Maurice Montal

Abstract Cnidarian envenomations cause a burning-pain sensation of which the underlying mechanisms are unknown. Activation of TRPV1, a non-selective cation channel expressed in nociceptive neurons, leads to cell depolarisation and pain. Here, we show \textit{in vitro} and \textit{in vivo} evidence for desensitization-dependent TRPV1 activation in cnidarian envenomations. Cnidarian venom induced a nociceptive reactivity, comparable to capsaicin, in laboratory rats, which could be reduced by the selective TRPV1 antagonist, BCTC. These findings are the first to explain at least part of the symptomology of cnidarian envenomations and provide insights into the design of more effective treatments for this global public health problem.

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Keywords: Cnidaria; TRPV1; Envenomation; Pain; Desensitization

1. Introduction

Increased frequency and magnitude of jellyfish blooms worldwide, together with increased occupational and recreational exposure of at-risk coastal populations to cnidarian envenomations, underscore the need for better clinical-management tools for the treatment of such stings. Limited success in post-envenomation pain relief has been achieved with the empirical use of topical vinegar or hot-water immersion\textsuperscript{[1–2]}, we tested whether TRPV1 activation was responsible for the painful symptoms associated with cnidarian envenomation.

2. Materials and methods

2.1. Preparation of crude venom extracts

Tentacles, from \textit{Aiptasia pulchella}, \textit{Cyanea capillata}, \textit{Physalia physalis} and \textit{Chironex fleckeri} were cut into small pieces and suspended in 50–60 ml 10\% acetic acid per 1.5–2 g of tentacles. The mixture was stirred overnight at room temperature with a magnetic stirrer, then centrifuged at 40000 \(\times\) g for 1 h. Nearly all of the supernatant was recovered by careful decantation or removal by a syringe. The supernatant was concentrated by rotatory evaporation to about 10\% of its original volume, diluted with 5–6 volumes of deionized water and freeze-dried. Freeze-dried samples were dissolved again in ND96. Solutions were titrated to pH 7.4 with NaOH.

2.2. Electrophysiological recordings

cRNA transcripts were synthesized from \textit{Xenopus} linearized VR1 cDNA templates using T7 RNA polymerase (Ambion). Oocytes, harvested from anaesthetized female \textit{Xenopus laevis} frogs as previously described\textsuperscript{[7]}, were injected with 0.5–5 ng TRPV1 cRNA. Two to seven days after injection, two-electrode voltage-clamp recording was performed. Currents were measured in ND96 solution using a protocol of ~90 mV during 400 s. Tail current experiments in Fig. 3 were measured at a holding of 0 mV, test steps from –100 to +160 mV for 200 ms. The recording chamber was perfused at a rate of 2 ml min\textsuperscript{–1} with a ND-96 solution containing (in mM) 96 NaCl, 2 KCl, 1.8 CaCl\textsubscript{2}, 5 Hepes, pH 7.4. Temperature of the perfusate was kept at 22 ± 1°C and controlled using a SC-20 dual-in-line heater/cooler (Warner Instruments) and pH was kept at 7.4. As previously described\textsuperscript{[7]}, capsaicin (2 \(\mu\)M) was used as an agonist and capsazepine (10 \(\mu\)M) as an antagonist of TRPV1. Capsaicin and capsazepine were purchased from Sigma, and anandamide from Tocris. In \textit{in vitro} experiments, following venom concentrations are used: 150 mg crude freeze-dried \textit{C. capillata} venom was dissolved in 50 ml ND96; 215 mg freeze-dried \textit{P. physalis} was dissolved in 50 ml ND96; 170 mg freeze-dried \textit{C. fleckeri} venom was dissolved in 30 ml ND96 and few hundred micromgrams of crude \textit{C. capillata} venom was dissolved in 5 ml ND96. Ten milligram \textit{BmK} venom was dissolved in 40 ml ND96.

To control the expression level of the TRPV1 channels, we always started our experiments with a first activation of TRPV1 with 2 \(\mu\)M capsaicin. Application of capsaicin (or anandamide) opens the channels. This activation gives an inward non-selective cation current.

2.3. In vivo studies

Male Sprague Dawley rats (Harlan, Eystrep, Germany) weighing between 270 and 310 g, were maintained in a climate-controlled environment on a 12 h light/dark cycle at a temperature of 22 ± 1°C. All experiments were carried out during the light phase. During housing water and food were available \textit{ad libitum}. The animals were habituated to the experimental room for at least 1 h before the start of the...
experiment. Animals were used only once. All tests were performed according to guidelines of the Institutional Ethical Committee for Animal Experiments and guidelines for animal research according to IASP. In in vivo experiments, 75 mg venom was dissolved in 0.5 ml sterile water and a dose of 40 mg/kg was injected.

The formalin test is a chemical assay of nociception. At 30 min after sc injection of the test compound rats were given a single injection of formaldehyde (50 μl, 1.75%) in the plantar hypodermis of the skin of the left hind paw. Within seconds the rats started to flinch the paw rapidly, this is the phase I response that is measured during the first 10 min. The following phase II is measured during 50 min and represents a more tonic state. This biphasic flinch behaviour in the rat following the formalin injection was measured in an Automated Nociception Analyser® (UC, San Diego, CA, USA). Therefore the animal was placed in a Plexiglas cylinder mounted above a transmitter/receiver coils assembly. The system detects movement of a small metal band placed on the injected paw. A signal is generated as the band breaks the electromagnetic field of a loop antenna placed underneath the rat. The signal is processed through an algorithm that determines flinch activity using amplitude, zero voltage crossing and duration. The total number of flinches observed during a selected time period was calculated by accumulating flinches of each individual animal over that time period and averaging the treatment groups. Activity of the compound was considered when the number of flinches was lower than 40 or 600 during phases I and II, respectively.

3. Results

We tested venom extracts (10% acetic acid tentacular extracts) of four cnidarian species (A. pulchella, C. fleckeri, P. physalis and C. capillata), representing each of the four classes in the phylum Cnidaria (Anthozoa, Cubozoa or sea wasps, Hydrozoa and Scyphozoa or jellyfishes). Although none of the four venoms tested had any significant effect of its own (Fig. 1A–D left), there was a clear allosteric effect on the [(n + 1) with (n > 0)] activation of the channel when the venom was applied with capsaicin (Fig. 1A–D right). We then tested whether the endogenous activator, anandamide [14,15] also induced this effect. That anandamide (10 μM) elicits the same allosteric effect (Fig. 1F) suggests future studies to further test the in vivo interplay of serum anandamide and venom constituents at the site of envenomation. Because stings of the scorpion Buthus martensi Karsch (BmK), a very well-studied preparation, are also accompanied by symptoms of redness and burning pain [16,17], we compared the effect of crude BmK venom under the same conditions, and found no effect on TRPV1.

![Fig. 1. Effects of Cnidaria and BmK venom on TRPV1. Traces from (A) Aiptasia pulchella (A.P.) venom (B) Chironex fleckeri (C.F.) venom (C) Physalia physalis (P.P) venom D. Cyanea capillata (C.C.) venom. (A)–(D) Left: crude venom in the absence of capsaicin (CAP) and capsazepine (CZP). (A)–(D) Right: Allosteric effect of venom with 2 μM capsaicin. (E) Left: Trace with lack of effect of BmK venom. (E) Right: Lack of effect of venom in the presence of capsaicin. (F) Allosteric effect of Cyanea capillata venom and 10 μM anandamide (ANA). Identical results were obtained independent of the order of CAP or venom administration.](image-url)
(Fig. 1E), indicating that the allosteric effect might be specific for cnidarian venoms. Crude venom of the green mamba (Dendroaspis angusticeps) also did not have any effect on TRPV1 (data not shown).

To clarify the mechanism of action of the venom and to investigate whether the observed venom effects were related to desensitization of TRPV1 [18,19], the effect of the venom at the peak current from the first and consecutive activations was tested. Desensitisation of TRPV1 can be described as channels becoming insensitive by repeated or continuous addition of an agonist (e.g. capsaicin). In voltage-clamp measurements it can be seen as smaller inward currents every application or during the application of the agonist without addition of an antagonist. By applying the venom on the first peak current, we ensured that there was virtually no desensitization of the channel (a situation comparable to Ca²⁺-free condition [18,19]). If the venom effect was indeed linked to desensitization, there should be no apparent effect of the venom on the peak current. If the venom was applied after the desensitization process had started or on the (n+1) activation of the TRPV1 channel, a desensitization-dependent venom effect would be expected.

The results of these experiments indicated that the venom was indeed without apparent effect during the first activation peak of TRPV1 (Fig. 2A). By contrast, the allosteric effect in which the desensitization-dependent component is being restored by the venom was clearly present when the venom was added during the desensitization phase or at the (n+1) activation (Fig 2B and C). Furthermore, we investigated if the venom could shift the TRPV1 activation curve (a frequently used trick of TRP channels [20]) and found that the open probability ($P_o$) calculated by fitting the tail current amplitudes was not significantly shifted along the voltage axis in the presence of capsaicin and venom as compared to capsaicin alone (Fig. 3). As such, these findings clearly indicate that the venom knocks down the desensitization of TRPV1, thus resulting in larger inward currents that would generate, in turn, the typical persistent burning-pain sensation.

To test the in vivo effects of cnidarian venoms and the possible therapeutic use of TRPV1 antagonists, we recorded the number of flinches for 4 min after intraplantar injection of capsaicin or venom in laboratory rats. The injected dose of crude C. capillata venom was chosen based on the number of flinches that was comparable with the number of flinches seen with 10 μg capsaicin. Fig. 4A and B show a significant increase of flinches when compared with the response after intraplantar injection of vehicle. To test whether a TRPV1 antagonist could inhibit the induced nociceptive reactions, 40 mg/kg of 4-(3-trifluoromethylpyridin-2-yl)piperazine-1-carboxylic acid (5-trifluoromethylpyridin-2-yl)amide (BCTC), a high-affinity TRPV1 antagonist [21], was injected subcutaneously 1 hour before intraplantar injection of capsaicin or C. capillata venom. As shown in Fig. 4A, a significant decrease in mean pain reaction (from 76.0 to 4.2, $P < 0.05$, n = 6) was observed when rats were injected with capsaicin, following pretreatment with BCTC. BCTC also significantly decreased the crude venom-induced mean number of flinches (from 65.8 to 14.0, $P < 0.05$, n = 6) (Fig. 4B). Injection of control vehicle did not significantly affect the reaction after intraplantar capsaicin or venom injection. The dose needed to decrease the venom-induced number of flinches by 50% ranged between 10 and 40 mg/kg (data not shown). The analgesic effect of BCTC was maximal at a dose
In vivo effects of Cyanea capillata venom with and without TRPV1 antagonist. (A) Number of flinches during 4-min observation period after left-hindpaw plantar injection of capsaicin (black) and vehicle (left diagonals). Pretreatment by subcutaneous injection of 40 mg/kg BCTC (white) or vehicle (right diagonals) and 1 h later the number of flinches and bites were observed after an intraplantar injection of capsaicin ($n = 6$). The effect of capsaicin with BCTC was significantly different from the effect of capsaicin alone. The BCTC vehicle did not give a significant difference as compared with venom alone. (B) Same tests as in A. Here, the effect of the venom was tested instead of capsaicin. The effect of venom with BCTC was significantly different from the effect of venom alone. The BCTC vehicle did not give a significant difference as compared with venom alone. In these experiments, the serum concentrations of anandamide or other endogenous TRPV1 activators were not determined.

of 40 mg/kg, with no further decrease in flinches when higher doses were injected. In all cases of capsaicin and venom, there was a baseline effect of a few flinches over the 4-min observation period. This indicates that other channels may also be involved in the induction of the nociceptive behaviour, although their relative contribution is presumably insignificant since the induced pain effect was reduced by 79% with BCTC, a selective TRPV1 antagonist.

Additional rat tests in which BCTC treatment is tested on a non-TRPV1 mediated pain behaviour (formalin test), BCTC did not show any analgesic effect. In phase I (first 10 min observation period) as well as in phase II (50 min observation) there was no significant decrease in pain response of the BCTC treated rats. In addition, BCTC did not show any side effects such as lethargy that may affect the flick/lick assay.

Taken together, these in vivo results do not give 100% prove that TRPV1 is the main pain inducing mechanism, but keeping in mind the experiences in the empiric treatment, it gives us a good idea in what might be happening in the in vivo situations and they provide supportive evidence that the use of TRPV1 antagonists as analgesics in cnidarian envenomations warrants consideration for clinical trials.

4. Discussion

In conclusion, we identified TRPV1 as a key component in the signal-transduction pathway of cnidarian envenomation. These newfound data provide a pathophysiologic basis for the symptomology of cnidarian stings. Although the active substance(s) in these venoms is (are) yet not fully identified (i.e., whether peptide or bioactive small molecule) and the precise site of interaction still remains to be elucidated (i.e., on the TRPV1 itself vs. the signal-transduction pathway), our discovery provides important and novel insights into the design of more effective treatments for cnidarian envenomations: on the one hand, TRPV1 blockers can possibly be used as therapeutics, just as atropine is used as an antidote for organophosphate-type intoxications on the muscarinic ACh receptor [22]; on the other hand, one might speculate that TRPV1 activators may also be useful in the treatment of cnidarian stings as they could counteract the venom-induced down regulation of the desensitization. As a consequence, an inverse relationship exists between the degree of desensitization and the size of the inward currents. As a corollary, therefore, the burning-pain sensation should diminish once the inward currents become smaller. It is clear that further studies are needed to pinpoint the selectivity ratio of cnidarian venoms between the different members of the large TRP family and to demonstrate if the human TRPV1 orthologue behaves in the same manner as the rat clone. Nonetheless, our in vitro and in vivo experiments have shown for the first time a clear link between cnidarian envenomation-associated pain and TRPV1 activation via ‘knock-down’ of desensitization that brings more effective treatments for the worldwide problem of cnidarian envenomations within reach.

Acknowledgements: We thank Dr. Carina Östman for providing tentacles of C. capillata, Dr. Olgas Castaneda for P. physalis and Dr. Phil Alderslade for C. fleckeri. We also thank Dr. David Julius for providing the rat TRPV1 clone, and we are very grateful to Dr. Theo Meert, Dr. Hilde Vermeirsch, Ria Biermans (J&J PRD, Belgium) for their support of the in vitro studies. Finally, we thank Sarah Debayee for making the TRPV1 RNA and Dr. Frank Bosmans, Dr. Chantal Maertens, Dr. Elke Vermassen, Yousra Abdel-Mottaleb, Thomas Vandenbriesche, Bert Billen and Dr. Richard Yanagihara for their critical reading of the manuscript. This work was supported in part by the FWO-Vlaanderen grant G.0330.06; the K.U. Leuven (Grant OT-05-64); and grants from the National Institute of Neurological Disorders and Stroke, National Institute of Health (US54NS039406) and the Hawaii Community Foundation (20011908).

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