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Kininogenase activity of *Thalassophryne nattereri* fish venom

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Abstract

Accidents caused by the venomous fish *Thalassophryne nattereri* are characterized by edema, intense pain and necrosis at the site of the sting. This study assessed the nociceptive and edematogenic activities of *T. nattereri* venom after injection into the mouse hindpaw and determination of the paw licking duration and weight. Subplantar injections of the venom (0.1–6 μg) induced a dose-related increase of the paw licking time and paw swelling with maximal values at 3 μg (209.5 ± 57.5 s and 135.0 ± 6.8 mg, respectively). Pretreatment of mice with either indomethacin (10 mg/kg, i.p.), a cyclooxygenase inhibitor, dexamethasone (1 mg/kg, s.c.), a steroid anti-inflammatory agent, cyproheptadine (1 mg/kg, i.p.), antagonist of serotonin receptors or L-NAME (100 mg/kg, s.c.), inhibitor of nitric oxide syntase, did not affect the venom-induced nociceptive and edematogenic responses. Injection of the opioid analgesic fentanyl (0.1 mg/kg, s.c.) reduced the paw licking time induced by 1 μg venom by 84% of control, without affecting the paw swelling. Both nociceptive and edematogenic responses were reduced after treatment with a specific tissue kallikrein inhibitor (TKI, 100 mg/kg, i.p.) by 78% and 24% from control values, respectively. Administration of a specific plasma kallikrein inhibitor ($PKSI_{527,}$ 100 mg/kg, s.c.) did not affect the venom-induced nociceptive response, but it decreased the paw edema by 15% from control. After injection of the angiotensin-converting enzyme inhibitor captopril (100 mg/kg, i.p.) the venom-induced nociceptive end edematogenic responses were increased by two-fold. The role of kallikreins possibly present in the venom was further assessed by hydrolysis of human kininogen and kininogen-derived synthetic peptides, showing the release of kallidin (Lys-bradykinin). The hydrolysis was inhibited by metal chelating agents but not by serino-, aspartyl- or cysteino-proteinase inhibitors. The data suggest that a protease with tissue-kallikrein-like activity plays a major role in nociception and edema induced by *T. nattereri* venom and this should be considered to achieve efficient treatments for human accidents with this venom.

Keywords: *Thalassophryne nattereri*; Edema; Nociception; Specific tissue kallikrein inhibitor; Lys-bradykinin

Abbreviations: PBS, phosphate buffered saline; EDDnp, *N*-[2,4-dinitrophenyl]-etilenedianine; TFA, trifluoroacetic acid; L-NAME, *N*ω-nitro-L-arginine methylester; TKI, phenylacetyl-FSR-EDDnp; DMSO, dimethyl sulfoxide; TLCK, tosyl lysyl chloromethyl ketone; TPCK, tosyl phenylalanyl chloromethyl ketone; PMSF, phenyl methyl sulphonyl fluoride; EDTA, ethylenediaminetetraacetic acid; TRIS, tris(hydroxymethyl)aminomethane

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1. Introduction

Animal venoms are usually very complex mixtures of toxic proteins and peptides that evolved in certain species all along animal phylogeny. These toxins bind with high affinity to physiological targets of the preys/predators causing immobilization, necrosis or death. Although, fish venoms have evolved to interact with sea animals, it is observed that human accidents also occur. The accidents caused by fishes are frequent and represent a public health problem in some regions of Brazil, particularly in the north and northeast states where the accidents are caused by *Thalassophryne nattereri* fish, popularly known as "niquim".

Human accidents occur by contact with the fish spines located on their dorsum and both sides of the head, and connected to the venom glands. Envenoming symptoms include severe edema and pain followed by a fast settling necrosis, both in human victims and experimental animals [1]. The number of accidents occurring in Brazil is uncertain. It is estimated that hundreds of accidents occur every year and the incidence is underestimated because patients seldom look for medical care due to its lack of efficacy. Current treatment of *T. nattereri* accidents includes anti-inflammatory and analgesic drugs, antibiotics and immersion of the lesioned tissue in warm water. These procedures do not reduce the acute symptoms of the accidents being effective only for prevention of secondary infections. Most accidents occur in the fishing communities and, due to the lack of efficient therapy, it may take weeks or even months for complete recovery of the victims.

T. nattereri venom is composed of proteins endowed with proteolytic and myotoxic properties, but devoid of phospholipase A_2 activity [2]. Analysis of its local effects showed a myotoxic effect with muscle damage and difficult regeneration [3]. Blood flow at microvessels was also impaired with stasis and presence of thrombi in venules, focal transient constrictions in arterioles, and increased vascular permeability. Venom lacked a direct pro-coagulant activity, but exerted a strong cytolytic action on platelets and endothelial cells in vitro [4]. A better understanding of the mechanisms regulating the acute-inflammatory events could lead us to the development of efficient therapeutic strategies.

This study aimed to examine the nociceptive and edematogenic activities of *T. nattereri* venom in mice and to determine the effects of antagonists of major mediators involved in both responses. Our results showed that the venom-induced nociception and edema were not affected by either steroidal and non-steroidal anti-inflammatory agents or a serotonin inhibitor, but they were reduced after administration of a specific tissue kallikrein inhibitor. We also showed the presence of tissue-kallikrein-like activity in the venom suggesting that a protease plays a major role in edema and nociception induced by *T. nattereri* venom.

2. Materials and methods

2.1. Animals

Male Swiss mice (18–22 g) were housed in a room with controlled temperature (22 ± 2 °C) and lighting (lights on from 6:00 a.m. to 6:00 p.m.), with free access to laboratory chow and tap water. All experiments were performed between 10:00 a.m. and 5:00 p.m. and the animals were cared in accordance with the ethical guidelines of the International Association for the Study of Pain [5]. The experiments were conducted under approval of the Instituto Butantan Animal Investigation Ethical Committee.

2.2. Venom

The venom was collected from fresh specimens of *T. nattereri* from the openings of the spines by applying pressure at their bases. The venom was either immediately used or kept frozen at −20 °C. For pharmacological tests, venom solutions were prepared in phosphate buffer saline (PBS: NaCl 135 mM, KCl 2.7 mM and phosphate buffer 10 mM, pH 7.2). Protein content was determined by the method of Bradford [6], using a standard curve with known concentrations of bovine serum albumin (sigma) as absorbance reference. Venom samples were further diluted in PBS for the appropriated concentration corresponding to the dosages used in each experiment.

2.3. Evaluation of nociception and edema

For nociceptive tests, each mouse was kept in an observation chamber (acrylic box, 12 cm × 12 cm × 12 cm) mounted with a mirror at a 45° angle beneath the floor [7]. After a 10 min adaptation period, the animals were injected with the venom (0.1–6 μg, s.c.) into the intraplantar region of the hind foot paw in a fixed volume of PBS (30 μL). The contralateral paw (control) was injected with an equal volume of the vehicle. Each animal was then returned to the observation chamber and the amount of time spent licking or biting each hind paw was recorded for 30 min and taken as the index of nociception [8,9]. After 2 h venom injection, the animals were killed by cervical dislocation under light ether anesthesia and both hind paws were cut off at the ankle joint and weighed. The difference in weight (milligrams) between the hind paws was used as an index of paw edema [10].

The effective anti-nociceptive dose of indomethacin was determined in the acetic acid-induced writhing test in mice as previously reported [11]. Mice were treated with indomethacin (10 mg/kg, i.p.), 30 min before injection of 1.2% acetic acid (0.1 mL/10 g, i.p.) [12] and the number of abdominal constrictions was counted cumulatively for 30 min. The tested anti-inflammatory dose of dexamethasone (1.0 mg/kg, s.c.) was determined after 60 min treatment of mice on the ear edema induced by topical

application of 10 μL croton oil solution (2.5%) as detailed elsewhere [11].

2.4. Enzymatic assays

The peptidase activity of *T. nattereri* venom was determined using internally-quenched fluorogenic substrates containing sequences derived from human kininogen as described previously [13]. The hydrolysis of the fluorogenic peptidyl substrates at 37 °C in tris-buffer pH 7.4 containing NaCl 100 mM was followed by measuring the fluorescence at $\lambda_{em} = 420$ nm and $\lambda_{ex} = 320$ nm in a Hitachi F-2000 spectrofluorometer. The 1 cm path-length cuvette containing 2 mL of the buffer was placed in a thermostatically controlled cell compartment for 5 min before the venom solution was added followed by the substrate addition, and the increase in fluorescence with time was continuously recorded for 5–10 min. In assays using different inhibitors, a pre-incubation time in the presence of the respective inhibitor was applied before the substrate addition. The slope was converted into mols of hydrolyzed substrate per minute based on the fluorescence curves of standard peptide solutions before and after total enzymatic hydrolysis. The concentration of the peptide solutions was obtained by colorimetric determination of 2,4-dinitrophenyl group (17,300 M^{-1} cm^{-1}, extinction coefficient at 365 nm). The venom concentrations for initial rate determinations were chosen at a value intended to hydrolyze less than 5% of the substrate present in the reaction. The inner-filter effect was corrected using an empirical equation as previously described by Araujo et al. [13].

2.5. Peptide synthesis

The internally quenched fluorogenic (IQF) peptides containing EDDnp attached to glutamine and *ortho*-aminobenzoyl/dinitrophenol groups as donor–acceptor pairs were synthesized by solid-phase strategy. An automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM eight system from Shimadzu) was used for the synthesis of all the peptides by the Fmoc-procedure. The final de-protected peptides were purified by semi-preparative HPLC using an Econosil C-18 column (10 μm, 22.5 mm × 250 mm), and a two-solvent system: (A) TFA/H_2O (1:1000) and (B) TFA/acetonitrile (ACN)/H_2O (1:900:100). The peptide was eluted at a flow rate of 5 mL/min with a 10–60% gradient of solvent B over 30 or 45 min. Analytical HPLC was performed using an Ultrasphere C-18 column (5 μm, 4.6 mm × 150 mm) with solvent systems A and B at a flow rate of 1 mL/min and a 10–80% gradient of B over 20 min. The HPLC column eluates were monitored by their absorbance at 220 nm (Shimadzu UV–vis detector) and by fluorescence emission at 420 nm following excitation at 320 nm (Shimadzu RF-353 fluorescence detector). The molecular weight and purity of synthesized peptides were checked by MALDI-TOF mass spectrometry (TofSpec-E, Micromass) and/or peptide sequencing using a protein sequencer PPSQ-23 (Shimadzu Tokyo, Japan).

2.6. Determination of cleaved bonds

The cleaved bonds were identified by isolation of the fragments by HPLC and either by comparing the retention times of the produced fragments with synthetic peptides encompassing the expected hydrolysis products or by molecular mass determined by MALDI-TOF mass spectrometry.

2.7. Bradykinin detection by radioimmunoassay (RIA)

T. nattereri venom (3 μg) was incubated with human high or low molecular mass kininogen (200 nM) in 50 mM tris-buffer, pH 7.41, 0.1 M NaCl in a final volume of 100 μL for 10 min at 37 °C. Kinin was extracted in ethanol (four times the reaction's final volume) for 10 min at 70 °C. Solutions were freeze-dried and dissolved in 200 μL of egg albumin buffer (0.1% egg albumin in 0.01 M phosphate buffer, pH 7.0, 0.14 M NaCl, 0.1% NaN_3, 30 mM EDTA, 3 mM *ortho*-phenantroline). Aliquots (50 μL) were incubated with 100 μL of antibody anti-Bradykinin (1:80,000) [14] and 100 μL of [^{125}I]-labeled Tyr-bradykinin for 20 h at 4 °C. Four hundred microliters of 0.1% bovine γ-globulin in 0.01 M phosphate buffer, pH 7.0, 0.14 M NaCl, 0.15 0.1% NaN_3 and 800 μL of 25% polyethyleneglycol 6000 solution were added to the samples, which were incubated for 10 min at 4 °C. Finally, the samples were centrifuged at 2000 × *g* for 20 min at 4 °C; the supernatants were removed and the pellets submitted to radiation counting [15].

2.8. Drugs and chemicals

The following drugs were used: fentanyl (Jassen Farmacêutica), dexamethasone (Decadron–Prodome), indomethacin (Indocid–MSD), cyproheptadine, L-NAME, and captopril. TKI was obtained by peptide synthesis in solution using anhydride procedures and *tert*-butyloxycarbonyl-amino acids, purified in silica gel and characterized by HPLC as previously described [16,17]. $PKSI_{527}$ was provided by Dr. Yoshio Okada, Faculty of Pharmacy Sciences, Kobe-Gakuin University, Kobe, Japan. Drugs solutions were administered 30 min before the venom, except dexamethasone and captopril, which were given, 60 and 120 min, respectively, before venom injections. All drugs were dissolved in sterile saline (NaCl 0.9%), except indomethacin and cyproheptadine which were dissolved in sodium bicarbonate (5%) and dimethyl sulfoxide (DMSO), respectively; TKI was dissolved in Tween 80 (40:1) and further diluted in saline. In enzymatic assay, the following inhibitors were used: trypsin-like serine proteases (TLCK), chymotrypsin-like serine proteases (TPCK), all serine

proteases (PMSF), cysteine proteases (E-64), aspartyl proteases (pepstatin A), and metalloproteinases (EDTA and *o*-phenantroline).

2.9. Statistical analysis

All results were presented as means ± S.E.M. of at least six animals in each group. Differences among data were determined using one way analysis of variance (ANOVA) followed by Dunnett's test. Differences between two means were determined using unpaired student's *t*-test. Data were considered different at $p < 0.05$.

3. Results

3.1. Nociceptive and edematogenic effects of *T. nattereri* venom

Plantar injection of *T. nattereri* venom (0.1–6 μg) into the mouse right hind-paw induced a dose-related increase of the paw licking duration that reached its maximum at 3 μg (209.5 ± 13.9 s), and was slightly diminished at the highest dose (Fig. 1a). After 30 min treatments, the mean paw weights increased proportionately to the dose up to 3 μg (from 35.0 ± 6.0 to 135.0 ± 2.8 mg) and stabilized thereafter, reflecting the associated venom-induced nociception and paw edema (Fig. 1b).

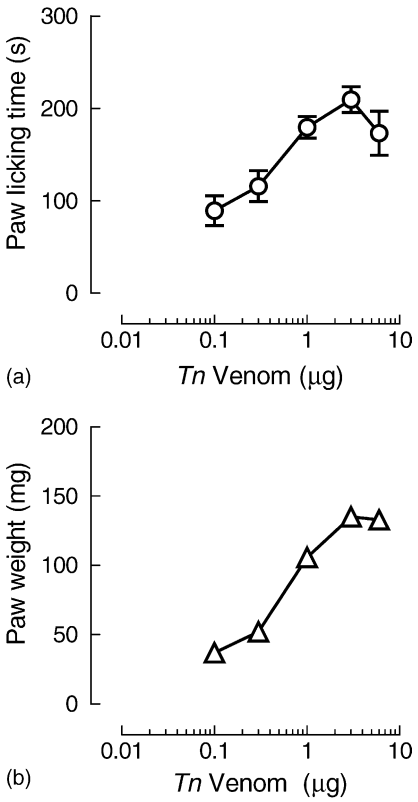

Fig. 1. Dose–response relationships of the nociceptive effect (a) and paw swelling (b) induced in mice by subplantar injections of single doses of *T. nattereri* venom (0.1–6 μg) into the hindpaw. Symbols and vertical bars are means ± S.E.M. of the effects recorded in 6–10 animals for each dose.

To assess the mechanisms involved in both nociceptive and edematogenic responses, the venom effect was tested at a dose (1 μg) that induced submaximal levels of nociceptive response and paw edema, in the presence of antagonists of the major related pharmacological pathways. These antagonists were tested at doses effective as anti-nociceptive and anti-inflammatory in the same animal model [11].

3.2. Effects of antagonists of nociceptive and inflammatory mediators

Previous treatment of mice with the opioid analgesic fentanyl (0.1 mg, s.c., dorsal region) reduced the paw licking time induced by injection of 1 μg of *T. nattereri* venom by 84% of control (from 185.0 ± 21.0 to 29.6 ± 3.6 s, $n = 6$), without changing the paw swelling (from 128.1 ± 7.5 to 129.2 ± 9.7 mg, $n = 6$).

In those mice treated with indomethacin (1 mg/kg, s.c.), a non-steroidal anti-inflammatory agent, the venom-induced paw licking time (218.0 ± 40.6 s, $n = 6$) and swelling (117.7 ± 9.1 mg) induced by 1 μg venom were not significantly different compared to control values (181.0 ± 18.7 s and 122.8 ± 6.2 mg, respectively). At the same dose indomethacin reduced the acetic acid-induced writhing in mice by 52% of control (from 18.5 ± 3.0 to 8.8 ± 2.1 writhes/30 min ($n = 6$).

Treatment of mice with dexamethasone (1 mg/kg, s.c.), a steroidal anti-inflammatory drug did not influence the venom-induced nociceptive response (207.0 ± 23.7 s) or the paw edema (114.8 ± 10.9 mg). An equal dose of dexamethasone, however, was effective in reducing the mouse ear edema induced by croton oil by 82% (from 8.8 ± 1.0 to 1.6 ± 0.6 mg, $n = 6$).

The paw licking time and edema induced in mice by 1 μg venom were not either affected by treatment of mice with cyproheptadine (1 mg/kg, i.p.), an antagonist of serotonin receptors (124.5 ± 13.6 s; 107.8 ± 7.6 mg, $n = 6$), or L-NAME (100 mg/kg, s.c.), a nitric oxide synthase inhibitor, (145.8 ± 8.3 s; 96.1 ± 7.3 mg, $n = 6$) compared to control values (149.0 ± 16.5 s and 106.2 ± 4.9 mg, respectively).

3.3. Effects of kallikrein inhibitors

Treatment of mice with a specific tissue kallikrein inhibitor (TKI, 100 mg/kg, i.p.), at a dose previously shown to reduce the formalin-induced neurogenic and inflammatory pain in mice [11], reduced the venom-induced paw licking time and edema by 78% and 24% from control values (162.7 ± 38.4 s and 105.5 ± 19.9 mg, $n = 12$), respectively (Fig. 2). In contrast, pretreatment with $PKSI_{527}$ (100 mg/kg, s.c.), a plasma kallikrein specific inhibitor did not affect the paw licking time, while it

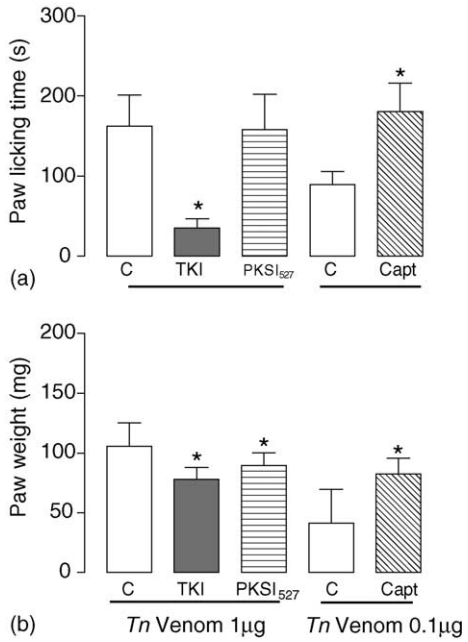

Fig. 2. Effects of specific inhibitors of tissue kallikrein (TKI, 100 mg/kg, i.p., injected 30 min before), plasma kallikrein ($PKSI_{527}$, 100 mg/kg, s.c., injected 30 min before) and the angiotensin-converting enzyme inhibitor captopril (Capt, 100 mg/kg, i.p., 120 min before) on the nociceptive response in (a), and paw swelling in (b), induced by subplantar injections of *T. nattereri venom* (*Tn* 1–0.1 μg) in mice. Columns and vertical bars are means ± S.E.M. of six animals in each group. (*) Different from corresponding control (C) ($p < 0.05$).

slightly reduced the paw edema (15%) induced by *T. nattereri* venom compared to control (106.2 ± 4.9 mg) (Fig. 2). A lower dose of TKI (30 mg/kg, i.p.) did not change the venom-induced nociceptive (147.0 ± 26.2 s) and inflammatory responses (89.2 ± 6.1 mg).

To evaluate a possible involvement of the kallikrein-kinin system in the venom-induced local effects, mice were pretreated with the angiotensin-converting enzyme inhibitor, captopril (100 mg/kg, i.p.). This inhibitor prevents degradation of bradykinin, thus inducing potentiation of kallikrein effects. As shown in Fig. 2, administration of captopril (3 mg/kg, s.c.) increased the paw licking time and paw swelling induced by 0.1 μg *T. nattereri* venom in mice by two-fold (Fig. 2).

3.4. *T. nattereri venom cleaves synthetic substrates from human kininogen*

To verify the presence of kinin releasing enzymes in the venom of *T. nattereri*, the enzymatic activity of the venom on synthetic substrates derived from human kininogen sequences was evaluated. Three internally quenched fluorescent peptides containing the sequence related to the bradykinin region of human kininogen were used to assess the substrate specificity requirements: (1) Abz-MISLMK**RPQ**-EDDnp, (Abz-Met-Ile-Ser-Leu-Met-Lys-Arg-Pro-Gln-EDDnp) corresponding to bradykinin *N*-terminal, (2) Abz-**GFSPFR**SSRQ-EDDnp (Abz-Gly-Phe-Ser-Pro-Phe-Arg-Ser-Ser-Arg-Gln-EDDnp), corresponding to bradykinin *C*-terminal, and (3) Abz-LGMISLMK**RPPGWSPFR**SSRIW-NH_2 (Abz-Leu-Gly-Met-Ile-Ser-Leu-Met-Lys-Arg-Pro-Pro-Gly-Trp-Ser-Pro-Phe-Arg-Ser-Ser-Arg-Ile-Trp-NH_2) peptide containing the entire bradykinin sequence. Hydrolysis of the two first peptides was observed by increase in fluorescence levels. Furthermore, HPLC analysis of the reaction products revealed fragments with the same retention time as the authentic synthesized fragments correspondent to cleavage of peptide (1) at the M–K bonds, the peptide (2) in R–S bond, and peptide (3) releasing KRPPGWSPFR (Lys-Arg-Pro-Pro-Gly-Trp-Ser-Pro-Phe-Arg), which corresponds to Lys-bradykinin or Kallidin (Table 1). The last fragment (from peptide 3) was detected using tryptophane (W) as fluorescent group and further analyzed by mass spectroscopy, confirming the molecular mass of Kallidin (Table 1).

The hydrolysis of the synthetic peptides 1, 2 and 3 was evaluated as described above in the presence of inhibitors of the major groups of proteolytic enzymes. The enzymatic activity was inhibited by metal-chelating agents (EDTA

Table 1
Cleavage sites of the hydrolysis by *T. nattereri* venom of the synthetic substrates derived from human kininogen sequence, determined by high performance liquid chromatography (HPLC) and by mass spectrometry (MS)

Substrate	Detected fragments (HPLC retention time)	MW (calculated)	Observed ion (*m/z*)
Abz-LGMISLM↓KRPQ-EDDnp	Abz-LGMISLM[a]	–	–
	KRPQ-EDDnp[a]	–	–
Abz-GFSPFR↓SSRQ-EDDnp	Abz-GFSPFR[a]	–	–
	SSRQ-EDDnp[a]	–	–
Abz-LGMISLM↓KRPPGWSPFR↓SSRI-NH_2[c]	KRPPGWSPFR[b]	1226.7	1227
	Abz-LGMISLM[a]		

(↓) Indicates the cleavage site.

[a] Cleavage sites determined by comparison of the retention time in the HPLC analysis of the reaction products with the corresponding synthetic fragments (Abz-LGMISLM, KRPQ-EDDnp, SSRQ-EDDnp, etc.).

[b] Fragment isolated in the HPLC analysis and submitted to MS analysis.

[c] To facilitate the detection (following the triptofan fluorescence) and the isolation of this fragment the Phe residue at this position (in the natural kininogen sequence) was replaced by a Trp residue (underlined residue) in the synthetic peptide assayed.

Table 2
Kinin-release by *T. nattereri* venom measured by radioimmunoassay

Sample	Kininins (pg)
HK	200
HK + Plasma kallikrein	16,000
HK + *T. nattereri* venom	240
LK	530
LK + Tissue kallikrein	13,500
LK + *T. nattereri* venom	6,300

T. nattereri venom (3 μg) was incubated with human high (HK) or low (LK) molecular mass kininogen (200 nM) in 50 mM tris-buffer, pH 7.41, 0.1 M NaCl for 10 min at 70 °C.

and *o*-PHE). In contrast, PMSF, TLCK, TPCK, E-64, and Pepstatin-A did not present inhibitory effect on action of whole venom.

3.5. Kinin release by T. nattereri venom

The ability of the venom to generate kinins from natural kininogen was evaluated by radioimmunoassay, incubating the venom with human high or low molecular mass kininogen. The results presented in Table 2 show that incubation of the venom with high molecular mass kininogen resulted in kinin levels similar to controls. In contrast, when incubated with human low molecular mass, kininogen venom induced the release of kinin (6300 pg compared to 13,500 pg released by tissue-kallikrein enzyme), thus, confirming the existence of venom peptidases with substrate specificity and activity similar to tissue-kallikrein enzymes.

4. Discussion

The main symptoms of *T. nattereri* envenomation include local edema and excruciating pain that develop a few minutes after the accident. Current treatment for *T. nattereri* accidents using anti-inflammatory drugs is ineffective [1]. Thus, a better understanding of the mechanisms regulating the acute inflammatory events could lead us to the development of more efficient therapeutic strategies. This study investigated the nociceptive and edematogenic activities of the poisonous fish *T. nattereri* in mice and the influence of major nociceptive and inflammatory mediators on both responses.

Injection of *T. nattereri* venom into the mouse foot paw reproduced the edema and nociception observed in human accidents. Significant inhibition of the nociceptive response was obtained after treatment with the opioid analgesic fentanyl. These drugs are known to produce analgesic effect as κ and μ agonists at spinal and supraspinal levels [18].

Pretreatment with the anti-inflammatory agents dexamethasone (steroidal) or indomethacin (non-steroidal) was ineffective on the venom-induced nociception and edema, indicating that eicosanoids generated from the arachidonic acid are not involved. These results may explain why the clinical use of these drugs has never led to any effect on patients stung by *T. nattereri*. Moreover, pretreatment with either cyproheptadine, a serotonin antagonist, or L-NAME, a nitric oxide synthase inhibitor, did not affect the nociceptive and edematogenic responses, excluding thus a major role of serotonin and nitric oxide as inflammatory mediators in the local effect of *T. nattereri* venom. In addition, inflammatory signs measured by cytokine release and cell migration in the response to *T. nattereri* venom injury in mouse foot paw were very discrete [19], suggesting a dissociation of classical inflammatory response and the symptoms of envenomation, including edema and nociception.

Both nociceptive and edematogenic responses of the venom were reduced by the tissue kallikrein inhibitor phenylacetyl-FSR-EDDnp (TKI), an antagonist previously shown to exert anti-nociceptive and anti-inflammatory actions in experimental models involving injuries related to kinin formation by tissue kallikrein [11]. In contrast, the plasma kallikrein inhibitor $PKSI_{527}$ did not influence the venom-induced nociceptive response, but it slightly decreased the paw edema. These findings indicated that pain and edema induced by *T. nattereri* venom could be associated with the kallikrein products. The role of kinins was further confirmed after inhibition of the angiotensin-converting enzyme with captopril, which increased both edema and nociceptive responses induced by the venom.

Kinins are well known mediators of inflammation. In this report, we show that kallikreins appear to be major mediators of nociception and important components on edema, independently of classical inflammatory pathways. The role of kinins in venom-induced edema was suggested previously for wasp stings. However, in this case, histamine, serotonin and lipoxigenases derivatives were involved [20]. Kinins were also reported to induce hyperalgesia after injection of *Bothrops asper* snake venom, and the effect was also inhibited by dexamethasone, suggesting the participation of arachidonic acid derivatives [21].

Rocha e Silva et al. and coworkers [22] described for the first time a kinin-releasing enzyme using as model the venom of *Bothrops jararaca*. Since then, the presence of kallikrein-like enzymes has been extensively described in a large number of different animal venoms [23]. Most of these studies, however, are related to the hypotensive effects of bradykinin. Recent studies also reported the presence of kallidin-releasing enzymes from *Bitis arietans*, *Trimeresurus elegans*, and *Phoneutria nigriventer* [24–28]. In this study, we also characterized the presence of a tissue-kallikrein-like enzyme in *T. nattereri* venom. Using in vitro enzymatic assays, venom samples hydrolyzed the human kininogen-derived synthetic peptides releasing Lys-bradykinin; additionally, it also released kinins when pre-incubated with low molecular weight kininogen.

A series of protease inhibitors were used to verify the nature of the kinin-releasing enzyme presented in

T. nattereri venom. Surprisingly, the classical serinoproteinase inhibitors did not interfere with peptide cleavage. Aspartyl and cysteine-peptidase inhibitors also failed in reducing enzyme activity. On the other hand, metal-chelating agents as EDTA and *ortho*-Phenanthroline inhibited the hydrolysis of kininogen-derived peptides. This suggests that the kallikrein-like activity of *T. nattereri* venom could be attributed to metalloproteinases. However, this is in disagreement with the current classification of kallikreins, which are classified as serinoproteinases. The effect of chelating agents could also be explained by the necessity of metal ions for stabilization of the enzyme structure, which may occur independently of the class of proteinase. In order to characterize the *T. nattereri* venom tissue-kallikrein-like enzyme, the component is currently being isolated and sequenced in our laboratory and this further work will certainly explain the nature of the enzyme.

In conclusion, the presented data indicate that nociception and edema induced by *T. nattereri* venom follow a peculiar inflammatory mechanism where kallikrein products play an important role and classical inflammatory pathways appear to exert minor influence. These findings must be considered in order to achieve efficient treatment for human victims of this fish.

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