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Why myotoxin-containing snake venoms possess powerful nucleotidases?

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ABSTRACT

The venom of the snake *Bothrops asper* causes muscle necrosis, pain and inflammation. This venom contains myotoxins which cause an increase in intracellular Ca^{2+} concentration and release of K^+ and ATP from myotubes. ATP is a key danger molecule that triggers a variety of reactions, including activation of the innate immune response. Here, using ATP-luciferase bioluminescence imaging technique, we show for the first time *in vivo*, that the purified myotoxins induce rapid release of ATP, whilst the complete venom of *B. asper* does at a very small extent. This apparent contradiction is explained by the finding that the venom contains powerful nucleotidases that *in vivo* convert ATP into ADP, AMP and Adenosine. These findings indicate that high concentrations of adenosine are generated by the double action of the venom and provide the experimental basis to the suggestion that *in situ* generated adenosine plays an important role in envenomation via its hypotensive, paralyzing and anti-coagulant activities.

1. Introduction

Snake bite envenomation has a major impact on human health in tropical and sub-tropical regions of the world [1,2]. Snake venoms may cause death, but more frequently they cause permanent disabilities secondary to prominent tissue damage [3–6]. However, despite the large effect on public health, their importance is not sufficiently appreciated and the mechanisms of pathogenesis of the alterations which follow envenomation are not sufficiently understood. The majority of snake bite envenomations in Central and South America are caused by species of the genus *Bothrops*, including the very dangerous lance-head vipers *B. asper* and *B. atrox*. These venoms cause pain, hemorrhage, hypotension, and myonecrosis, together with inflammation. A major role in the pathophysiology of envenomations by *B. asper* is played by myotoxins which rapidly induce local myonecrosis, provoking a prominent inflammatory response and permanent tissue damage. We have previously studied in detail the effect of *B. asper* myotoxins on myotubes and *ex vivo* muscles and found that they cause Ca^{2+} entry and release of K^+ and ATP [7–10]. We have also shown that the released ATP diffuses around the site of toxin application and extends beyond the area of damage [10]. Indeed, ATP is known to exert a large variety of pharmacological effects [11–13]. Our experimental work was performed both with C2C12 cultured mouse myotubes and excised hind limb muscles. A consequent extension of these studies is that of assessing the venom effects *in vivo*. By imaging the luciferin emitted chemiluminescence, here we show that *B. asper* myotoxins injected into the hind limbs of mice also induce ATP release *in vivo*. However, on the contrary, little ATP was detected at the site of injection of the whole venom. This apparent contradiction led us to investigate the possible presence of adenine-nucleotidases in this venom, and here we report that these enzymes are present and highly active; not only this explains the ATP *in vivo* imaging data, but also provides an explanation for the evolutionary advantage of the presence of nucleotidases in all those snake venoms that also have myotoxins.

2. Materials and methods

2.1. Venoms and toxins

The venom of *B. asper* consisted of a pool obtained from more than 40 adult specimens collected in the Atlantic region of Costa Rica and kept at the serpentarium of Instituto Clodomiro Picado, University of Costa Rica; venom was lyophilized and stored at −20 °C. Myotoxins I and II were isolated from *B. asper* venom by ion-exchange chromatography on CM-Sephadex C-25 as described [14,15] followed by RP-HPLC on a C8 semi-preparative column (10 × 250 mm; Vydac) eluted at 2.0 ml/min with a 0–70% acetonitrile gradient containing 0.1% trifluoroacetic acid, during 30 min, on an Agilent 1200 instrument monitored at 215 nm. Homogeneity of the final preparations was assessed by analytical reverse-phase

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HPLC on a C4 column (4.6 × 150 mm) using a gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid (v/v). Venom samples were dissolved in 10 mM Hepes and 150 mM NaCl with 50% glycerol and sterilized by filtration through 0.22 μm GV Durapore® (Millipore).

2.2. Animals

Adult mice (C57 Bl6 strain) weighing 25–30 g were used. All experimental procedures involving animals were carried out in accordance with the Italian Animal Welfare Act and were approved by the local authority veterinary service.

2.3. In vivo ATP imaging

In vivo bioluminescent imaging was performed with an ultra low-noise, high sensitivity cooled CCD camera mounted on a light–tight imaging chamber (IVIS Lumina System, Caliper, Perkin Elmer). Tracking, monitoring and quantification of signals were controlled by the acquisition and analysis software Living Image. Mice were anesthetized with a continuous flux of isoflurane, positioned in the instrument chamber and injected with a 50 μl syringe fitted with a 29 gauge needle (Hamilton). For each mouse, the right leg was injected intramuscularly with a mixture composed of the reporter solution (luciferase–luciferine mix, Promega) and the toxin or the venom indicated, in a total volume of 20 μl. The contralateral leg was used as control, i.e. it was injected with the reporter solution plus the vehicle used to solubilize toxins. Two mice per each experimental run were monitored immediately after injections; luminescent images were obtained with constant exposure time periods of 5 min for a total time of 30 min; regions of interest were defined manually around the site of injection for determining total photon flux (photons per second).

2.4. Colorimetric assay of orthophosphate

ATPase, ADPase and 5′-nucleotidase activities were determined according to previously described methods [16–18], using a colorimetric assay for orthophosphate liberation from nucleotides. *B. asper* venom was diluted in incubation buffer (0.1 M glycine–NaOH, pH 8.9), in the range of 1.25–640 μg/ml. Samples (150 μl) of each venom dilution were added in duplicate to Eppendorf tubes. Incubation buffer alone was used as a blank. The same volume of 1 mM ATP (disodium salt, Sigma, USA), 1 mM ADP (sodium salt, Sigma, USA) or 1 mM AMP (sodium salt, Sigma, USA) dissolved in incubation buffer containing 3.8 mM $MgCl_2$, was subsequently added to the venom solutions and blanks and incubated for 15 min at 37 °C. Duplicates of orthophosphate solutions (KH_2PO_4, ranging from 1.88 to 30 nmol) were used for the construction of standard curves. The color reagent (81.1 mM ascorbic acid, 3 mM ammonium molybdate, and 0.5 M H_2SO_4) was added (700 μL for each tube) and then incubated at 37 °C for 1 h. Absorbance was determined at 820 nm using a Lambda 25 UV/V is spectrophotometer (Perkin Elmer) and values were plotted versus venom concentration. The amount of venom that liberated 15 nmol of orthophosphate was used for comparison. Specific activities of nucleotidases on ATP, ADP and AMP were calculated and expressed in μmol of orthophosphate/min/mg of venom. The mean values of three independent experiments were used.

3. Results

3.1. Injection of B. asper myotoxins causes ATP release in vivo

Lys-49 (Mt-II) and Asp-49 (Mt-I) myotoxins induce the release of ATP from cultured myotubes and excised muscle [10], which then displays its various functions [11–13]. It remained to be demonstrated whether this ATP release does occur *in vivo* as well. This type of analysis can be performed in animal models with the reporter system luciferase–luciferin. A previous work used the injection of stably transfected cells expressing the luciferase gene on their surface; in the presence of ATP, the administration of luciferin triggers a chemiluminescent reaction of luciferase, and the photons emitted can be detected by an appropriate imaging apparatus [19]. Other studies used different techniques, for example electroporation to administrate luciferase to cells [20]. In preliminary experiments, we found that the reporter system luciferase–luciferin adsorbs onto the muscle cells and reports efficiently changes in extracellular ATP levels. We took advantage of this finding and, consequently, injected the luciferase–luciferin mixture in the mouse hind legs together with toxins or venoms. We injected in the mouse hind limbs 50 μg of Mt-II myotoxin, an amount much lower than that present in *B. asper* venom: a single bite is estimated to inject milligrams of venom [21], and Lys-49 myotoxins comprise about 20% of venom proteins [22]. Fig. 1 shows that this amount is sufficient to induce a rapid release of ATP. The contralateral leg was injected with carrier solution and the differential effect is evident by simply examining the chemiluminescent signal (panel A of Fig. 1); the relative quantification is reported in panel B of Fig. 1. ATP runs down in about 10 min following dilution into the body fluids and owing to the activity of the ecto-ATPases present on cell surfaces. Panel C of Fig. 1 shows the effect of the other major myotoxin present in *B. asper* venom, the Asp-49 myotoxin Mt-I which acts via its PLA_2 activity and is less active than Mt-II on myotubes in culture [8,9]. Also Mt-I causes a rapid ATP release *in vivo*, but the extent of this release is lower than that caused by Mt-II, with a remarkable agreement of between the results obtained in cell cultures and the present results obtained *in vivo*. The absolute value of the extent of the effect varies from sample to sample but this is corrected by the internal ratio between one leg and the opposite one. Ratios are reported in panels B and D of Fig. 1. The injection of 100 μg of Mt-I produces an effect similar to that of 50 μg of Mt-II; in addition, the ATP release is less prolonged than in the case of Mt-II.

3.2. Injection of B. asper venom causes little ATP release in vivo

From these data, the fact that the two myotoxins are present together in *B. asper* venom and act synergistically in inducing Ca^{2+} entry from the medium into muscle cells [9], we were expecting that the injection of the venom would induce a large ATP release *in vivo*. On the contrary, the injection of 150 μg of BaV induced a very small ATP release as compared with that released by the isolated myotoxins (Fig. 2, panel A). As myotoxins comprise approximately 30% of total venom proteins [22], their content in the venom dose used here is lower with respect to the experiment with purified toxins, but higher venom doses could not be used because they caused a strong local damage which saturated the ATP reporter system and that did not allow for an appropriate measure. Another way to compare the effect of toxins and venom that also overcomes the variability among animals is to determine the internal ratio between the total photons emitted from the treated leg to the control leg. Panel B of Fig. 2 shows that both toxins generate an emission four times higher than the control, for the venom the ratio is only 1.3, thus confirming the lower effect induced by the venom.

3.3. B. asper venom contains powerful nucleotidases

A possible way to explain this apparent paradox is to assume that ATP released by the myotoxins is hydrolyzed as soon as it comes out from the damaged muscle cells. Indeed, it was recently