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NPP-BJ, a nucleotide pyrophosphatase/phosphodiesterase from *Bothrops jararaca* snake venom, inhibits platelet aggregation

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ABSTRACT

Enzymes of the pyrophosphatase/phosphodiesterase family have multiple roles in extracellular nucleotide metabolism and in the regulation of nucleotide-based intercellular signaling. Snake venoms contain enzymes that hydrolyze nucleic acids and nucleotides, but their function is poorly understood. Here we describe for the first time the isolation and functional characterization of a soluble phosphodiesterase from *Bothrops jararaca* venom, which shows amino acid sequence similarity to mammalian nucleotide pyrophosphatase/phosphodiesterase 3 (NPP3), and inhibits ADP-induced platelet aggregation. The enzyme, named NPP-BJ, showed an apparent molecular mass of 228 kDa by size exclusion chromatography. NPP-BJ exhibited nuclease activity as well as pyrophosphatase and phosphatase activities, preferentially hydrolyzing nucleoside 5′-triphosphates over nucleoside 5′-diphosphates, but was not active upon nucleoside 5′-monophosphates. Depending on the substrate used, dithiothreitol and EDTA differently inhibited the catalytic activity of NPP-BJ. Platelet aggregation induced by ADP was also abrogated by NPP-BJ, whereas thrombin-induced platelet aggregation was only slightly attenuated. However, polyclonal antibodies raised against NPP-BJ could not abolish the lethal activity of *B. jararaca* venom. Altogether, these results show that NPP-BJ has a minor contribution to the lethal activity of this venom, but interferes with mechanisms of ADP-induced platelet aggregation.

1. Introduction

Snake venoms are an abundant and complex source of peptides, proteins and enzymes with medical and biological interest. The jararaca (*Bothrops jararaca*) is a snake of wide territorial distribution in the southeast Brazil, and 98% of the snake accidents are caused by *B. jararaca* in São Paulo State (Santoro et al., 2008). *Bothrops* venoms are very toxic to animals and human beings, and contain a variety of toxins that disturb hemostasis, including proteins and enzymes that impair platelet function (Sano-Martins and Santoro, 2003). The characterization of toxins that act on blood platelets has focused on searching compounds with a direct action on platelet receptors, either inhibiting or activating platelets (Sano-Martins et al., 2003). Enzymes that act on platelets indirectly, producing mediators that interfere in platelet aggregation, have had minimal interest, except for L-amino acid oxidases (Takatsuka et al., 2001).

Snake venoms are a rich source of nucleases and nucleotidases (de Roodt et al., 2003a; Sales and Santoro, 2008). These enzymes act directly on mono- and/or polynucleotides, liberating nucleosides and nucleotides, which remarkably control several biological systems (Ralevic and Burnstock, 1998), including platelet function (Haslam and Cusak, 1981). Nucleotides play such a role in the control of

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hemostasis that platelets store large amounts of several nucleotides in dense bodies (Goetz et al., 1971), whose content is secreted during platelet activation. Guanosine, inosine, cytidine and uridine nucleotides have been reported to interfere in platelet function (Packham et al., 1972, 1974), but adenosine and their nucleotides (ATP, ADP and AMP) – whose purinergic receptors P2 ($P2Y_1$, $P2Y_{12}$ and $P2X_1$) and P_1 (A_{2a}) are present in platelet membrane (Ralevic et al., 1998; Gessi et al., 2000; Kunapuli et al., 2003) – deserve special attention, inasmuch as the increase in nucleotide concentration in the extracellular milieu drastically interfere in platelet function, both *ex vivo* and *in vivo* (Packham et al., 1972, 1974; Soslau and Youngprapakorn, 1997; Enjyoji et al., 1999; Koszalka et al., 2004).

Among the enzymes that degrade nucleic acids and nucleotides in snake venoms, phosphodiesterases have been extensively studied. Various names exist in scientific literature to designate phosphodiesterases (EC 3.1.4.1): exonuclease, 5′-exonuclease, 3′-exonuclease, phosphodiesterase I, ADPase and DNase. Previous to the current technology to amplify and sequence DNA, snake venom phosphodiesterases have been used as tools to characterize and sequence nucleic acids (Holley, 1966; Ho and Gilham, 1973). In fact, venom phosphodiesterases promote the hydrolysis of phosphodiester bonds, with the successive and gradual removal of units of 5′-mononucleotides, starting from the 3′ extremity of a polynucleotide chain. These enzymes catalyze the hydrolysis of single and double strands DNA, oligonucleotides, ribosomal and transfer RNA, and nucleoside 5′-tri- and diphosphates. However, phosphodiesterases show a low activity on the *p*-nitrophenyl phosphate, a synthetic substrate for phosphomonoesterases (alkaline phosphatases) (Mackessy, 1998). Phosphodiesterase activity has been already described in several snake venoms (Mackessy, 1998), including *Bothrops* venoms (Tan and Ponnudurai, 1991; Sales et al., 2008), and they have been isolated from *Bothrops atrox* (Williams et al., 1961; Björk, 1963; Frischauf and Eckstein, 1973; Philipps, 1976) and *Bothrops alternatus* (Valério et al., 2002) venoms. Purified phosphodiesterases from the aforementioned venoms possess an apparent molecular mass of 130 and 105 kDa, respectively, and they are inhibited by EDTA (Frischauf et al., 1973; Philipps, 1976; Valério et al., 2002). Despite reports of isolation of various snake venom phosphodiesterases and their widespread use as a tool for studying nucleic acids, exclusively the total amino acid composition has been published so far (Dolapchiev et al., 1980; Ouyang and Huang, 1986; Sugihara et al., 1986; Mori et al., 1987). Besides, published papers have dealt almost exclusively with their biochemical characterization, regardless of the biological activities of phosphodiesterases during envenomation. As a unique exception, five phosphodiesterases isolated from *Crotalus* and *Vipera* snakes were reported to show low toxicity for mice and cats (Russell et al., 1963). However, the contribution of phosphodiesterases for the pathogenesis of snake envenomation is unclear, although the disturbances in extracellular levels of nucleotides have been postulated to contribute to the death of preys (Mackessy, 1998; Aird, 2002).

Inasmuch as *B. jararaca* venom contains nucleotidase activity (Sales et al., 2008), and considering that envenomation by this snake result in hemorrhagic disturbances in human beings (Sano-Martins et al., 1997; Santoro and Sano-Martins, 2004; Santoro et al., 2008), we hypothesized that phosphodiesterases might contribute to the toxicity and hemostatic disturbances evoked by this venom, insofar as the alteration in extracellular levels of purines might interfere with the steady state of homeostasis. To test this hypothesis, we isolated and characterized a phosphodiesterase from *B. jararaca* venom, and investigated its ability to impair platelet function as well as its participation in the lethal activity of *B. jararaca* venom. This phosphodiesterase was named NPP-BJ as it showed comparable enzymatic activity to members of the mammalian nucleotide pyrophosphatase/phosphodiesterase (NPP) family, and its partial amino acid sequence showed remarkable similarity to NPP3.

2. Materials and methods

2.1. Materials

A pool of lyophilized crude venom of 30 adult specimens of *B. jararaca* was supplied by the Laboratory of Herpetology, Institute Butantan, and maintained at −20 °C until the moment of use. Nucleotides, apyrase (A6257), DNAse I (D4527) and ribonuclease A (R5500) from bovine pancreas, DNA from calf thymus (D3664), bis(*p*-nitrophenyl) phosphate, thymidine 5′-monophosphate *p*-nitrophenyl ester, *p*-nitrophenyl phosphate, SCH-58261, bovine thrombin and cold water fish skin gelatin were purchased from Sigma (USA). All other reagents were of analytical grade or better.

2.2. Animals

All procedures involving the use of animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and were approved by the Ethical Committee for the Use of Animals of Institute Butantan.

2.3. Purification of NPP-BJ

NPP-BJ was purified by size exclusion and affinity chromatography on blue-sepharose and oligo-dT cellulose (GE Healthcare, Sweden). All chromatographic procedures were accomplished at room temperature on a ÄktaPrime chromatography system (GE Healthcare, Sweden), and the eluates were monitored continuously at 280 nm. Lyophilized *B. jararaca* crude venom (500 mg) was diluted in 10 ml of buffer A (50 mM Tris–HCl, pH 7.5), and centrifuged at 10,000 × *g* for 15 min at 4 °C. Two aliquots of 5 ml each were applied under the same conditions on a HiPrep 26/60 Sephacryl S-300 HR column (Amersham Biosciences), previously equilibrated in buffer A. Samples were eluted at a flow rate of 1.5 ml/min, and 10 ml fractions were collected. Fractions were tested for their ability to liberate orthophosphate from AMP, ADP and ATP, as well as to hydrolyze bis(*p*-nitrophenyl) phosphate (see below) (Fig. 1a). Fractions containing phosphodiesterase and 5′-nucleotidase activity were pooled and concentrated in Amicon stirred cells (Millipore, USA), using 3 kDa ultrafiltration discs (Millipore, USA), to the final volume of 17 ml.