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Influence of temperature upon paralyzing and myotoxic effects of bothropstoxin-I on mouse neuromuscular preparations

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Abstract

Bothropstoxin-I (BthTX-I), from *B. jararacussu* venom, is a phospholipase A_2 (PLA_2) homologue devoid of enzymatic activity. Besides inducing severe myonecrosis, BthTX-I promotes paralysis of both directly and indirectly evoked contractions in isolated neuromuscular preparations. We applied an experimental paradigm in order to characterize the steps involved in the toxic effects of BthTX-I on mouse neuromuscular junction. Myotoxicity was assessed by microscopic analysis of extensor digitorum longus muscles; paralyzing activity was evaluated through the recording of isolated contractions indirectly evoked in phrenic-diaphragm preparations. After 90 min at 35 °C, BthTX-I induced complete and irreversible paralysis, and damaged $30.3 \pm 2.7\%$ of muscle fibers. In contrast, no effect was observed when tissues were incubated with BthTX-I at 10 °C for 60 min and subsequently washed with toxin-free solution and maintained at 35 °C. These results indicate that the binding of BthTX-I to the cellular tissue surface is very weak at low temperature and that an additional factor is necessary. However, when tissues were submitted to BthTX-I (10 °C for 60 min), and the temperature was elevated to 35 °C, omitting the washing step, it was observed muscle paralysis and damage in $39.04 \pm 4.2\%$ of muscle fibers. These results indicate that a temperature-dependent step is necessary for BthTX-I to promote both its myotoxic and paralyzing activities.

Keywords: Bothropstoxin-I; Myonecrosis; Muscle paralysis; Myotoxin; Phospholipase A_2; Lys49

1. Introduction

Phospholipases A_2 (PLA_2s) constitute a prominent group of myotoxins found in venoms from snakes of the Viperidae family [1–4]. These myotoxins have been classified in two different types: the Asp49 PLA_2s, which catalyze the hydrolysis of the ester bond in the

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sn-2 position of glycerophospholipids; and the variant Lys49 PLA2s, or "PLA_2-like" proteins, which are devoid of enzymatic activity, but retain a membrane-damaging activity via a non-hydrolytic mechanism [4–8].

Bothropstoxin-I (BthTX-I) is a basic homodimeric Lys49-PLA_2 myotoxin isolated from *Bothrops jararacussu* venom [9,10]. Despite its lack of enzymatic activity [11,12], BthTX-I induces extensive myonecrosis [1–3,9] and promotes paralysis of both directly and indirectly evoked contractions in nerve-muscle preparations [13–15]. However, the relationship between the myonecrosis and the muscle paralysis induced by BthTX-I has not been fully examined, and further studies are necessary to evaluate whether these effects are manifestations of the same toxic mechanism.

To achieve a deeper understanding of this matter, in this work we adapted a classic experimental paradigm [16] to study the steps underlying the paralyzing and the myotoxic effects of BthTX-I. This paradigm has been successfully used to identify the sequence of events involved in both the neuromuscular blockade induced by neurotoxic PLA_2s [17,18] and the cytolytic effect of some myotoxic PLA_2s [19,20]. In this work, paralyzing activity was evaluated through the recording of indirectly evoked contractions of phrenic-diaphragm (PD) preparations, whereas myotoxicity was assessed by light microscopic analysis of extensor digitorum longus (EDL) muscles.

2. Material and methods

2.1. Materials

BthTX-I was donated by IPEN/CNEN (São Paulo, Brazil). It was isolated from *Bothrops jararacussu* venom according to procedures described by Spencer et al. [21]. All other reagents were of analytical grade. BthTX-I was dissolved in physiological saline solution (PSS, 0.9% NaCl).

2.2. Animals

Adult male mice weighing 25–30 g were maintained under a 12 h light–dark cycle (lights on at 07:00 h) in a temperature-controlled environment ($22 \pm 2\,^{\circ}C$) for at least 10 days prior to the experiments. Food and water were freely available. Animal procedures were in accordance with the guidelines for animal care prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA.

2.3. Evaluation of paralyzing activity

Animals were killed by exsanguinations and the PD preparations were removed and mounted vertically in a conventional isolated organ-bath chamber containing 15 ml of physiological solution of the following composition (mmol/l): NaCl, 135; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; $NaHCO_3$, 15; Na_2HPO_4, 1; glucose, 11. This solution was bubbled with carbogen (95% O_2 and 5% CO_2). The preparations were attached to isometric force transducers (Grass, FT03), for recording the twitch tension. The transducer signal outputs were amplified and recorded on computer via a transducer signal conditioner (Gould, 13-6615-50) with an AcquireLab Data Acquisition System (Gould). The resting tension was 2 g. Indirect contractions were evoked by supramaximal pulses (0.2 Hz, 0.5 ms) delivered from an electronic stimulator (Grass-S88K) and applied on the phrenic nerve by means of a suction electrode. The time course for muscle paralysis was determined by the amplitude (%) of twitches relative to the initial amplitude.

2.4. Evaluation of myotoxic activity

The EDL muscles were isolated and transferred to chambers containing 5 ml of physiological solution with the same composition as that of the myographic study. This solution was bubbled with carbogen (95% O_2 and 5% CO_2). After exposure to the toxin, as described in Section 2.5, EDL muscles were removed from the bath and frozen in liquid nitrogen. Transverse sections (8 μm thick) were cut out at $-20\,^{\circ}C$ in a cryostat and stained with haematoxylin and eosin (HE) prior to examination by light microscopy [22]. Morphological damage was quantified by counting the number of fibers with lesions expressed as a percentage of the total number of cells (myonecrosis index) in three non-overlapping, non-adjacent areas of each muscle.

2.5. Experimental paradigm

In preliminary experiments, BthTX-I (1 μM) was added to the tissue bath while the temperature was