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Influence of temperature upon effects of crotoxin and gamma-irradiated crotoxin at rat neuromuscular transmission☆

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Abstract

The influence of temperature upon the effects of crotoxin (CTX), from *Crotalus durissus terrificus* venom, and gamma-irradiated (^{60}Co, 2000 Gy) crotoxin (iCTX) was studied in rat neuromuscular transmission 'in vitro'. Indirect twitches were evoked in the phrenic-diaphragm preparation by supramaximal strength pulses with a duration of 0.5 ms and frequency of 0.5 Hz. The phospholipase A_2 (PLA_2) enzymatic activity of CTX and iCTX was assayed against phosphadityl choline in Triton X-100. At 27°C, CTX (14 μg/ml) did not affect the amplitude of indirectly evoked twitches. However, at 37°C, CTX induced a time-dependent blockade of the neuromuscular transmission that started at 90 min and was completed within 240 min. iCTX (14 μg/ml) was inneffective on the neuromuscular transmission either at 27 or 37°C. The PLA_2 enzymatic activity of CTX at 37°C was 84 and that at 27°C was 27 μmol fatty acid released/min/mg protein, and that of the iCTX at 37°C was 39 μmol fatty acid released/min/mg protein. Thus, it was concluded that the mechanism of detoxification of CTX by gamma radiation at the neuromuscular level relies on the loss of its PLA_2 enzymatic activity.

Keywords: *Crotalus durissus terrificus*; Crotoxin; Gamma radiation; Neuromuscular transmission

1. Introduction

Ionizing radiation has been successfully employed to detoxify snake venoms without affecting their antigenic properties (Salafranca, 1973; Kankonkar et al., 1975; Herrera et al., 1986;

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Murata et al., 1990; Nascimento et al., 1996). Since snake venoms are employed as immunogens for the production of ophidic antisera, ionizing radiation could be a useful tool to produce atoxic immunogen using highly lethal proteins. In this way, our group has been studying the effects of gamma radiation on the immunogenic and toxic properties of crotoxin (CTX), the main toxin of *Crotalus durissus terrificus* venom (Murata et al., 1990; Nascimento et al., 1996; Gallacci et al., 1998).

CTX is a β-neurotoxin that possesses highly specific toxicity towards neuromuscular junctions. At this level, CTX acts primarily by causing a triphasic change (depression, facilitation and final blockade) of acetylcholine release from the motor nerve terminal (Chang and Lee, 1977; Hawgood and Smith, 1977). Secondarily, CTX also induces a postsynaptic blockade of neuromuscular transmission by stabilizing a desensitized state of the nicotinic receptor to acetycholine (Vital Brasil, 1966; Bon et al., 1979).

CTX is composed of two noncovalently linked subunits, a basic protein, phospholipase A_2 (PLA_2), and an acidic subunit, crotapotin (Habermann and Breithaupt, 1978; Bon et al., 1986). Crotapotin, although devoid of pharmacological intrinsic activity, acts as a chaperone of PLA_2 subunit, enhancing its toxicity and specificity to the synaptic membrane sites (Bon and Délout, 1992). There are evidences that the enzymatic activity of the PLA_2 subunit plays an important role in the neurotoxic effect of CTX (Hawgood and Smith, 1977; Marlas and Bon, 1982; Su and Chang, 1984).

We have previously demonstrated that gamma radiation reduces the major neurotoxic effect of crotoxin, i.e. the blockade of the skeletal neuromuscular transmisssion (Gallacci et al., 1998). In order to advance our understanding of the mechanism of detoxification of CTX by gamma radiation, we investigated the influence of temperature upon the effects of CTX and gamma-irradiated crotoxin (iCTX) in the phrenic-diaphragm preparation (P-D) of rats.

2. Materials and methods

Crotalus durissus terrificus crude air-dried venom was purchased from Instituto Butantan (São Paulo, Brazil). CTX was purified from this venom by gel filtration on Sephadex G-75 (Pharmacia), followed by isoelectric pH precipitation. The Bradford method was used for protein determination and purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Souza-Filho et al., 1992). Gamma radiation with ^{60}Co was performed with a GAMMACELL 220 source (produced by the Atomic Energy Commission of Canada, Ltd.). A dose of 2000 Gy was applied at the rate of 400 Gy/h, using 2 mg/ml CTX in 0.15 M NaCl, adjusted to pH 3.0 with 0.1 M HCl for solubility purposes (Gallacci et al., 1998).

Adult male rats weighing 200–250 g were housed in groups of five and maintained under a 12 h light–dark cycle (lights on: 07:00 h) in a temperature-controlled environment (22 ± 2°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 Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA. The animals were killed with ether anesthesia immediately before the P-D preparation removal. Each preparation was mounted vertically in a conventional isolated organ bath chamber containing 50 ml physiological solution of the following composition: NaCl, 135 mmol/l; KCl, 5 mmol/l; $MgCl_2$, 1 mmol/l; $CaCl_2$, 2 mmol/l; $NaHCO_3$, 15 mmol/l; Na_2HPO_4, 1 mmol/l; glucose, 11 mmol/l. This solution was gassed with O_2 (95%) + CO_2 (5%) and kept at 27 or 37°C. The polygraphic recording of muscle tension was performed by means of an isometric transducer. Indirect twitches were evoked by supramaximal strength pulses with a duration of 0.5 ms and frequency of 0.5 Hz.

PLA_2 enzymatic activity against egg yolk L-alpha-phosphatidyl choline was determined using a Radiometer pHM82 pH meter equipped with a TTT 80 titrator, an ABU 80 auto burette and a TTA 80 titration assembly. Fatty acids released by the reaction were titrated to pH 8.0 with 0.04

M NaOH under nitrogen at 37°C in an unbuffered reaction mixture. Substrate was prepared using 90 mg L-alpha-phosphatidyl choline, dried under nitrogen, and then suspended by sonication in 9 ml of 12.5 mM Triton X-100 (estimated molecular weight of 646), using a Virsonic Cell Disrupter, Model 16-850 (Virts Company, Garbener, NY, USA). When the suspension was completed, 1.0 ml of 0.1 M $CaCl_2$ was added and the substrate–$CaCl_2$ mixture was then briefly resonicated. This yielded a roughly 2:1 molar ratio of Triton X-100:phospholipid. PLA_2 activity was assayed using 1.25 μg protein/2 ml substrate/20 min. Activity was expressed in micromoles of fatty acids released per minute per milligram of protein.

Data were analysed by analysis of variance for two-way classification and post-hoc tests were subsequently performed using the Tukey–Kramer test with the significance level set at $P < 0.05$.

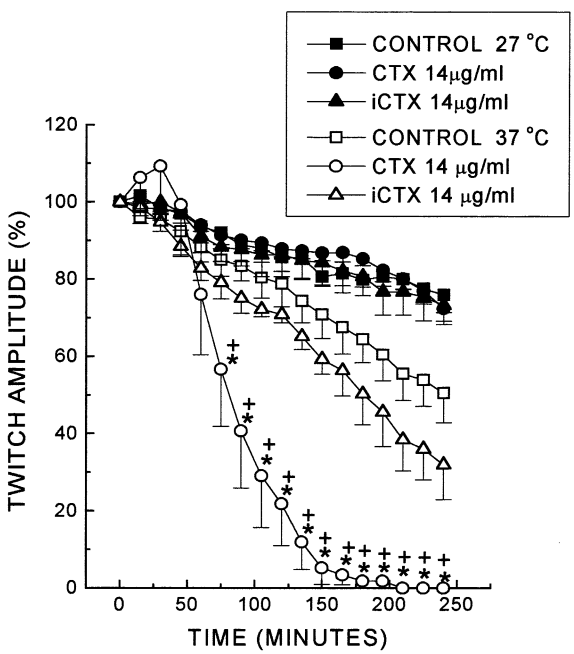

Fig. 1. Effect of temperature, 27 and 37°C, on the rate of action of crotoxin (CTX) and irradiated crotoxin (iCTX), and on the indirectly evoked twitch amplitude of phrenic-diaphragm preparation of rats. The ordinate represents the (%) amplitude of twitches relative to the initial amplitude. The abscissa indicates the time (min) after addition of CTX or iCTX to the organ bath. Each point is the mean ± S.E.M. of at least four muscles. *, $P < 0.05$ versus control at the respective temperature; +, $P < 0.05$ versus iCTX concentration at the respective temperature.

3. Results

As illustrated in Fig. 1, CTX (14 μg/ml) did not affect the twitch amplitude at 27°C. However, at 37°C, this compound induced a time-dependent blockade of the neuromuscular transmission that started at 90 min and was completed within 240 min. Differently from CTX, iCTX was without effect on the neuromuscular transmission either at 27 or 37°C.

The PLA_2 enzymatic activity of CTX at 37°C was 84 and at 27°C was 27 μmol fatty acid released/min/mg protein, and that of the iCTX at 37°C was 39 μmol fatty acid released/min/mg protein, when assayed against phosphadityl choline in Triton X-100.

4. Discussion

As suggested by Hawgood and Smith (1977), the influence of temperature upon the CTX-induced neuromuscular blockade strongly indicates the involvement of the enzymatic activity of PLA_2 subunit in the CTX neurotoxiticy. Indeed, an irreversible inactivation of PLA_2 enzymatic activity with *p*-bromophenacyl bromide completely abolishes the toxic and pharmacological activities of CTX (Jeng and Fraenkel-Conrat, 1978; Marlas and Bon, 1982). Another argument for the conjunction of PLA_2 enzymatic activity with toxicity stems from the Ca^{2+} requirements of both effects. It has been demonstrated that the blockade of the mouse diaphragm and of the cholinergic response of electroplaques is also prevented when the PLA_2 enzymatic activity is reversibly inhibited by replacing Ca^{2+} by Sr^{2+} ions in the physiological medium (Hawgood and Smith, 1977; Marlas and Bon, 1982). These observations altogether demonstrate that the PLA_2 enzymatic activity is a necessary condition for the CTX neurotoxic action.

Distinct from CTX, iCTX was without effect on neuromuscular transmission either at 27 or 37°C. These findings may be attributed to the

decrease of the enzymatic activity of the PLA_2 subunit of CTX, since it is largely known that this activity is responsible for CTX neurotoxicity. In fact, the assay of CTX and iCTX PLA_2 enzymatic activity has provided evidence that iCTX exhibits about one-half of the PLA_2 enzymatic activity of CTX. In view of this, it is conceivable that the decreased PLA_2 enzymatic activity plays an important role in the gamma radiation-induced detoxification of CTX at the neuromuscular level.

One might suggest that gamma radiation detoxifies CTX by dissociating PLA_2 and crotapotin subunits since they have to be present together for the complete neurotoxic effect of CTX (Rübsamen et al., 1971; Horst et al., 1972). Nevertheless, irradiation of CTX leads to aggregation and generation of lower molecular weight breakdown products (Nascimento et al., 1996). The aggregation products retain at least part of the higher-ordered structure of CTX, and many of its original antigenic and immunological properties. Moreover, compared with CTX, aggregates are less myotoxic and largely devoid of PLA_2 enzymatic activity.

In conclusion, the results reported here constitute the first evidence that the mechanism of detoxification of CTX by gamma radiation at the neuromuscular level relies on the loss of its PLA_2 enzymatic activity.

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