

Crotoxin, the major toxin from the rattlesnake *Crotalus durissus terrificus*, inhibits ³H-choline uptake in guinea pig ileum

L.S. Kattah¹,
M.M. Santoro¹,
C.R. Diniz^{1,2} and
M.E. De Lima¹

¹Laboratório de Venenos e Toxinas Animais, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

²Fundação Ezequiel Dias, Belo Horizonte, MG, Brasil

Abstract

We examined the effect of crotoxin, the neurotoxic complex from the venom of the South American rattlesnake *Crotalus durissus terrificus*, on the uptake of ³H-choline in minces of smooth muscle myenteric plexus from guinea pig ileum. In the concentration range used (0.03-1 μM) and up to 10 min of treatment, crotoxin decreased ³H-choline uptake by 50-75% compared to control. This inhibition was time dependent and did not seem to be associated with the disruption of the neuronal membrane, because at least for the first 20 min of tissue exposure to the toxin (up to 1 μM) the levels of lactate dehydrogenase (LDH) released into the supernatant were similar to those of controls. Higher concentrations of crotoxin or more extensive incubation times with this toxin resulted in elevation of LDH activity detected in the assay supernatant. The inhibitory effect of crotoxin on ³H-choline uptake seems to be associated with its phospholipase activity since the equimolar substitution of Sr²⁺ for Ca²⁺ in the incubation medium or the modification of the toxin with p-bromophenacyl bromide substantially decreased this effect. Our results show that crotoxin inhibits ³H-choline uptake with high affinity (EC₂₅ = 10 ± 5 nM). We suggest that this inhibition could explain, at least in part, the blocking effect of crotoxin on neurotransmission.

Key words

- Crotoxin
- Choline uptake
- Guinea pig ileum
- *Crotalus durissus terrificus*
- Phospholipase A₂

Correspondence

M.E. De Lima
Laboratório de Venenos e Toxinas
Animais
Departamento de Bioquímica e
Imunologia, ICB, UFMG
Av. Antônio Carlos, 6627
Caixa Postal 486
30161-970 Belo Horizonte, MG
Brasil
Fax: +55-31-441-5963
E-mail: delima@icb.ufmg.br

Research supported by CNPq,
CAPES, FAPEMIG and PRPq-UFMG.

Received March 3, 1999
Accepted June 21, 2000

Crotoxin from the venom of the South American rattlesnake *Crotalus durissus terrificus* (1) is a complex of two different subunits: a basic and weakly toxic phospholipase A₂, component B (CB), and an acidic, non-toxic protein, component A (CA) (2). Crotoxin exerts its pathophysiological action by blocking neuromuscular transmission. It acts primarily at the presynaptic level by altering neurotransmitter release (3,4) al-

though it is also able to act postsynaptically by stabilizing the acetylcholine receptor in an inactive state (5). Crotoxin blocks acetylcholine release induced by *Tityus serrulatus* venom in guinea pig ileum (6) and the response of smooth muscle myenteric plexus to field electrical stimulation (7,8).

In the present study we determined the effect of crotoxin on the transport of choline by innervated longitudinal muscle minces of

guinea pig small intestine. We found that crotoxin partially inhibited ^3H -choline uptake and propose that this may be related to the neuromuscular blockade caused by the toxin. Parts of the present data have appeared previously in different form (9).

The ^3H -choline uptake assays were carried out by the method of Pert and Snyder (10), with minor modifications. Briefly, fragments of smooth muscle myenteric plexus were weighed and minced with scissors into small pieces, and samples (about 40 mg of tissue) in triplicate were suspended in 1.0 ml depolarizing buffer (Tyrode solution containing 50 mM KCl, with equimolar reduction of NaCl), at 37°C for 15 min upon agitation, to release endogenous acetylcholine. After washing with 1 ml Tyrode solution (composition: 136 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl_2 , 5.5 mM glucose, 10 mM Tris, and 20 μM paraoxan, pH 7.4), minces were incubated for 10 min in 1 ml of normal buffer at 37°C, with or without crotoxin. One μCi ^3H -choline (12 nM) in 1.0 ml of normal or depolarizing incubation buffer was added to the sample and the incubation prolonged for 10 min more. Incubations were stopped by the rapid addition of ice-cold Tyrode solution with 1.0 μM unlabeled choline and by centrifugation (13,000 rpm, 5 min) at 4°C using a Marathon 13K/H microcentrifuge (Marathon, Pittsburg, PA, USA). Nonspecific or passive uptake was defined as uptake occurring at 4°C. The ^3H -choline uptake is expressed as cpm or as percentage of control at 37°C. The tissue was solubilized with 50 μl 1 M NaOH for 40 min at 60°C. After dilution with 1 ml Tyrode and homogenization, 250- μl samples were counted by liquid scintillation spectrometry in a liquid scintillation counter (LKB/Walack, Bromma, Sweden).

All experiments were performed on preparations obtained from male and female guinea pigs (250-600 g). The animals were anesthetized with CO_2 , killed by a blow to the head and bled. The entire small intestine was

rapidly removed and washed with Tyrode solution at 37°C. The longitudinal muscle strips containing adherent (Auerbach's) myenteric plexus were obtained by the method of Paton and Zar (11).

In some experiments, the supernatants were used for the determination of lactate dehydrogenase (LDH) activity according to Kubowitz and Ott (12). Briefly, an aliquot from the supernatant (150 μl) was placed in a cuvette containing LDH assay buffer (50 mM phosphate, 0.6 mM sodium pyruvate and 0.2 mM NADH). The absorbance (365 nm) was recorded at 1, 2 and 3 min using a Shimadzu UV-160A spectrophotometer and the enzymatic activity was calculated. One unit of enzymatic activity is defined as that producing oxidation of 1 μM NADH/min at 25°C. Total lysis, corresponding to 100% LDH in minces, was determined after treatment with 1% Triton X-100.

The alkylation by p-bromophenacyl bromide was performed at 30°C in 1.5 ml of 100 mM sodium cacodylate buffer, pH 5.8, and 100 mM NaCl. The phospholipase isolated from the crotoxin complex (15 μM) was preincubated for 1 h under these conditions before the reaction was started by adding 15 μl of 8 mM p-bromophenacyl bromide in acetone. After incubation at 30°C for 60 min, excess reagent was eliminated by dialysis against water. The crotoxin complex was reconstituted by adding CA (in 1.5 ml 50 mM Tris-HCl, pH 7.4, 0.1 M NaCl) to CB (1.5 CA:1.0 CB) treated with p-bromophenacyl bromide. All assays were done in triplicate and repeated at least three times ($N = 3$).

Data were analyzed statistically by the Tukey test, Dunnett test, Student *t*-test, and F test (13). The test performed for each experiment is described in the legends to the figures.

^3H -Choline uptake and the release of LDH, a cytoplasmic enzyme marker for tissue integrity, were monitored at different time intervals (Figure 1A and B). The levels of LDH measured in samples treated for 20

min with 0.3 μ M crotoxin were similar to those of the control and amounted to about 20% (Figure 1B), while the inhibitory effect on ³H-choline uptake under the same conditions was about 50%. After a longer incubation time (e.g., 120 min) with 0.3 μ M crotoxin (the concentration used in most experiments), ³H-choline uptake was drastically reduced to values similar to those measured at 4°C (Figure 1A, inset), while LDH activity increased to 40% in the supernatant. Crotoxin (0.3 μ M) inhibited ³H-choline uptake after 2-4 min of incubation, whereas the increase in LDH activity was detected several minutes later (data not shown). The calculated EC₂₅ (10 \pm 5 nM) showed a high affinity of crotoxin for this preparation (data not shown). Different controls (inset in Figure 1) were regularly performed in all experiments and included incubations at 4°C and at 37°C in normal and in depolarizing medium. About 20-30% uptake was observed in normal medium at 4°C. These values were interpreted as passive uptake. In depolarizing medium the choline transporter was inhibited as described (14). The difference between total uptake measured at 37°C and passive uptake measured at 4°C corresponds to high affinity choline transport. This type of transport is characteristic of nerve cells (10). The total uptake at 37°C was considered as 100%.

To determine if the phospholipase activity from crotoxin was implicated in the inhibition of ³H-choline uptake, two different experiments were carried out. The modification of crotoxin with p-bromophenacyl bromide, which inactivates the phospholipase A₂ activity of crotoxin (15), decreased the inhibitory effect of the toxin from 50% to about 20% (Figure 2A). The equimolar substitution of Sr²⁺, a competitive inhibitor of Ca²⁺ for phospholipase activity, for Ca²⁺ with the addition of 1 mM EGTA in the medium also decreased this effect of the toxin (Figure 2B). Note that in both conditions crotoxin still showed a partial, but not significant inhibitory effect. The existence

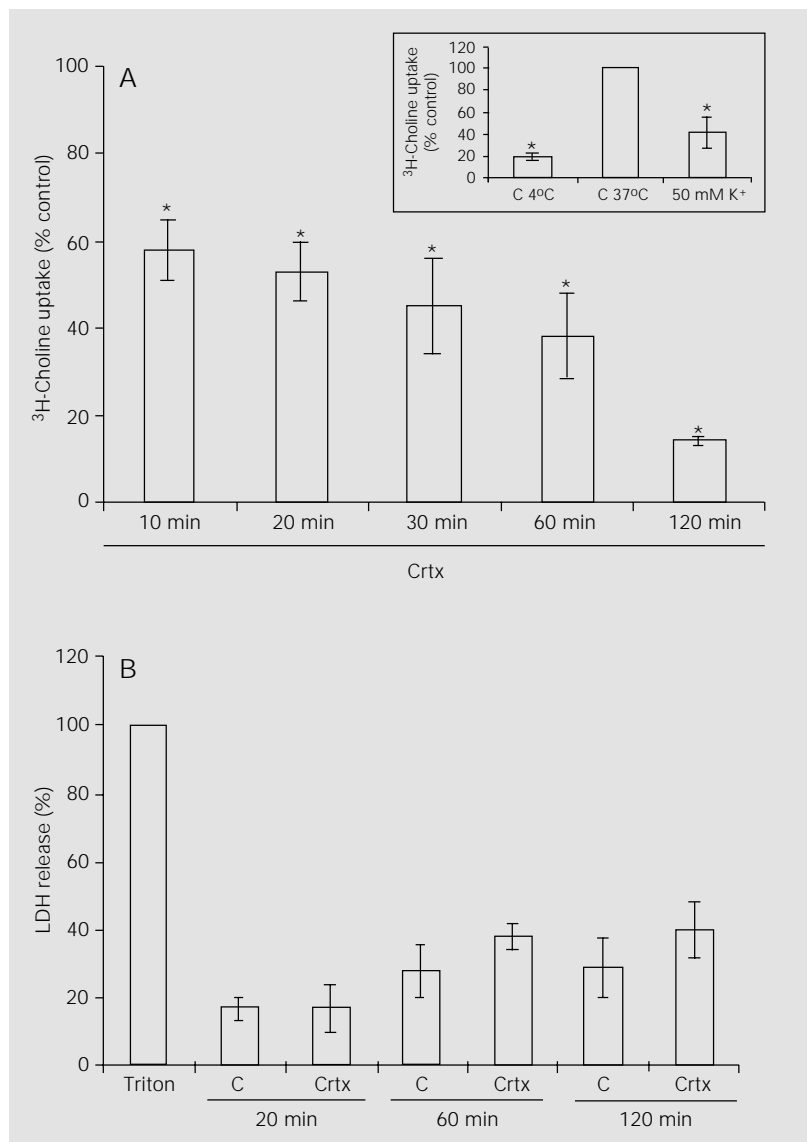


Figure 1 - Effect of pre-incubation time with crotoxin (Ctx) on ³H-choline uptake inhibition and lactate dehydrogenase (LDH) release. A, Minces of neuromuscular preparation (40 mg in triplicate) were pre-incubated for different periods of time at 37°C in Tyrode solution in the presence of 0.3 μ M Ctx. ³H-Choline (12 nM) was added and the uptake measured after 10-min incubation. Values are reported as percentage of total uptake (control at 37°C) and represent the mean and standard deviation of the mean of 3 different experiments. *P<0.05 compared to control (uptake at 37°C). Analysis of variance (P = 0) showed that there was a difference between treatments. Using the F test for repeated measures and calculating the reverse Helmert contrasts we observed that: control>10' (P = 0.012), 10'>20' (P = 0.014), 20'>30' (P = 0.009), 30'>60' (P = 0.024) and 60'>120' (P = 0.006). Inset: Control (C) values obtained in 3 different experiments at 37°C (100% uptake) in normal and depolarizing medium (50 mM K⁺) and at 4°C in normal medium, for 10 min after the addition of ³H-choline (12 nM). *P<0.05 compared to control at 37°C (t-test). B, LDH released in the control condition (C) and upon incubation with Ctx (0.3 μ M) for different periods of time at 37°C. The LDH released by the preparation treated with 1% Triton X-100 was taken as 100%. Values (mean and standard deviation of the mean of 3 different experiments) were not significant compared to the respective controls (P>0.05).

of a residual phospholipase activity of crotoxin under our experimental conditions could explain this result. The inhibitory effect of crotoxin on the release of acetylcholine induced by tityustoxin in this same neuromuscular preparation has also been suggested to depend on its phospholipase activity (6). The inhibitory effect of some fatty acids on choline uptake has already been shown in rat brain synaptosomes (16) and was confirmed in preliminary experiments using our preparation (data not shown). This effect also argues in favor of a role of the phospholipase activity of crotoxin on the inhibition process. The fatty acids released

by crotoxin phospholipase activity on membrane phospholipids may be implicated in the inhibition of choline uptake.

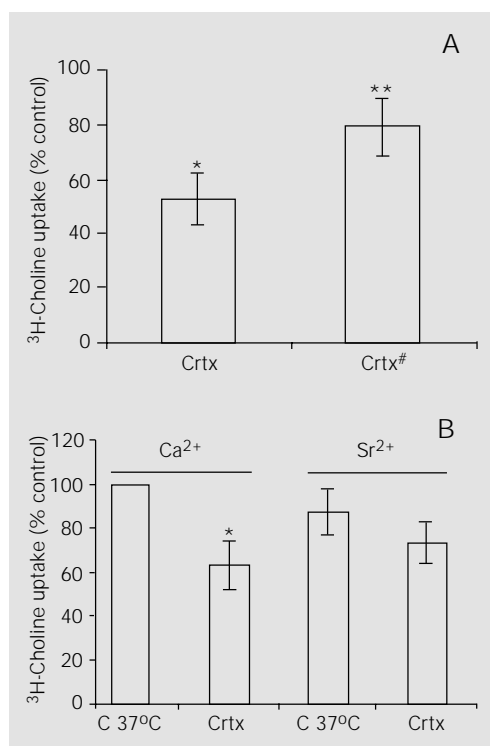
The role of phospholipase A₂ activity from crotoxin in neurotransmission inhibition at the presynaptic (4,17) and postsynaptic (18) level has been widely supported.

The precise mechanism involving blockade of neurotransmission by crotoxin, responsible for death after the bite of *C. durissus terrificus*, still is an unsolved question. Studies with β -neurotoxins suggest that the effects observed are related to the phospholipase activity of the toxins or may result from the interactions of toxins with acceptors on biological membranes (19). Toxins that act presynaptically such as β -bungarotoxin, notexin and taipoxin inhibit the release of acetylcholine and are potent inhibitors of the high affinity choline transport system in a membrane preparation derived from the cholinergic endings of the Torpedo electric organ (20). β -Bungarotoxin also inhibits high affinity choline uptake in a synaptosomal preparation of rat cerebral cortices. The presynaptic inhibition of neurotransmitter release seems to be the main effect explaining the neuromuscular blockade induced by crotoxin. In summary, our results indicate that the inhibition of high affinity choline uptake may constitute an important effect of crotoxin, contributing to the neuromuscular blockade it induces.

Acknowledgments

The authors thank Dr. Consuelo Latorre Fortes Dias for providing the subunits of crotoxin, Dr. Sérgio Vale A. Campos for reading the manuscript, and Carla Adriana Ferreira, Júlio C. dos Reis and Zulema J. de Oliveira for technical assistance.

Figure 2 - Inhibition of the phospholipase activity of crotoxin (Crtx) decreases its effect on ³H-choline uptake. A, Pre-incubation of minces (40 mg, triplicate) in normal Tyrode solution for 10 min at 37°C with 1 μ M crotoxin modified with p-bromophenacyl bromide (Crtx[#]) (see Methods) or with native toxin (Crtx). Values represent the mean and standard deviation of the mean of 3 different experiments. *P<0.05 compared to the control at 37°C. **P<0.05 compared to the native crotoxin. Analysis of variance showed that there was a difference between treatments (P = 0). Multiple comparisons based on the confidence intervals for paired differences using the Tukey test (total level of significance = 5%) showed: control (C)>Crtx; C>Crtx[#], and Crtx[#]>Crtx. B, Mince (40 mg, triplicate) were pre-incubated in normal Tyrode solution (with Ca²⁺) or in modified Tyrode solution (Sr²⁺ was substituted for Ca²⁺ at equimolar concentration and 1 mM EGTA was added), in the absence or in the presence of 0.3 μ M Crtx, as indicated, for 10 min at 37°C. ³H-Choline (12 nM) was added and the uptake measured after 10-min incubation. Values represent the mean and standard deviation of the mean of 3 different experiments. *P<0.05 compared to the control at 37°C incubated with Ca²⁺ (Student t-test).



References

1. Slotta KH & Fraenkel-Conrat HL (1938). Schlangengifte, III: Mitteilung: Reinigung und Kristallisation des Klapperschlangengiftes. *Berichte der Deutschen Chemischen Gesellschaft*, 71: 1076-1081.
2. Hendon RA & Fraenkel-Conrat H (1971). Biological roles of the two components of the crotoxin. *Proceedings of the National Academy of Sciences, USA*, 68: 1560-1563.
3. Vital Brazil O & Excel BJ (1970). Action of crotoxin and contractin from the venom of *Crotalus durissus terrificus* (South American rattlesnake) on the frog neuromuscular junction. *Journal of Physiology*, 212: 34-35.
4. Hawgood B & Smith J (1977). The presynaptic action of crotoxin at the murine neuromuscular junction. *Journal of Physiology*, 266: 91-92.
5. Bon C, Changeux JP, Jeng TW & Fraenkel Conrat H (1979). Postsynaptic effects of crotoxin and of its isolated subunits. *European Journal of Biochemistry*, 99: 471-481.
6. De Lima ME & Diniz CR (1985). Crotoxin inhibits the release of acetylcholine induced by *Tityus serrulatus* scorpion venom. *Toxicon*, 23: 588 (Abstract).
7. Anadón A & Martínez-Larrañaga MR (1985). Effects of crotoxin on autonomic neuromuscular transmission in the guinea pig myenteric plexus and vas deferens. *Toxicon*, 23: 963-972.
8. Muniz ZM & Diniz CR (1989). The effect of crotoxin on the longitudinal muscle-myenteric plexus preparation of the guinea pig ileum. *Neuropharmacology*, 28: 741-747.
9. Kattah LS, Ferreira CAG, Santoro MM, Diniz CR & De Lima ME (1998). Crotoxin inhibits the uptake of ³H-choline in guinea pig ileum. XXVII Meeting of the Sociedade Brasileira de Bioquímica e Biologia Molecular, May 23-26, Caxambu, MG, Brazil (Abstract).
10. Pert CB & Snyder HS (1974). High affinity transport of choline into the myenteric plexus of guinea pig intestine. *Journal of Pharmacology and Experimental Therapeutics*, 191: 102-108.
11. Paton WDM & Zar MA (1968). The origin of acetylcholine released from guinea-pig intestine and longitudinal muscle strips. *Journal of Physiology*, 194: 13-33.
12. Kubowitz F & Ott P (1943). Isolierung und Kristallisation eines Gärungsferments aus Tumoren. *Biochemische Zeitschrift*, 314: 94-117.
13. Hsu JC (1996). *Multiple Comparisons. Theory and Methods*. Chapman & Hall, New York.
14. Simon RJ & Kuhar MJ (1976). High affinity choline uptake: ionic and energy requirements. *Journal of Neurochemistry*, 27: 93-99.
15. Radvanyi F & Bon C (1982). Catalytic activity and reactivity with p-bromophenacyl bromide of the phospholipase subunit of crotoxin. Influence of dimerization and association with the noncatalytic subunit. *Journal of Biological Chemistry*, 257: 12616-12623.
16. Saltarelli MD, Yamada K & Coyle JT (1990). Phospholipase A₂ and ³H-hemicholinium-3 binding sites in rat brain: a potential second-messenger role for fatty acids in the regulation of high-affinity choline uptake. *Journal of Neuroscience*, 10: 62-72.
17. Chang CC & Su MJ (1978). The mechanism of potentiation of the presynaptic effect of phospholipase A₂ by the crotoprotoxin component of the crotoxin complex. *Toxicon*, 16: 402-405.
18. Marlas G & Bon C (1982). Relationship between the pharmacological action of crotoxin and its phospholipase activity. *European Journal of Biochemistry*, 125: 157-165.
19. Kini RM & Evans HJ (1989). A model to explain the pharmacological effects of snake venoms phospholipase A₂. *Toxicon*, 28: 613-635.
20. Dowdall MJ, Fohlman J & Eaker D (1977). Inhibition of high-affinity choline transport in peripheral cholinergic endings by presynaptic snake venom neurotoxins. *Nature*, 269: 700-702.