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Toxicon 41 (2003) 823–829

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Inflammatory oedema induced by phospholipases A_2 isolated from *Crotalus durissus* sp. in the rat dorsal skin: a role for mast cells and sensory C-fibers

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Received 27 September 2002; accepted 11 February 2003

Abstract

The ability of the phospholipases A_2 (PLA_2s) from *Crotalus durissus cascavella*, *Crotalus durissus collilineatus* and *Crotalus durissus terrificus* venoms and crotapotin to increase the vascular permeability in the rat skin as well as the contribution of both mast cells and sensory C-fibers have been investigated in this study. Vascular permeability was measured as the plasma extravascular accumulation at skin sites of intravenously injected ^{125}I-human serum albumin. Intradermal injection of crotalic PLA_2s (0.05–0.5 μg/site) in the rat skin resulted in dose-dependent increase in plasma extravascular whereas crotapotin (1 μg/site) failed to affect this response. Co-injection of crotapotin (1 μg/site) did not modify the increased vascular permeability induced by the PLA_2s (0.05–0.5 μg/site). Previous treatment (30 min) of the animals with cyproheptadine (2 mg/kg, i.p.) markedly reduced PLA_2 (0.5 μg/site)-induced oedema. In rats treated neonatally with capsaicin to deplete neuropeptides, the plasma extravasation induced by all PLA_2s (0.5 μg/site) was also significantly reduced. Similarly, the tachykinin NK_1 receptor antagonist SR140333 (1 nmol/site) significantly reduced the PLA_2-induced oedema. In addition, the combination of SR140333 with cyproheptadine further reduced the increased plasma extravasation by PLA_2 from *C. d. cascavella* venom, but not by PLA_2 from *C. d. terrificus* and *C. d. collilineatus* venoms. Our results suggest that increase in skin vascular permeability by crotalic PLA_2s is mediated by activation of sensory C-fibers culminating in the release of substance P, as well as by activation of mast cells which in turn release amines such as histamine and serotonin.

Keywords: Neurogenic inflammation; Mast cells; Phospholipase A_2; Venoms; Sensory fibers

1. Introduction

Secretory phospholipases A_2 (PLA_2s) are a broad group of enzymes intensely studied due to their potential involvement in the production of inflammatory mediators, such as prostaglandins, prostacyclin, thromboxane A_2, leukotrienes and platelet-activating factor. In recent years, several additional forms of PLA_2s have been discovered (Six and Dennis, 2000). At the present time, they are divided into 11 groups based on their structure similarity and localization. Venom PLA_2s are comprised into three main groups, namely type I (purified from Elapidae and Hydrophidae venoms), type II (purified from Crotalidae venoms) and type III (purified from bee and wasp venoms). Type II PLA_2s can be subdivided into Asp-49 PLA_2s and Lys-49-like proteins, the former of which contains an

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0041-0101/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0041-0101(03)00037-0

aspartic acid residue at the calcium binding site and high enzymatic activity whereas the latter contains lysine at the position 49 and very little or no enzymatic activity (Ownby et al., 1999). Venom PLA_2s cause local inflammatory effects characterized mainly by increase in vascular permeability (Cirino et al., 1989; Wang and Teng, 1990; Moreno et al., 1992; Landucci et al., 1998, 2000a,b; Chaves et al., 1998) and leukocyte infiltration into tissues (Vadas et al., 1989; Bomalaski et al., 1991; Lomonte et al., 1993; Castro et al., 2000) which take place by mechanisms dependent and independent of the catalytic activity.

Crotoxin is the main neurotoxic component isolated from *Crotalus durissus* sp. venom, representing approximately 50% of the venom protein. Crotoxin consists of a reversible protein complex composed of two non-identical sub-units, a basic phospholipase A_2 (PLA_2) and an acidic non-enzymatic component named crotapotin (Rubsamen et al., 1971; Hendon and Fraenkel-Conrat, 1976). The crotapotin component is reported to be pharmacologically inactive and is thought to act as a chaperon protein for PLA_2 increasing the biological activities of this enzyme (Habermann and Breithaupt, 1978). There are several isoforms of crotoxin that are believed to be a consequence of random association of the PLA_2 with crotapotin, possibly resulting from a post-translation modification of a unique precursor of crotoxin or expression of different mRNA (Faure et al., 1991, 1994). The objective of this study was first to examine the ability of the novel described PLA_2s from *Crotalus durissus cascavella* and *Crotalus durissus collilineatus* venoms (Beghini et al., 2000; Ponce-Soto et al., 2002) to increase the vascular permeability in the rat skin in absence and presence of crotapotin in comparison with that of *C. d. terrificus* venom (Landucci et al., 1994). Second, to explore the contribution of mast cells and sensory C-fibers to the increased permeability induced by these PLA_2s.

2. Materials and methods

2.1. Measurement of rat dorsal skin oedema

Experiments were performed in male Wistar rats (200–300 g). All experiments were carried out in accordance with the guidelines for animal care of the State University of Campinas (UNICAMP). The animals were anaesthetised with sodium pentobarbitone (50 mg kg^{-1}, given i.p.) and maintenance doses were administered when required. Local plasma protein extravasation was measured in the shaved dorsal rat skin, in response to intradermally injected PLA_2s or other agents (100 μl/site in Tyrode solution), according to Brain and Williams (1985). Agents were injected in a random order, according to a balanced site pattern. Plasma protein extravasation was measured by the accumulation of intravenously injected (i.v.) ^{125}I-human serum albumin (^{125}I-HSA; 2.5 μCi rat^{-1}) with Evan's blue dye (25 mg kg^{-1}) to act as a visual marker. Antagonists and other test agents were injected as required by specific protocols. At the end of the accumulation period (30 min), a cardiac blood sample (5 ml) was taken and the rats killed by anaesthetic overdose. The blood samples were centrifuged at 8000*g* for 10 min to obtain a plasma sample. The injected sites were punched out and counted for radioactivity, with the plasma samples in a γ-counter. Plasma extravasation was expressed as the volume (μl) of plasma accumulated at each skin site compared to total counts in 1 ml of plasma.

2.2. Neonatal treatment of rats with capsaicin

Neonates Wistar rats (7–8 g) were pretreated on the second day of life by a single subcutaneous (s.c.) injection of capsaicin (50 mg/kg) under ether anaesthesia. Control animals were pretreated with the corresponding volume (0.1 ml) of capsaicin-vehicle (1:1:8; Ethanol:Tween 80:NaCl solution v/v), as previously described (Jancsó et al., 1977). Both male and female rats were used 60–70 days later.

2.3. Drugs

The PLA_2s and crotapotins used in our study were isolated in house according to previous studies (Landucci et al., 1994; Beghini et al., 2000; Ponce-Soto et al., 2002). They show similar molecular mass, hydrophilicity and hydrophobicity for all the venoms used. Capsaicin, cyproheptadine, compound 48/80 and substance P were purchased from Sigma Chemical Co. (St Louis, MO, USA) ^{125}I-Human serum albumin was radio-labelled in IPEN/CENEN-USP (São Paulo, Brazil). Sodium pentobarbitone was purchased from Rhone Merieux (Dublin, Ireland). SR140333 ((*S*)1-{2-(3(3-4-dichlorophenyl)-1-(3-iso-propoxyphenylacetyl) piperidine-3-yl)ethyl}-4-phenyl-1-azoniabicyclol(2.2.2) octone, chloride) and SR48968 ((*S*)-*N*-methyl-*N*-(4-(4-acetylamino-4-phenylpiperidino)-2-(3,4,-dichlorophenyl)buty)benzamide) were provided by Sanofi Recherche (Montpellier, France). Crotalic PLA_2s and test-agents were stored at −20 °C and diluted with modified Tyrode solution prior to use. The Tyrode composition was (in mM): NaCl, 137; KCl, 2.7; $MgCl_2$, 0.5; NaH_2PO_4, 0.4; $NaHCO_3$, 11.9 and glucose, 5.6.

2.4. Statistical analysis

Results are presented as mean values ± SEM for *n* experiments. The values were analysed by Student's unpaired *t* test or ANOVA followed by Bonferroni's modified *t* test. $P < 0.05$ was taken as significant.

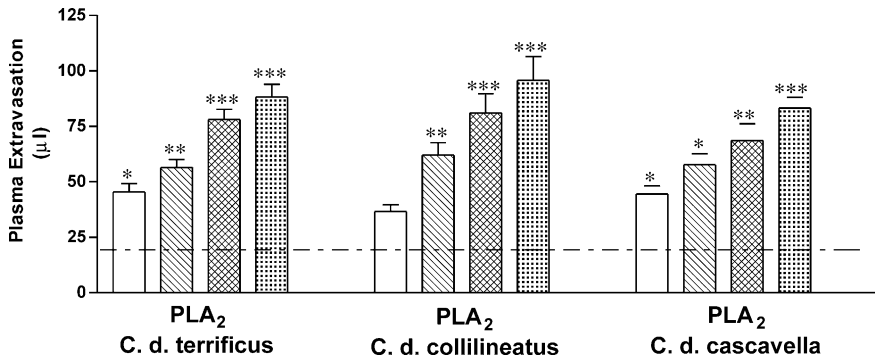

Fig. 1. Rat skin plasma protein extravasation induced by PLA_2s from *C. d. terrificus*, *C. d. collilineatus* and *C. d. cascavella* venoms at the doses of 0.05 (open columns), 0.1 (hatched columns), 0.25 (cross-hatched columns) and 0.5 μg/site (dotted columns). Results are expressed as μl plasma extravasated per site and each point represents the mean ± SEM of 7–12 animals. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to Tyrode (dotted line).

3. Results

3.1. Effect of intradermal injection of crotalic PLA_2s and crotapotin

Intradermal injection of PLA_2s from venoms of *C. d. durissus*, *C. d. cascavella* or *C. d. collilineatus* (0.05–0.5 μg/site; $n = 5$) in the rat dorsal skin induced a dose-dependent plasma extravasation in the rat dorsal skin, which was significantly different from that achieved by i.d. administration of Tyrode (Fig. 1).

Intradermal injection of crotapotin (isolated from *C. d. terrificus* venom) had no significant effect on the plasma protein extravasation (21.5 ± 2.5, 33.7 ± 5.1 and 35.6 ± 8.0 μl for 0.3, 1.0 and 3.0 μg/site, respectively), compared to Tyrode (21.7 ± 2.4, $n = 5$). Crotapotin isolated from *C. d. cascavella* or *C. d. collilineatus* venoms also failed to significantly affect the plasma extravasation (23.5 ± 5.5 and 23.6 ± 6.2 μl for 1.0 μg/site, respectively; $n = 5$) compared to Tyrode.

In addition, crotapotin (1 μg/site; isolated from *C. d. terrificus* venom) did not significantly modify the plasma extravasation induced by *C. d. terrificus* venom PLA_2 (0.05–0.5 μg/site; Table 1). Similarly, the crotapotins (1 μg/site) isolated from *C. d. cascavella* or *C. d. collilineatus* venoms had no significant effect on the plasma extravasation induced by their correspondent PLA_2 (Table 1). The combination of the crotapotin (1 μg/site) isolated from *C. d. terrificus* venom with the PLA_2 (0.5 μg/site) isolated from *C. d. cascavella* or *C. d. collilineatus* venoms (or other mixture of crotapotin with non-correspondent PLA_2s) also failed to affect the plasma extravasation (not shown; $n = 5$).

3.2. Role of mast cells

In rats pretreated (30 min before) with cyproheptadine (2 mg/kg, i.p.), a histamine H_1 and 5-HT receptor antagonist, the plasma extravasation evoked by the mast cell degranulator compound 48/80 (0.5 μg/site) was nearly abolished (76.2 ± 11.8 and 14.4 ± 4.9 μl, for control and treated-sites, respectively; $n = 5$), whereas that of substance P (1 nmol/site) was not significantly changed (56.3 ± 4.2 and 48.4 ± 7.0 μl, for control and treated-sites, respectively; $n = 4$). In these cyproheptadine-treated rats, the plasma extravasation in response to PLA_2s

Table 1
Lack of effect of crotapotin (CA; 1.0 μg/site) on the skin oedema induced by phospholipases A_2 (PLA_2) from *C. d. terrificus*, *C. d. cascavella* and *C. d. collilineatus* venoms (0.05–0.5 μg/site)

μg/site	*C. d. terrificus* PLA_2		*C. d. cascavella* PLA_2		*C. d. collilineatus* PLA_2	
	−CA	+CA	−CA	+CA	−CA	+CA
0.05	54.2 ± 7.0	61.2 ± 8.1	54.6 ± 3.6	61.6 ± 5.6	44.7 ± 5.3	63.1 ± 5.2
0.1	65.0 ± 5.4	66.3 ± 5.4	66.5 ± 2.9	68.4 ± 7.9	48.2 ± 6.0	57.4 ± 4.5
0.25	82.0 ± 5.0	84.0 ± 9.0	83.8 ± 8.9	72.0 ± 9.7	76.4 ± 2.4	83.7 ± 8.8
0.5	73.3 ± 4.5	64.8 ± 7.4	77.7 ± 1.6	92.2 ± 3.4	87.4 ± 7.6	61.8 ± 5.3

Each PLA_2 was co-injected with its correspondent crotapotin. Results are expressed as mean values ± SEM of five rats.

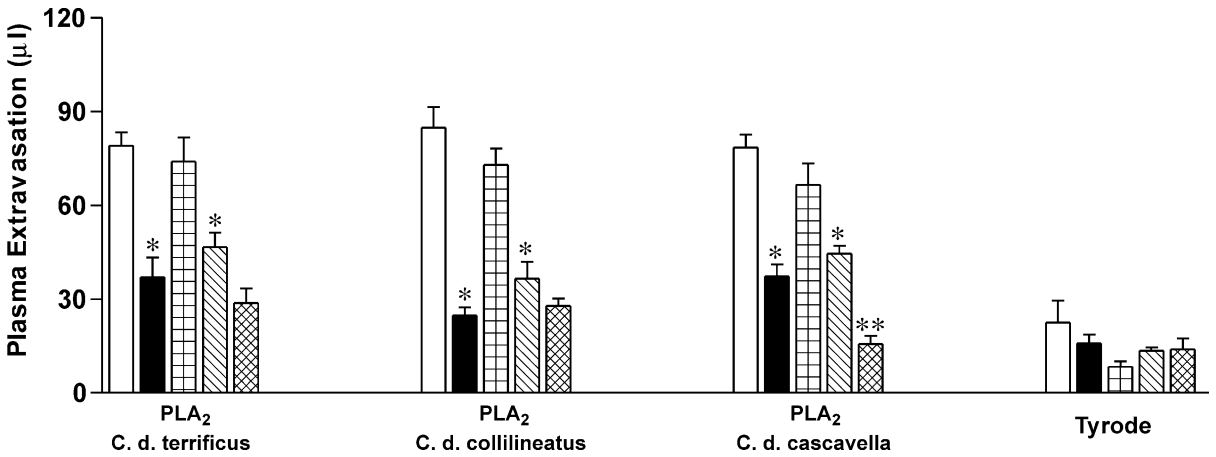

Fig. 2. Inhibitory effect of cyproheptadine (2 mg/kg, i.p.; solid columns), SR48968 (1 nmol/site; squared columns), SR140333 (1 nmol/site; hatched columns) or combination of cyproheptadine (2 mg/kg) plus SR140333 (1 nmol/site; cross-hatched columns) on plasma protein extravasation induced by PLA_2 (0.5 μg/site) from *C. d. terrificus*, *C. d. collilineatus* and *C. d. cascavella* venoms. Results are expressed as μl plasma extravasated per site and each point represents the mean ± SEM of 4–9 animals. $^*P < 0.01$, compared to untreated rats. $^{**}P < 0.05$ compared to rats treated with either cyproheptadine or SR140333.

from *C. d. terrificus*, *C. d. cascavella* and *C. d. collilineatus* venoms (0.5 μg/site each; $n = 9$) was reduced by 53.3, 74.8 and 52.6%, respectively ($P < 0.01$; Fig. 2).

3.3. Effect of tachykinin NK_1 receptor antagonist and of capsaicin pretreatment

The plasma extravasation evoked by substance P (1 nmol/site; 74.5 ± 9.9 μl) was markedly reduced ($P < 0.01$) by the co-injection with the tachykinin NK_1 receptor antagonist SR140333, at a dose of 1 nmol/site (24.8 ± 5.5 μl; $n = 5$). At this dose of SR140333, the plasma extravasation induced by PLA_2s from *C. d. terrificus*, *C. d. cascavella* and *C. d. collilineatus* venoms (0.5 μg/site each) was significantly inhibited (Fig. 2; $n = 5$). The tachykinin NK_2 receptor antagonist SR48968 (1 nmol/site, $n = 5$) affected neither the plasma extravasation induced by substance P (not shown) nor by PLA_2 from *C. d. terrificus*, *C. d. cascavella* and *C. d. collilineatus* venoms (Fig. 2; $n = 4$). In addition, the combination of SR140333 (1 nmol/site) with cyproheptadine (2 mg/kg, i.p.) did not further reduce the increased plasma extravasation by PLA_2 from *C. d. terrificus* and *C. d. collilineatus* venoms, except for *C. d. cascavella* venom PLA_2, where a greater inhibition was observed (Fig. 2). Additionally, the increased plasma extravasation induced by compound 48/80 (0.5 μg/site) was significantly ($P < 0.05$) reduced by SR140333 (1 nmol/site; 61.1 ± 4.2 and 32.5 ± 2.9 μl for control and treated-sites, respectively; $n = 4–6$).

The role of capsaicin sensitive primary afferent neurones in response to crotalic PLA_2s in dorsal skin was tested in rats treated at neonatal stage with capsaicin to deplete neuropeptides. Fig. 3 shows that a significant inhibition of plasma extravasation in capsaicin-pretreated rats was observed in response to all crotalic PLA_2s (0.5 μg/site) as compared to the response seen in control rats. The substance P (1 nmol/site)-induced plasma extravasation was unmodified in capsaicin-pretreated rats as compared to the values seen in control rats ($n = 5$).

4. Discussion

Our results demonstrate that secretory PLA_2s isolated from venoms of *C. d. terrificus*, *C. d. cascavella* and *C. d. collilineatus* are able to increase the microvascular permeability in the rat dorsal skin by mechanisms involving activation of local mast cells and sensory C-fibers. Previous studies have demonstrated that other snake venom PLA_2s are able to activate mast cells in vivo (Cirino et al., 1989; Wang and Teng, 1990; Moreno et al., 1992) and in vitro (Lau and Roche, 1997; Landucci et al., 1998, 2000a,b; Castro et al., 2000) increasing plasma extravasation in the rat due to release of histamine and serotonin; however, no studies until now have reported the involvement of capsaicin-sensitive primary afferent fibers (C-fibers; Holzer, 1998) as target for snake venom PLA_2s.

The non-adrenergic non-cholinergic innervation is primarily constituted by capsaicin-sensitive sensory C-fibers that are found beneath and within the epithelium, around blood vessels and submucosal glands. It is well established that acute electrical or chemical (capsaicin) stimulation of sensory nerve fibres leads to neuropeptide release (e.g. tachykinins such as substance P and also calcitonin gene related peptide) that can mediate local neurogenic inflammatory effects in the innervated tissues (Escott and Brain,

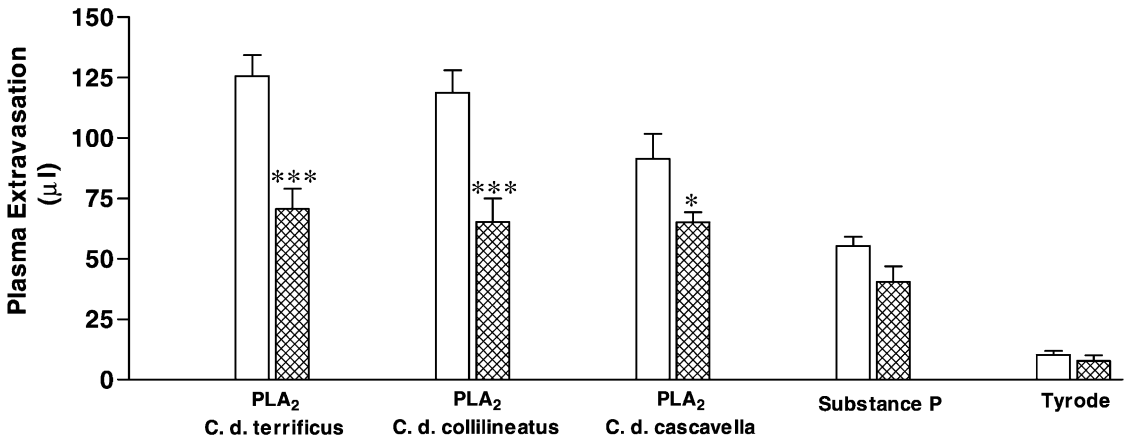

Fig. 3. Effect of neonatal capsaicin-pretreatment on plasma protein extravasation induced by PLA_2s (0.5 µg/site) from *C. d. terrificus*, *C. d. collilineatus* and *C. d. cascavella* venoms. Response to venom PLA_2s in control and capsaicin-pretreated rats is showed by the open and cross-hatched columns, respectively. The results are expressed as the mean ± SEM of five rats. $^*P < 0.05$, $^{***}P < 0.01$ compared to vehicle.

1993). Capsaicin (8 methyl-*N*-vanillyl-6-nonenamide) is a pungent ingredient contained in a wide variety of red peppers of the genus *Capsicum* and excites a subset of primary sensory neurons with somata in dorsal root ganglion or trigeminal ganglion via the VR1 receptors, a subgroup of the transient receptor potential family of ion channels (Szallasi and Blumberg, 1999; Gunthorpe et al., 2002). Capsaicin stimulates the peripheral terminals of C-fibers causing the release of the neuropeptides, thus initiating the cascade of neurogenic inflammation. When given to newborn rats, capsaicin degenerates the neurons located in dorsal root ganglion (Jancsó et al., 1977) and, therefore, has largely been used to identify capsaicin-sensitive neuronal pathways and to explore their contributions to evaluate sensory neuron mechanisms (Holzer, 1998). Substance P and NK_1 receptor agonists act primarily via the tachykinin receptors to mediate vasoactive responses in post-capillaries venules. They are potent mediators of increased permeability and, as a consequence, oedema formation (Brain, 1996). Our findings that plasma extravasation induced by all crotalic PLA_2s was significantly reduced in capsaicin-pretreated rats indicate that part of their exudative response requires integrity of the sensory fibers supplying the cutaneous microcirculation, and suggest that neurogenic mediators (mainly substance P) may be released in response to PLA_2s. It is thus likely that such proteins act directly (or indirectly) to activate sensory neurons to release the tachykinin NK_1 receptor agonist substance P. This is further supported by our results showing that SR140333, a selective NK_1 receptor antagonist (Emonds-Alt et al., 1993), significantly inhibited the PLA_2s-induced oedema.

It has been demonstrated that substance P-induced plasma extravasation results from a direct action of this peptide on the endothelium of postcapillary venules (tachykinin NK_1 receptors) and on the release of histamine and serotonin from mast cells. Mast cell activation is an intermediate step in the sensory nerve-mediated neurogenic responses (Foreman et al., 1983). It has been attributed mainly to the close anatomical contact between sensory nerves and mast cells (Botchkarev et al., 1997). Substance P can induce in vivo and in vitro mast cell degranulation, resulting in the local release of the vasoactive amines (Devillier et al., 1989; Holzer, 1998). Activation of mast cells by substance is triggered by receptor-independent mechanisms involving direct interaction with G proteins, and reflects electrostatic interaction between positive charges of this peptide with the negative charges of the mast cell surface (Ferry et al., 2002). A previous study showed that lower doses of substance P causes an oedema dependent on the direct activation of NK_1 receptors, whereas at higher doses this peptide evokes oedema through the release of secondary mediators, possibly from mast cells (Walsh et al., 1995). This suggests that mast cell degranulation by these peptides may account for the inflammatory actions of PLA_2s, a proposal corroborated by our findings that treatment of the animals with cyproheptadine markedly inhibited these inflammatory responses. It is known that mast cell-derived agents (i.e. histamine, 5-HT and tryptase) are potentially able to stimulate sensory neurons to release substance P (Saria et al., 1984) further amplifying the inflammatory response (Steinhoff et al., 2000). However, we cannot ascertain from our study whether in vivo activation of sensory C-fibers by the PLA_2s reflects a direct action of these proteins in the nerve endings or via mast cells to stimulate neuropeptide release, or both of these mechanisms. The reduction by SR140333 of plasma extravasation induced by compound 48/80 (a potent mast cell activator) in the rat skin may suggest the mast cell constituents play an important role in PLA_2-induced oedema. At this point, it is unclear why concomitant blockade of tachykinin NK_1 and histamine/serotonin receptors by the use of SR140333 and

cyproheptadine did not further reduce the increased plasma extravasation by PLA_2 from *C. d. terrificus* and *C. d. collilineatus* venoms, but did so only with the PLA_2 from *C. d. cascavella*.

Crotapotin is a protein naturally found complexed with a basic PLA_2, and the resulting complex (crotoxin) is responsible for the potent neurotoxic activity present in the rattlesnake venoms. Crotapotin may act as chaperon protein for the PLA_2, increasing the lethal neurotoxic effects of PLA_2s (Bon, 1982). Previous studies demonstrated that crotapotin increases the lethal potency of agkistrodotoxin (a β-neurotoxin PLA_2) and modifies the pharmacological effects of this toxin on *Torpedo* synaptosomes (Choumet et al., 1993). Our results showed that crotapotin did not significantly modify the plasma extravasation of crotalic PLA_2s in the rat skin. This is surprising since our previous studies showed that crotapotin inhibits the rat paw oedema induced by PLA_2s from both *Naja Naja* and *Apis mellifera* venoms (Landucci et al., 2000) as well as the carrageenin-induced rat paw oedema (Landucci et al., 1995). Nevertheless, in vitro interaction of purified PLA_2 and crotapotin restoring the native crotoxin complex with the same potency and biological actions has been proven to be difficult to demonstrate. Provided crotapotin inhibits the in vitro enzymatic activity of different venom PLA_2s (Landucci et al., 2000), including that from *C. d. terrificus* (personal communication), we may speculate that activation of sensory C-fibers and/or mast cells by the PLA_2s takes place by mechanisms independent of their catalytic activity. The existence of M (muscle)- and N (neuronal)-type receptors for PLA_2 have been identified and cloned in both experimental animal and humans, thus suggesting the existence of novel physiopathological functions for mammalian secretory PLA_2 unrelated to their enzymatic acitvity (Lambeau and Lazdunski, 1999). Whether venom PLA_2s increase vascular permeability by binding to one (or both) of these receptors in sensory terminals and/or mast cells remains to be elucidated.

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