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Inflammatory Oedema Induced by the Lys-49 Phospholipase A_2 Homologue Piratoxin-I in the Rat and Rabbit

EFFECT OF POLYANIONS AND *P*-BROMOPHENACYL BROMIDE

Elen C. T. Landucci,†‡ Rogerio C. de Castro,* Marcos Toyama,† Jose R. Giglio,§ Sergio Marangoni,† Gilberto De Nucci* and Edson Antunes**

DEPARTMENTS OF *PHARMACOLOGY AND †BIOCHEMISTRY, UNICAMP, 13081–970 CAMPINAS, SP, BRAZIL; AND §FACULTY OF MEDICINE, USP, RIBEIRÃO PRÊTO (SP), BRAZIL

ABSTRACT. Piratoxin-I (PrTX-I) is a Lys-49 phospholipase (PLA_2) homologue, isolated from *Bothrops pirajai* snake venom, that has no phospholipase activity. In this study, we investigated the *in vivo* oedematogenic activity of PrTX-I in both the rat and the rabbit as well as the ability of PrTX-I to activate rat mast cells *in vitro*. In the rat paw and skin, PrTX-I (3–100 μg/paw) induced a dose-dependent oedema that was associated with extensive mast cell degranulation. The involvement of mast cells in PrTX-I-mediated oedema formation in the rat was further confirmed by the findings that this protein significantly activated rat peritoneal mast cells *in vitro*, causing the release of [^{14}C]5-hydroxytryptamine ([^{14}C]5-HT; 51 ± 1%). In the rabbit, PrTX-I (10–100 μg/site) also induced dose-dependent skin oedema formation that was not affected by either mepyramine (a histamine H_1 receptor antagonist) or cyproheptadine (1.0 μg/site), indicating that mast cells do not play a role in this animal species. The bradykinin B_2 receptor antagonist Hoe 140 (0.5 μg/site) and the platelet-activating factor (PAF) receptor antagonist WEB 2086 (200 μg/site) also failed to affect the PrTX-I-induced rabbit skin oedema, ruling out the involvement of kinins and PAF. The PLA_2 inhibitor *p*-bromophenacyl bromide greatly reduced the PrTX-I-induced oedema in both the rat and the rabbit, and also inhibited the rat *in vitro* mast cell activation induced by this PLA_2 homologue. The polyanions heparin and dermatan sulphate efficiently prevented oedema formation in both species, and heparin inhibited PrTX-I-induced rat mast cell degranulation. Our results are consistent with the suggestion that the cationic charge of PrTX-I plays a major role in the inflammatory responses induced by this PLA_2 homologue. BIOCHEM PHARMACOL **59**;10:1289–1294, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. phospholipases A_2; proteoglycans; *p*-bromophenacyl bromide; mast cell; vascular permeability

PLA_2s[‖] are known to hydrolyze the *sn*-2 ester bonds in phosphoglyceride molecules, generating proinflammatory substances including monoacylglycerides, eicosanoids, and PAF. Secretory PLA_2s obtained from snake venoms have been employed widely as pharmacological tools to investigate the role of these enzymes in diverse models of experimental inflammatory processes (see Refs. 1 and 2). Early studies demonstrated that snake venom secretory PLA_2s cause local oedema formation in the rat as a result of the formation of vasoactive substances including histamine, serotonin, prostaglandins, and kinins [3]. Although the mode of action of these proteins is still controversial, their proinflammatory effects are mostly dependent on their catalytic activity [4, 5]. However, the findings that snake venoms in the Viperidae family contain the so-called Lys-49 PLA_2-like enzymes (see Ref. 6), which have no (or negligible) enzymatic activity, have called into question the importance of their enzymatic activity, since they are also very active in inducing different pharmacological effects. Besides this, the cationic content of some PLA_2s is also believed to strongly modulate their inflammatory activities [7–9]. Hence, basic secretory PLA_2s are usually more potent than the acidic ones, and the polyanion heparin prevents PLA_2-mediated responses in certain biological models [10, 11]. We have demonstrated that PLA_2-like enzymes isolated from *Bothrops jararacussu* venom, such as bothropstoxin-I (Lys-49 PLA_2 with no phospholipase activity) and bothropstoxin-II (Asp-49 PLA_2 with little phospholipase activity) [12], are able to increase microvascular permeability in the rat by a mechanism involving mast cell degranulation [13]. Since heparin significantly reduces the mast cell activation by bothropstoxins both *in*

‡ Corresponding author: Dra. Elen C. T. Landucci, Department of Pharmacology, Faculty of Medical Sciences, UNICAMP, P.O. Box 6111, 13081–970 Campinas (SP), Brazil. FAX (55) 19-289–2968; E-mail: landucci@bestway.com.br

‖ *Abbreviations*: PLA_2, phospholipase A_2; PrTX-I, piratoxin-I; [^{14}C]5-HT, [^{14}C]5-hydroxytryptamine; PAF, platelet-activating factor; and p-BPB, *p*-bromophenacyl bromide.

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vivo and *in vitro*, it has been suggested that the cationic charge of these substances plays a major role in their mode of action [13].

PrTX-I, a Lys-49 PLA_2 homologue (13.6 kDa) recently isolated from *B. pirajai* snake venom [14], has been found to be devoid of catalytic and proteolytic activities [15]. In an attempt to further understand the proinflammatory action of snake venom PLA_2s, we have investigated the oedematogenic activity of PrTX-I in both rats and rabbits *in vivo*. The mast cell degranulating activity of this secretory PLA_2 homologue was also examined using rat peritoneal mast cells, as assessed by the release of [^{14}C]5-HT.

MATERIALS AND METHODS

Venom and Reagents

PrTX-I was isolated and purified by HPLC as previously described [14, 15]. p-BPB, EDTA, compound 48/80, cyproheptadine, mepyramine, dermatan sulphate, and Evans blue dye were obtained from the Sigma Chemical Co. ^{125}I-Human serum albumin (50 μCi/mL, 20 mg albumin/mL) and [^{14}C]5-HT were obtained from Amersham International. Pentobarbitone sodium (Sagatal) was obtained from Rhône Mérieux, and heparin from Roche. All the salts were obtained from Merck.

Measurement of Rat Paw Oedema

Male Wistar rats (120–150 g) were used. Hind paw oedema was induced by a single subplantar injection (0.1 mL) of the PrTX-I [dissolved in 0.9% (w/v) sterile saline]. Paw volume was measured immediately before the injection of PrTX-I and at selected times thereafter using a hydroplethysmometer (model 7150, Ugo Basile). The results were expressed as the increase in paw volume (mL) calculated by subtracting the basal volume. In some cases, the area under the time–course curve (AUC, mL · min) was calculated using the trapezoidal rule.

Measurement of Local Oedema Formation in Rabbit and Rat Skin

Local oedema formation was measured in both male Wistar rats (180–220 g) and male New Zealand White rabbits (1.5–2.5 kg) as the local accumulation of i.v. injected ^{125}I-human serum albumin into skin sites as described previously [16]. Rats were anaesthetized by i.p. injection of sodium pentobarbitone (Sagatal, 30–40 mg/kg). ^{125}I-Human serum albumin (10 μCi/kg) and Evans Blue dye [1.5 mL/kg, 2.5% (w/v)] were injected via the tail vein. Test agents were made up in Tyrode's bicarbonate solution and injected in volumes of 100 μL into the shaved dorsal skin according to a balanced site pattern with two replicates per agent. After a 30-min accumulation period, a 5-mL cardiac blood sample was taken into heparin, and the animal was killed by a barbiturate overdose. Rabbits were anaesthetized with Sagatal (30–40 mg/kg) injected via the marginal ear vein, and maintenance doses were given when required. ^{125}I-Human serum albumin (2 μCi/kg) and Evans blue dye [0.5 mL/kg, 2.5% (w/v)] were injected by the same route. The agents under test were made up in sterile saline and intradermally injected in 100-μL volumes into the shaved dorsal skin according to a balanced site pattern with four to six replicates per dose. After 30 min, a 5-mL cardiac blood sample was taken into heparin, and the animal was killed by a Sagatal overdose. For both rabbits and rats, the dorsal skin was removed, and the injection sites were punched out (15 mm diameter) and counted for radioactivity in a γ counter. Oedema formation at each site was expressed as plasma volume, calculated from the counts in 1 mL of plasma.

Rat Mast Cell Isolation and Incubation

Mast cells were obtained from 4–6 male Wistar rats (200–300 g) by injecting 10 mL of Krebs–Ringer phosphate solution (pH 7.3) into the peritoneal cavity. The abdomen was massaged carefully, and the fluid was withdrawn and placed in polypropylene tubes prior to centrifugation at 300 *g* for 5 min at room temperature. The cell suspension was washed twice in Krebs–Ringer phosphate and was incubated subsequently with 40 nCi of [^{14}C]5-HT/mL at 37° for 60 min. Then the cells were washed three times and resuspended in 6.5 mL of Krebs–Ringer phosphate [17]. Aliquots of the peritoneal cell suspension (0.5 mL) were warmed at 37° for 20 min prior to stimulation (final volume of 1.0 mL) with PrTX-I or compound 48/80. After 20 min at 37°, the cells were centrifuged (300 *g*, 10 min), and the supernatant was removed for the determination of released [^{14}C]5-HT. Krebs–Ringer solution (1.0 mL) was added to the cell pellet to release residual [^{14}C]5-HT. The [^{14}C]5-HT concentrations were determined by measuring the luminescence in a β counter. [^{14}C]5-HT release was expressed as a percentage of the total cellular content of the amine. All values were corrected for the spontaneous [^{14}C]5-HT release that occurred in the absence of stimulus.

Modification of the Protein with p-BPB

Native PrTX-I (900 μL) was dissolved in 0.2 M Tris–HCl, pH 8.0, containing 1.4 mM EDTA and mixed with 100 μL of p-BPB to give a final inhibitor concentration of 0.08 mg/mL. The mixture was incubated for 20 hr at 37°. The non-reacted inhibitor was removed by ultrafiltration using an Amicon model 8 MC apparatus and a filter with a cutoff of 3 kDa. The ultrafiltration process resulted in a protein loss of approximately 32%.

Statistical Analysis

Data are presented as means ± SEM and were analyzed by ANOVA and application of the Bonferroni corrected *P* value for multiple comparisons. $P < 0.05$ was considered to indicate significance.

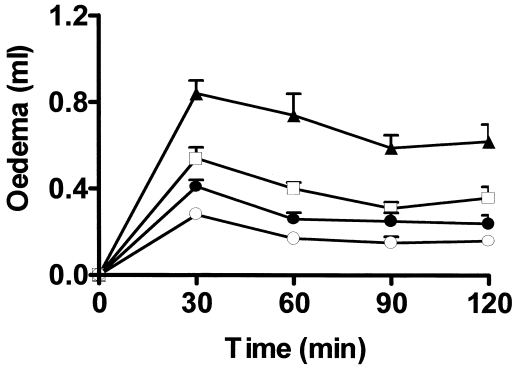

FIG. 1. Dose-dependent rat paw oedema induced by PrTX-I at doses of 3 (○; N = 5), 10 (●; N = 5), 30 (□; N = 5), and 100 (▲; N = 15) μg/paw. The rat paw oedema is expressed as the increase in volume (mL) of the injected paw compared with its basal volume. Vertical bars indicate SEM.

RESULTS

Rat Paw Oedema

Figure 1 shows that subplantar injection of PrTX-I (3–100 μg/paw) induced a dose-dependent rat paw oedema (N = 5–15). Pretreatment of the animals with the histamine/5-HT receptor antagonist cyproheptadine (2 mg/kg, i.p., 30 min before injection; N = 5) virtually abolished oedema formation induced by this PLA_2 homologue (Fig. 2). In another experiment, the oedema induced by the mast cell degranulating agent compound 48/80 (3 μg/paw) was reduced by 78% ($P < 0.01$) in the cyproheptadine-treated animals (0.87 ± 0.08 and 0.19 ± 0.09 mL at 30 min for control and treated, respectively; N = 5), thus confirming the efficacy of the treatment.

p-BPB, a compound known to inhibit PLA_2 activity by alkylating the His-48 residue, greatly reduced the rat paw oedema induced by PrTX-I ($P < 0.01$; Fig. 2). This inhibition was specific for this protein, since the paw oedema induced by compound 48/80 (3 μg/paw) was not affected significantly when this agent was administered in the presence of p-BPB solution (2.2 ± 0.1 and 2.3 ± 0.3 mL · min, AUC for control and p-BPB, respectively; N = 5).

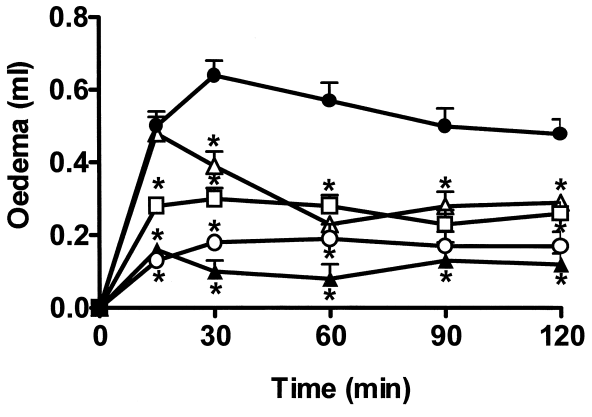

FIG. 2. Effect of cyproheptadine (2.0 mg/kg, i.p., 30 min before injection, N = 5; ○), p-BPB (p-BPB was incubated with PrTX-I as described in Materials and Methods, N = 5; ▲), dermatan sulphate (100 μg/paw, N = 5; △), and heparin (50 IU/paw, N = 5; □) on rat paw oedema induced by PrTX-I (100 μg/paw) compared with untreated animals (N = 15; ●). The oedema is expressed as the increase in volume (mL) of the injected paw compared with its basal volume. Each point represents the mean of N rats. Vertical bars indicate SEM. Key: (*) $P < 0.01$ compared with control values.

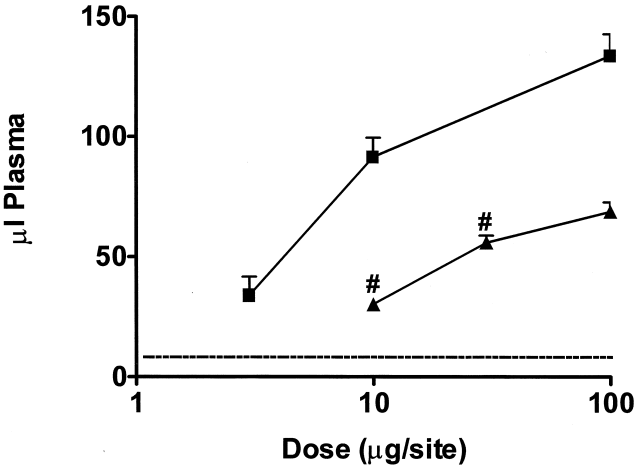

FIG. 3. Dose-dependent skin oedema induced by PrTX-I in both the rat (■, N = 4) and the rabbit (▲, N = 5). The dashed line represents the sites injected with Tyrode's solution. The skin oedema was measured 30 min after the intradermal injection of PrTX-I and is expressed as microliters of plasma protein extravasation. Vertical bars indicate SEM. Key: (#) $P < 0.05$ compared with the respective dose in rat skin.

The polyanions heparin (50 IU/paw; N = 5) and dermatan sulphate (100 μg/paw; N = 5) also significantly reduced the paw oedema induced by PrTX-I (Fig. 2).

Rat Skin Oedema

The intradermal injection of PrTX-I (3–30 μg/site, N = 4) caused a marked oedema formation in rat skin (Fig. 3). Previous treatment of rats with cyproheptadine (2 mg/kg, i.p., 30 min before PrTX-I; N = 4) virtually abolished the oedema formation induced by PrTX-I (Fig. 4). At this dose, cyproheptadine reduced by 83% ($P < 0.01$) the oedema induced by compound 48/80 (0.5 μg/site) in rat skin (data not shown).

p-BPB also significantly ($P < 0.01$) inhibited the rat skin oedema induced by PrTX-I (3 μg/site, N = 4; Fig. 4). This inhibitory effect of p-BPB was not observed with compound 48/80 (97 ± 3.5 and 90 ± 13 μL, plasma extravasation for 0.5 μg/site of compound 48/80 prepared in saline and p-BPB solution, respectively; N = 10). In addition, the PrTX-I (3 μg/site)-induced rat skin oedema was reduced significantly ($P < 0.01$) when this protein was co-injected with either heparin (25 IU/site, N = 4) or dermatan sulphate (30 μg/site, N = 5; Fig. 4).

Rabbit Skin Oedema

The intradermal injection of PrTX-I (10–100 μg/site, N = 5) caused dose-dependent oedema formation in rabbit skin

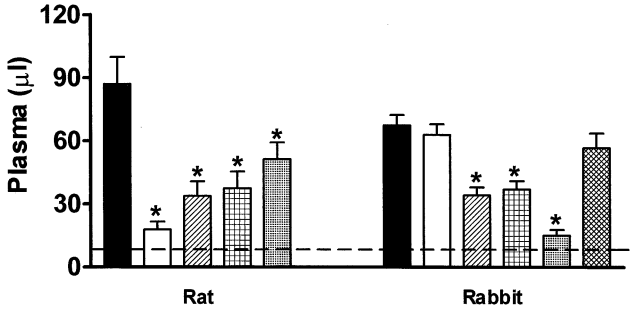

FIG. 4. Effect of cyproheptadine (1 μg/site, open columns), p-BPB (incubated with PrTX-I as described in Materials and Methods; hatched columns), heparin (25 IU/site; checked columns), and dermatan sulphate (30 μg/site; dotted columns) on oedema formation induced by PrTX-I (30 μg/site) in both rat and rabbit skin. Mepyramine (1 μg/site; cross-hatched column) was also tested in the rabbit skin. Control sites are shown by the solid columns. The dashed line represents the response induced by Tyrode's solution. p-BPB was employed as described in Materials and Methods. Skin oedema was measured 30 min after the intradermal injection of PrTX-I and is expressed as microliters of plasma protein extravasation. Each column represents the mean of 4–5 animals. Vertical bars indicate SEM. Key: (*) $P < 0.01$ compared with control values.

(Fig. 3), which was significantly smaller than the response in rats. As opposed to the findings in rats, neither cyproheptadine (1 μg/site, N = 5) nor the histamine H_1 receptor antagonist mepyramine (1 μg/site, N = 5) affected PrTX-I (30 μg/site)-induced oedema in rabbit skin (Fig. 4). The doses of cyproheptadine and mepyramine used in the rabbit were effective, since they significantly ($P < 0.05$) inhibited the histamine (3 μg/site)-induced oedema (100 ± 15, 42 ± 7, and 27 ± 2 μL, plasma extravasation for saline-, cyproheptadine-, and mepyramine-treated sites, respectively; N = 4). Compound 48/80 failed to increase vascular permeability significantly in this animal species (10.4 ± 1, 10 ± 1, and 13 ± 1.1 μL for 0.1, 0.3, and 1.0 μg/site, respectively) compared with saline-injected sites (9 ± 1 μL; N = 3).

In addition, neither the bradykinin B_2 receptor antagonist Hoe 140 (0.5 μg/site; N = 3) nor the PAF receptor antagonist WEB 2086 (200 μg/site, N = 3) affected PrTX-I (30 μg/site)-induced rabbit skin oedema (115 ± 12, 124 ± 2, and 125 ± 4 μL, plasma extravasation for Tyrode-, Hoe 140-, and WEB 2086-treated sites, respectively; N = 3). The doses of Hoe 140 and WEB 2086 employed were also effective, since the former inhibited by 85% ($P < 0.01$) the rabbit skin oedema induced by bradykinin (3 μg/site; N = 3), whereas the latter reduced by 78% ($P < 0.01$) the PAF (1.5 μg/site; N = 3)-induced oedema.

p-BPB (N = 4), heparin (25 IU/site, N = 4), and dermatan sulphate (30 μg/site, N = 5) significantly ($P < 0.01$) inhibited the rabbit skin oedema induced by PrTX-I (Fig. 4).

Rat Mast Cell Degranulation In Vitro

PrTX-I (100 μg/mL) (Table 1) and compound 48/80 (30 μg/mL) (data not shown) induced a significant release of [^{14}C]5-HT from rat peritoneal mast cells. p-BPB (N = 5) and heparin (50 IU/mL, N = 5) significantly ($P < 0.01$) inhibited the PrTX-I (100 μg/mL)-induced [^{14}C]5-HT release (Table 1) .

TABLE 1. Inhibitory effect of p-BPB and heparin (50 IU/mL) on the [^{14}C]5-HT release induced by PrTX-I (100 μg/mL) in rat peritoneal mast cells

Treatment	[^{14}C]5-HT (%)
PrTX-I	51 ± 1
PrTX-I + p-BPB	22 ± 0.9*
PrTX-I + Heparin	1.5 ± 0.3*

Data represent means ± SEM of 5 experiments, each of which was carried out in duplicate.
*$P < 0.01$, compared with PrTX-I alone.

DISCUSSION

Our present results show that the Lys-49 PLA_2 homologue PrTX-I markedly increased the vascular permeability in both rat paw and skin as well as in rabbit skin. In the rat, oedematogenic activity by PrTX-I involves extensive *in vivo* mast cell degranulation, and, hence, local release of vasoactive mediators such as histamine and 5-HT. This was confirmed by the findings that cyproheptadine virtually abolished PrTX-I-induced oedema in the rat, and also by the results showing the ability of this protein to activate rat mast cells *in vitro*, leading to the release of [^{14}C]5-HT in the cell supernatant.

Mast cell-mediated oedema formation in response to different secretory PLA_2s has been reported in a number of studies, but the mechanisms involved in cell activation are still controversial [18, 19]. Either the catalytic activity [4, 5, 20, 21] or the positive charge content [10] of the various secretory PLA_2s has been postulated as the main element implicated in their inflammatory activities. Our findings that PLA_2s devoid of (or with negligible) catalytic activity, such as PrTX-I (this study) and bothropstoxin-I and bothropstoxin-II [13], are able to activate rat mast cells both *in vivo* (paw and skin) and *in vitro* by a heparin-sensitive mechanism reinforce the proposal that the cationic charge contents of these substances are essential for their pharmacological action. Indeed, heparin is able to complex with the Lys-49 PLA_2 from *B. asper* venom through electrostatic interactions between the negatively charged groups of heparin and the cationic site (residues 115–129 with the contribution of lysines 36 and 38) of this PLA_2 [8, 9]. Similar findings were described for PLA_2s from porcine pancreas [22] and type II PLA_2 from both rats [10] and humans [23]. More recently, it was demonstrated that type II secretory PLA_2s do interact with proteoglycans in cell surfaces via their glycosaminoglycan domain [24]. Furthermore, interaction between positively charged substances with negatively charged cell membrane surfaces (mainly proteoglycans) has also been considered as the initial step in the transduction mechanism for a number of cationic

inflammatory substances other than basic PLA_2s, including amino acids, polyamino acids, and positively charged peptides [25–30]. Similar to heparin, the compound dermatan sulphate, a proteoglycan widely distributed in all tissues of vertebrates, also markedly reduced PrTX-I-induced oedema in both rat and rabbit. Since dermatan sulphate is devoid of anticoagulant activity, we may exclude the possibility that this activity is involved in the inhibitory action of these compounds.

p-BPB alkylates the histidine-48 residue located in the active site of the PLA_2s, thus inhibiting their enzymatic activity [31]. This compound has been employed largely to ascertain whether the pharmacological effects of these enzymes are due primarily to their enzymatic activity. Although PrTX-I is devoid of PLA_2 activity, p-BPB markedly inhibited the oedema formation induced by this secretory PLA_2 in both the rat and the rabbit, suggesting that the histidine-48 residue is also important for the pharmacological action. Similar findings were described for a Lys-49 myotoxin-II from *B. asper* venom [32] and for bothropstoxins [13]. The modification of PLA_2 by p-BPB causes either marked [33] or insignificant [34] conformational changes in other parts of this enzyme molecule. Nevertheless, our results indicate that this compound does not represent a good pharmacological tool to indicate involvement of catalytic activity of PLA_2s in their biological actions [13]. A previous study also pointed out that p-BPB modifies amino acid residues other than active-site histidines and should not be used as a selective inhibitor of PLA_2, since it inhibits a wide spectrum of enzymatic activities including yeast alcohol dehydrogenase, bovine pancreatic α-chymotrypsin, and human platelet phospholipase C [35].

Piratoxin-I also significantly increased cutaneous microvascular permeability in rabbit skin by a proteoglycan-sensitive mechanism unrelated to mast cell activation. The participation of mast cells in this animal species was excluded based on the failure of cyproheptadine and mepyramine to affect the PrTX-I-induced oedema, and also by the current knowledge that these cells are sparse in rabbits [36]. Compound 48/80, which is a powerful mast cell degranulator in rodents, also failed to cause oedema formation in rabbits. This may explain our results showing that PrTX-I-induced oedema in rat skin was significantly higher than in rabbit skin.

The lack of effect of Hoe 140 and WEB 2086 on PrTX-I-induced rabbit skin oedema also excludes the involvement of the vasoactive mediators kinins and PAF. Interestingly, the cationic substance polylysine causes a charge- and size-dependent local oedema formation in the rabbit by a mechanism possibly related to generation of an endothelial cell-derived vasodilator prostaglandin [37]. Whether PrTX-I increases permeability in the rabbit by acting directly in the endothelial cells or indirectly through interaction with other cell types is under investigation.

Different high affinity receptors for secretory PLA_2 have been identified and cloned in both experimental animals and humans [38, 39], thus suggesting the existence of novel pathophysiological functions for mammalian secretory PLA_2s, unrelated to their enzymatic activity ([38, 39]; see also Ref. 40). These receptors have been classified as M (muscle)- and N (neuronal)-types based on their secretory PLA_2-binding properties in different tissues. Labeled ligand studies have indicated that some secretory PLA_2s bind to both M- and N-type receptors (e.g. *Oxyuranus scutellatus* venom PLA_2-2), whereas others are specific for either M- (e.g. *O. scutellatus* venom PLA_2-1) or N- (e.g. bee venom) type receptors [39]. Whether PrTX-I increases vascular permeability by binding to one (or both) of these receptors is yet to be elucidated. Since PrTX-I-induced skin oedema is due to different mechanisms of action in the rat (mast cell-dependent) and the rabbit (mast cell-independent), it is likely that different receptors mediate the oedematogenic activity of this secretory PLA_2 homologue in both animal species.

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