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Biochemical Pharmacology 63 (2002) 65–72

Biochemical Pharmacology

Human neutrophil migration *in vitro* induced by secretory phospholipases A_2: a role for cell surface glycosaminoglycans

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Received 7 March 2001; accepted 21 May 2001

Abstract

The purpose of this study was to examine the ability of type I- (porcine pancreas and *Naja mocambique mocambique* venom), type II- (bothropstoxin-I, bothropstoxin-II, and piratoxin-I), and type III- (*Apis mellifera* venom) secretory phospholipases A_2 ($sPLA_2$s) to induce human neutrophil chemotaxis, and the role of the cell surface proteoglycans, leukotriene B_4 (LTB_4), and platelet-activating factor (PAF), in mediating this migration. The neutrophil chemotaxis assays were performed by using a 48-well microchemotaxis chamber. Piratoxin-I, bothropstoxin-I, *N. m. mocambique* venom PLA_2 (10–1000 μg/mL each), bothropstoxin-II (30–1000 μg/mL), porcine pancreas PLA_2 (0.3–30 μg/mL), and *A. mellifera* venom PLA_2 (30–300 μg/mL) caused concentration-dependent neutrophil chemotaxis. Heparin (10–300 U/mL) concentration-dependently inhibited the neutrophil migration induced by piratoxin-I, bothropstoxin-II, and *N. m. mocambique* and *A. mellifera* venom PLA_2s (100 μg/mL each), but failed to affect the migration induced by porcine pancreas PLA_2. Heparan sulfate (300 and 1000 μg/mL) inhibited neutrophil migration induced by piratoxin-I, whereas dermatan sulfate and chondroitin sulfate (30–1000 μg/mL each) had no effect. Heparitinase I and heparinase (300 mU/mL each) inhibited by 41.5 and 47%, respectively, piratoxin-I-induced chemotaxis, whereas heparitinase II and chondroitinase AC failed to affect the chemotaxis. The PAF receptor antagonist WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6*H*-thienol-[3,2-*f*] [1,2,4]-triazolo-[4,3-*a*] [1,4]-diazepine-2-yl]-1-(4-morpholynil)-1-propionate) (0.1–10 μM) and the LTB_4 synthesis inhibitor AA-861 [2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone] (0.1–10 μM) significantly inhibited the piratoxin-I-induced chemotaxis. Piratoxin-I (30–300 μg/mL) caused a concentration-dependent release of LTB_4. Our results suggest that neutrophil migration in response to $sPLA_2$s is independent of PLA activity, and involves an interaction of $sPLA_2$s with cell surface heparin/heparan binding sites triggering the release of LTB_4 and PAF.

Keywords: Phospholipase A_2; Neutrophil chemotaxis; *Flavobacterium heparinum* lyases; Leukotriene B_4; Platelet-activating factor; *N*-Formyl-methionyl-leucyl-phenylalanine

1. Introduction

PLA_2s play an important role in inflammatory processes since they provide precursors for pro-inflammatory lipid substances such as arachidonic acid-derived mediators and PAF [1]. $sPLA_2$s purified from snake and bee venoms, as well as from porcine pancreas, have largely been employed as pharmacological tools to investigate the role of these proteins in diverse physiopathological processes [2]. According to their primary structure, venom PLA_2s have been subdivided 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). The endogenous PLA_2s isolated from mammalian pancreatic juice have been classified as type I, whereas those purified from platelets and human synovial fluid have been classified as type II [1]. Furthermore, type II PLA_2s can be subdivided into Asp-49 PLA_2s and Lys-49-

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Abbreviations: PLA_2s, phospholipases A_2; $sPLA_2$, secretory PLA_2; MEM, Eagle's Minimum Essential Medium; HPF, high-power field; LTB_4, leukotriene B_4; PAF, platelet-activating factor; and fMLP, *N*-formyl-methionyl-leucyl-phenylalanine.

0006-2952/02/$ – see front matter
PII: S0006-2952(01)00841-3

like proteins. The former contains an aspartic acid residue at the calcium binding site and high enzymatic activity, whereas the latter contains lysine at position 49 and very little or no enzymatic activity. The presence of an aspartic acid residue at position 49 is believed to be crucial for calcium binding and thus for their catalytic activity [3,4].

The administration of different snake venom PLA_2s into animals *in vivo* evokes local inflammatory effects characterized mainly by an increase in vascular permeability and oedema formation, which take place by mechanisms dependent and independent of the catalytic activity [5–9]. Bothropstoxin-I and bothropstoxin-II are Asp-49 and Lys-49 type II PLA_2s, respectively, isolated from *Bothrops jararacussu* snake venom [10–12], whereas piratoxin-I is an Lys-49 type II PLA_2 isolated from *Bothrops pirajai* snake venom [13]. Although the Asp-49 PLA_2s usually contain high phospholipase activity, bothropstoxin-II has only low enzymatic activity, whereas bothropstoxin-I and piratoxin-I are completely devoid of enzymatic activity. Bothropstoxin-I, bothropstoxin-II [14], and piratoxin-I [15] markedly increase vascular permeability in rats through extensive mast cell degranulation, a mechanism dependent upon the cationic charge content of these PLA_2s. Recently, these proteins have been shown to promote *in vivo* rat pleural leukocyte infiltration by mechanisms unrelated to pleural mast cell activation and enzymatic activity [16]. In this study, we describe the ability of type I (from both porcine pancreas and *Naja mocambique mocambique* venom), type II (bothropstoxin-I, bothropstoxin-II, and piratoxin-I), and type III (from *Apis mellifera* venom) PLA_2s to induce *in vitro* human neutrophil chemotaxis. The role of cell surface proteoglycans mediating the PLA_2-induced neutrophil chemotaxis and the involvement of LTB_4 and PAF were also investigated in this study.

2. Materials and methods

2.1. PLA_2s and other drugs

Bothropstoxin-I (pI 8.2, MW 13,720), bothropstoxin-II (pI 8.2, MW 13,975), and piratoxin-I (pI 8.3, MW 13,825) were all isolated and purified in house according to previous studies [10,11,13]. Bothropstoxin-I and piratoxin-I have been shown to be enzymatically inactive (<1.0 nmol/min/mg), whereas bothropstoxin-II has residual enzymatic activity (1.02 nmol/min/mg), as monitored spectrophotometrically by the hydrolysis of a synthetic substrate 4-nitro-3-(octanoyloxy) benzoic acid, according to a previous study [17]. The PLA2s purified from N. m. mocambique venom (pI 8.8), *A. mellifera* venom (pI 10.5), and porcine pancreas (pI 6–7) were obtained from the Sigma Chemical Co.; they have an enzymatic activity of 79.2 ± 1.0, 9.0 ± 0.1, and 23.8 ± 0.7 nmol/min/mg, respectively, as measured using 4-nitro-3-(octanoyloxy) benzoic acid.

Heparitinase I, heparitinase II, chondroitinase AC, and heparinase were prepared from *Flavobacterium heparinum*, as previously described [18,19]. Heparan sulfate from bovine pancreas was prepared as described [20]. Chondroitin sulfate from whale cartilage was purchased from the Seikagaku Kogyo Co. fMLP, Histopaque, MEM, and dermatan sulfate were obtained from the Sigma Chemical Co. Heparin was obtained from Akzo Nobel Ltda. WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6*H*-thienol-[3,2-*f*] [1,2,4]-triazolo-[4,3-*a*] [1,4]-diazepine-2-yl]-1-(4-morpholynil)-1-propionate) was obtained from Boehringer.

2.2. Human neutrophil isolation

Blood from healthy donors was anticoagulated with 0.1 vol. of 3.8% (v/w) sodium citrate. Neutrophils were obtained by centrifugation of blood (300 *g* for 28 min at 21°) on a Ficoll-Hypaque gradient. The gradient zone containing the neutrophils was removed and washed once with MEM (pH 7.2). Contaminating erythrocytes were disrupted by hypotonic lysis. After centrifugation (300 *g* for 10 min at 21°), the resulting pellet was suspended in MEM supplemented with ovalbumin 0.1% (MEM/OVA). The final cell suspension (2.0×10^6 cells/mL) contained 95% neutrophils. Cell viability (>98%) was assessed by the trypan blue dye exclusion test.

2.3. Chemotaxis assay

The neutrophil migration assay was performed using a 48-well microchemotaxis chamber as previously described [21]. Briefly, 50 μL of the neutrophil suspension (2.0×10^6 cells/mL) was added to the upper compartment of the microchemotaxis chamber (Neuro Probe) and separated from the PLA_2s (dissolved in MEM) in the lower compartment by a polycarbonate filter (PVP-free; 25 × 80 mm; average pore size, 5 μm; Poretics Products –Osmonics). MEM was substituted for the chemotactic agents to measure random migration. fMLP (1×10^{-8} M, dissolved in MEM) was used as a positive control. The loaded chambers were incubated for 1 hr at 37° in a 5% CO_2 atmosphere. After incubation, the filters were removed, fixed in methanol, and stained with Diff-Quick (Baxter Healthcare Corp.). Chemotaxis was quantified by counting neutrophils that migrate completely through the filter in five random HPFs (1000×) per well. Triplicate wells were always run.

2.4. Influence of heparin and other glycosaminoglycans on human neutrophil chemotaxis

The influence of heparin (10–300 U/mL), heparan sulfate (0.03–1 mg/mL), dermatan sulfate (0.03–1 mg/mL), and chondroitin sulfate (0.03–1 mg/mL) on human neutrophil chemotaxis *in vitro* was studied by adding different concentrations of these substances to the lower or upper compartment of the microchemotaxis chamber, which contained the chemoattractant or neutrophils, respectively.

2.5. Incubation of human neutrophils with F. heparinum enzymes

Human neutrophils were incubated with heparitinase I, heparitinase II, chondroitinase AC, or heparinase (300 mU/mL each) for 30 min at 37° in a 5% CO_2 atmosphere. After incubation, the cells were washed, suspended in MEM/ovalbumin, and used for the chemotaxis assay. Control experiments where neutrophils were incubated with the enzyme vehicle ethylene diamine acetate (EDA) buffer (0.1 M) were also performed.

2.6. LTB_4 measurement

LTB_4 in the supernatants of neutrophils stimulated with fMLP (1×10^{-8} M), piratoxin-I (3–300 μg/mL), bothropstoxin-II (3–300 μg/mL), and *A. mellifera* venom PLA_2 (3–300 μg/mL) was measured using the Cayman Chemical LTB_4 EIA kit.

2.7. Statistical analysis

Data are presented as means ± SEM and were analyzed by ANOVA and the Dunnett's test. A P value of less than 0.05 was considered to indicate significance.

3. Results

3.1. Human neutrophil chemotaxis induced by different PLA_2s

Figure 1 shows that type II PLA_2s such as piratoxin-I and bothropstoxin-I (10–1000 μg/mL each) and bothropstoxin-II (30–1000 μg/mL each) caused concentration-dependent neutrophil migration, as compared with spontaneous migration. Similar results were observed for the type I PLA_2s such as those purified from porcine pancreas (0.3–100 μg/mL) and *N. m. mocambique* venom (10–1000 μg/mL) as well as for the type III PLA_2 (purified from *A. mellifera* venom, 30–300 μg/mL; Fig. 1).

3.2. Effect of heparin and other glycosaminoglycans

The human neutrophil migration induced by piratoxin-I, bothropstoxin-II, *N. m. mocambique* venom PLA_2, and *A. mellifera* venom PLA_2 (100 μg/mL each) was concentration-dependently inhibited by heparin (10–300 U/mL), as observed when heparin was incubated with the PLA_2s in the lower compartment of the microchemotaxis chamber (Fig. 2). Using the same experimental conditions, heparin failed to significantly affect neutrophil migration induced by the porcine pancreas PLA_2 (14.3 ± 1.3, 13.9 ± 0.9, 12.1 ± 0.8, 11.7 ± 0.9, and 10.7 ± 1.0 neutrophils/HPF, for control and 10, 30, 100, and 300 U/mL of heparin, respectively; N = 3). Incubation of neutrophils with heparin in the upper compartment of the microchemotaxis chamber also markedly inhibited piratoxin-I (100 μg/mL)-induced chemotaxis (25.0 ± 1.6, 26.0 ± 2.9, 13.8 ± 3.7, 12.2 ± 2.3, and 8.9 ± 1.4 neutrophils/HPF, for control and 10, 30, 100, and 300 U/mL of heparin, respectively; N = 4). For further studies, piratoxin-I was routinely used.

The incubation of the polyanion heparan sulfate (300–1000 μg/mL) in the lower compartment of the microchemotaxis chamber significantly inhibited the neutrophil migration induced by this PLA_2 (Table 1). Conversely, the polyanions dermatan sulfate and chondroitin sulfate (30–1000 μg/mL each) had no effect on the piratoxin-I-induced neutrophil migration (Table 1).

Incubation of heparin, heparan sulfate, dermatan sulfate, and chondroitin in the lower compartment of the microchemotaxis chamber affected neither spontaneous migration (N = 8; Table 2) nor fMLP (1×10^{-8} M)-induced neutrophil chemotaxis (N = 3; Table 2). Added in the upper compartment, heparin (300 IU/mL) also failed to affect spontaneous migration (4.7 ± 0.3 and 4.5 ± 0.2 neutrophils/HPF for control and treated, respectively) and fMLP-induced chemotaxis (49.5 ± 2.2 and 53.8 ± 8.9 neutrophils/HPF for control and treated, respectively).

3.3. Effect of the F. heparinum enzymes heparitinase I, heparitinase II, heparinase, and chondroitinase AC

Previous incubation (30 min at 37°) of either heparitinase I (300 mU/mL) or heparinase (300 mU/mL) with neutrophils significantly inhibited piratoxin-I (100 μg/mL)-induced chemotaxis (Fig. 3). At the same concentrations, heparitinase II and chondroitinase AC had no effect on neutrophil migration induced by piratoxin-I (Fig. 3). The spontaneous migration and fMLP (10^8 M)-induced neutrophil chemotaxis were not modified when neutrophils were pretreated with any of the *F. heparinum* enzymes (N = 3; Table 2).

3.4. Effect of WEB 2086 and AA-861 [2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone]

Previous treatment of neutrophils (37°, 30 min) with either the PAF receptor antagonist WEB 2086 (0.1–10 μM) or the LTB_4 synthesis inhibitor AA-861 (0.1–10 μM) concentration-dependently inhibited the piratoxin-I (100 μg/mL)-induced neutrophil chemotaxis (Fig. 4). The co-incubation of WEB 2086 with AA-861 (1 μM each) reduced by 75% ($P < 0.01$) the piratoxin-I-induced chemotaxis. Suppression of chemotaxis was observed when both compounds were co-incubated at a concentration of 10 μM each.

3.5. LTB_4 measurement

Incubation (37°, 1 hr) of neutrophils with piratoxin-I caused a concentration-dependent LTB_4 release in the cell supernatant (86.2, 186.6, and 506.7 pg/mL for 30, 100, and

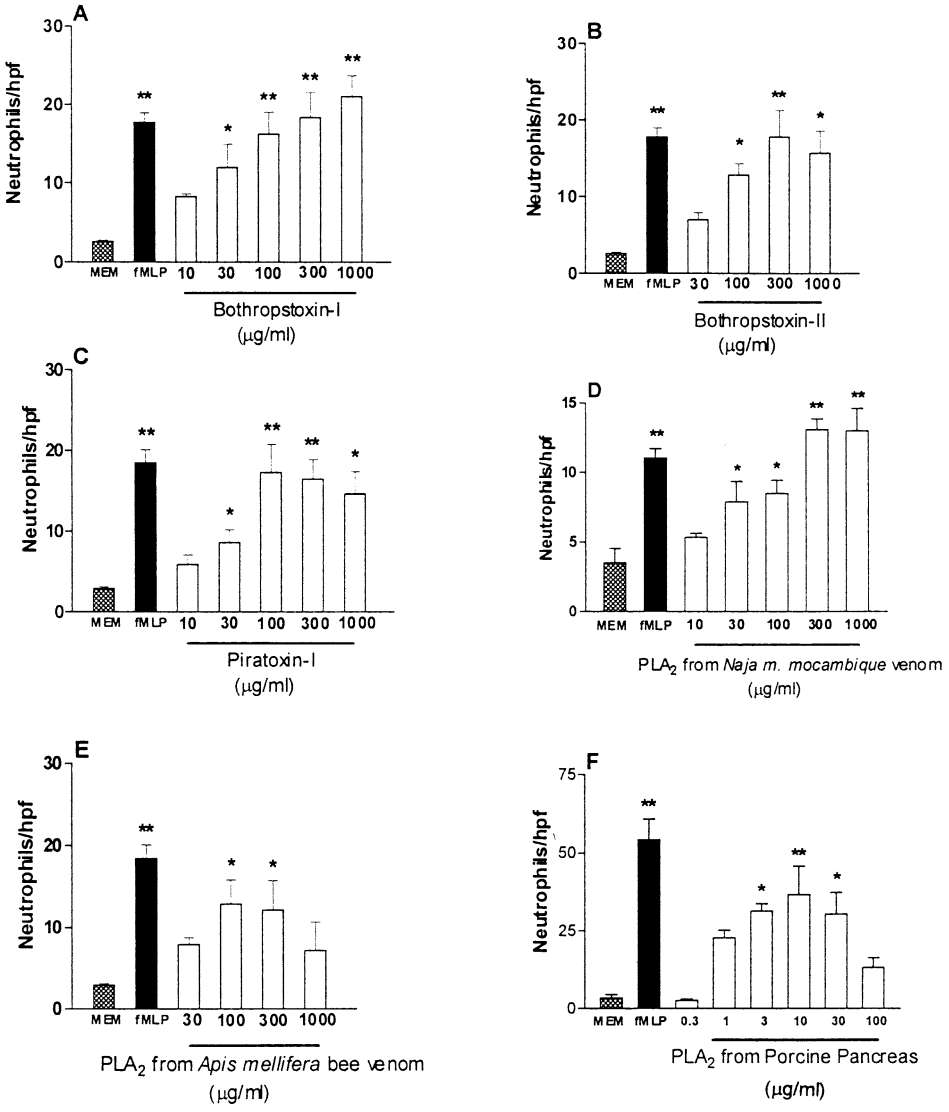

Fig. 1. Human neutrophil chemotaxis *in vitro* induced by bothropstoxin-I (10–1000 μg/mL; panel A), bothropstoxin-II (30–1000 μg/mL; panel B), piratoxin-I (10–1000 μg/mL; panel C), *N. m. mocambique* venom PLA_2 (10–1000 μg/mL; panel D), *A. mellifera* bee venom PLA_2 (30–1000 μg/mL; panel E), and porcine pancreas PLA_2 (0.3–100 μg/mL; panel F). fMLP (1×10^{-8} M)-induced neutrophil chemotaxis (solid bars) is also shown as a positive control. Data represent means ± SEM of 3 experiments (each in triplicate). Key: (*) $P < 0.05$, and (**) $P < 0.01$, compared with MEM.

300 μg/mL, respectively; N = 2 in duplicate) compared with basal release (6.7 pg/mL). Similar results were observed for bothropstoxin-II (85.0, 80.0, and 142.1 pg/mL for 30, 100, and 300 μg/mL, respectively; N = 2 in duplicate), *A. mellifera* venom PLA_2 (98.8, 331.7, and 426.0 pg/mL for 30, 100, and 300 μg/mL, respectively; N = 2 in duplicate), and fMLP (239.9 pg/mL for 1×10^{-8} M; N = 2 in duplicate).

4. Discussion

This study shows that all of the types of $sPLA_2$ assayed here (piratoxin-I, bothropstoxin-I, bothropstoxin-II, and those purified from *N. m. mocambique* and *A. mellifera* venoms and mammalian pancreas) were able to attract human neutrophils *in vitro* by a mechanism possibly involving interactions of these proteins with cell surface glycosaminoglycans of the heparin/heparan sulfate family that ultimately lead to the release of chemoattractant mediators such as LTB_4 and PAF.

Plasma membranes of mammalian tissues contain fixed anionic sites that confer an overall electronegative charge to these structures. These negative sites are constituted primarily by different sulfated glycosaminoglycans, depending on the cell type studied [22,23]. In polymorphonuclear leucocytes, the glycosaminoglycans were identified as heparan sulfate, chondroitin sulfate, and dermatan sulfate, with chondroitin sulfate being the principal component [24,25]. It is known that interaction of sulfated glycosaminoglycans

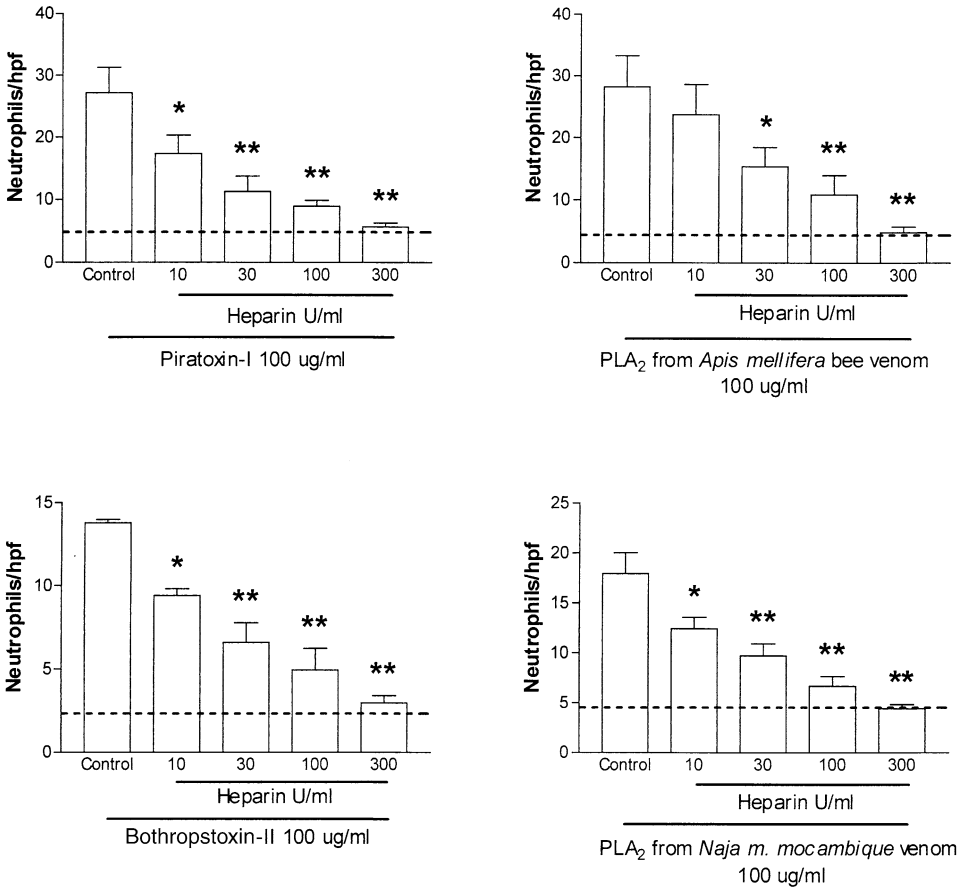

Fig. 2. Inhibitory effect of heparin (10–300 IU/mL) on human neutrophil chemotaxis induced by piratoxin-I (100 μg/mL), bothropstoxin-II (100 μg/mL), *A. mellifera* bee venom PLA_2 (100 μg/mL), and *N. m. mocambique* venom PLA_2 (100 μg/mL). The dashed lines represent spontaneous migration. Data represent means ± SEM of 3 experiments (each in triplicate). Key: (*) $P < 0.05$, and (**) $P < 0.01$, compared with the control (untreated cells).

with basic proteins triggers a wide range of biological responses [26–30], including the release of storage granule contents from neutrophils [31]. Our results showed that heparin (a sulfated glycosaminoglycan) concentration-dependently inhibited the neutrophil-induced chemotaxis when this molecule was incubated with $sPLA_2$s in the lower compartment of the microchemotaxis chamber. Incubation of heparin with neutrophils in the upper compartment also markedly inhibited piratoxin-I-induced chemotaxis. Although we have no further evidence to predict the exact mechanisms by which heparin inhibits $sPLA_2$-induced neutrophil chemotaxis, previous studies demonstrated that heparin binds to bothropstoxin [32] and to the Lys-49 PLA_2 from *Bothrops asper* venom [33,34] inhibiting their myotoxic activity. The increased vascular permeability induced by bothropstoxins and piratoxin-I in rats and rabbits is also inhibited by heparin [14,15]. It is suggested that heparin binds to a substrate binding site of PLA_2 that is not a part of

Table 1
Effect of the glycosaminoglycans heparan sulfate, dermatan sulfate, and chondroitin sulfate upon human neutrophil migration *in vitro* induced by piratoxin-I (100 μg/ml)

	Control	Neutrophils/HPF			
		Concentration of glycosaminoglycan			
		30 μg/mL	100 μg/mL	300 μg/mL	1000 μg/mL
Heparan sulfate	25.8 ± 2.2	23.0 ± 1.4	22.6 ± 1.1	17.5 ± 1.2*	10.6 ± 1.2*
Dermatan sulfate	21.6 ± 1.4	25.4 ± 2.2	30.4 ± 5.6	30.0 ± 6.2	27.3 ± 3.5
Chondroitin sulfate	24.8 ± 1.2	28.6 ± 1.9	28.8 ± 3.1	27.8 ± 3.2	22.4 ± 2.2

Chemotaxis is expressed as the mean number of migrated neutrophils per high-power field (HPF). Data represent means ± SEM of 3 experiments, each of which was carried out in triplicate.

* $P < 0.01$, compared with the control.

Table 2
Effect of different treatments on either spontaneous migration or fMLP (10^{-8}M)-induced human neutrophil migration

Treatments	Neutrophils/HPF	
	Spontaneous	fMLP
Control	3.9 ± 0.5	29.5 ± 3.0
Heparin (300 IU/mL)	4.0 ± 1.2	32.5 ± 6.6
Heparan sulfate (1 mg/mL)	5.8 ± 0.9	35.2 ± 2.0
Dermatan sulfate (1 mg/mL)	6.2 ± 1.5	33.7 ± 3.4
Chondroitin sulfate (1 mg/mL)	5.9 ± 1.0	30.5 ± 3.5
Control (EDA 0.1 M)	4.6 ± 1.8	41.1 ± 7.1
Heparinase	4.8 ± 0.2	47.0 ± 9.5
Heparitinase I	5.2 ± 1.0	45.4 ± 8.8
Heparitinase II	5.5 ± 0.8	44.3 ± 7.5
Chondroitinase AC	5.9 ± 0.7	45.9 ± 8.2

Chemotaxis is expressed as the mean number of migrated neutrophils per high-power field (HPF). Data represent means ± SEM of 3–8 experiments, each of which was carried out in triplicate.

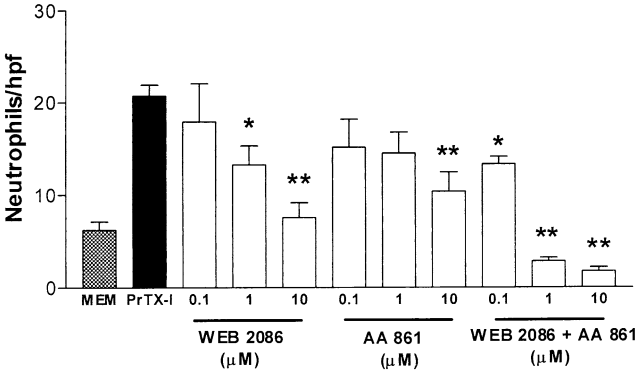

Fig. 4. Effect of the PAF receptor antagonist WEB 2086 (0.1–10 μM) and the lipoxygenase inhibitor AA-861 (0.1–10 μM) on human neutrophil chemotaxis induced by piratoxin-I (100 μg/mL). Data represent means ± SEM of 3 experiments (each in triplicate). Key: (*) $P < 0.05$, and (**) $P < 0.01$, compared with either control (untreated cells).

the enzymatic site [35,36]. This specific heparin-binding site is located in the carboxyl terminal region, and both electrostatic and non-electrostatic interactions are suggested to be involved in this binding [33,37]. We employed other glycosaminoglycans and showed that heparan sulfate (but not chondroitin sulfate and dermatan sulfate) inhibited neutrophil chemotaxis. This suggests that the interaction of members of the heparin/heparan sulfate family with PLA_2 is not based on a non-specific electrostatic interaction due to the basic character of the proteins, but rather a specific recognition component may be involved. This is in agreement with Lomonte *et al.* [34], who observed that heparan sulfate and low-molecular weight heparin, but not a variety

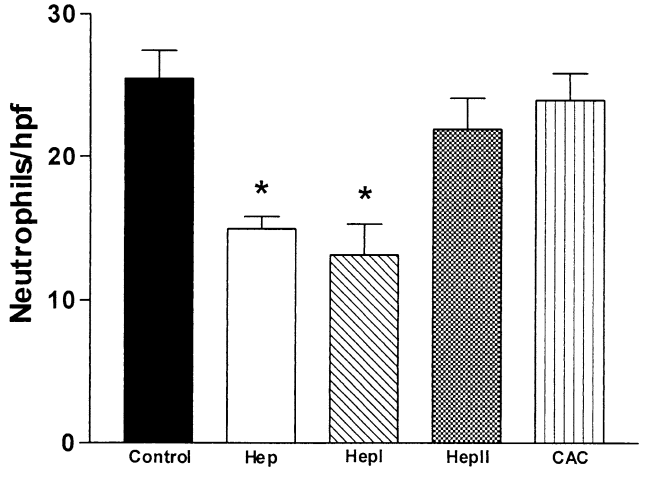

Fig. 3. Ability of heparinase and heparitinase I to inhibit piratoxin-I (100 μg/mL)-induced human neutrophil migration. Neutrophils were treated with heparinase, heparitinase I, heparitinase II, or chondroitinase AC (300 mU/mL) for 30 min at 37°. Data represent means ± SEM of 3 experiments (each in triplicate). Key: (*) $P < 0.01$, compared with either the control (untreated cells) or cells treated with EDA (medium for the enzymes). Abbreviations: Hep, heparinase; HepI, heparitinase I; HepII, heparitinase II; and CAC, chondroitinase AC.

of heparin-derived disaccharides or chondroitin sulfate, caused neutralization of PLA_2 from *B. asper* venom. The lack of effect of heparin on the neutrophil chemotaxis induced by porcine pancreas PLA_2 may reflect the fact that mammalian pancreatic PLA_2 (but not types II and III PLA_2) lack the characteristic carboxyl extension, and that they usually activate cells via specific receptors [1,38,39].

The findings that PLA_2 contains a heparin binding site that is distinct from its catalytic site suggest that heparin might regulate the interaction of PLA_2 with heparin-like molecules on cell surfaces [40]. Indeed, treatment of human neutrophils with either heparinase or heparitinase I (but not with chondroitinase AC and heparitinase II) resulted in a decreased number of migrated cells, reinforcing the suggestion that PLA_2-induced chemotaxis occurs via heparin/heparan sulfate binding with the neutrophil surface. Although the quantification of heparin/heparan sulfate on the neutrophil suspensions treated with *F. heparinum* enzymes could not be performed in our study, previous studies have indicated that such enzymes can selectively remove cell glycosaminoglycans from certain cell types including human neutrophils [41–43].

The PLA_2-induced neutrophil chemotaxis was associated with large amounts of LTB_4 in the supernatant of stimulated neutrophils, and was attenuated markedly by both the lipoxygenase inhibitor AA-861 and the PAF receptor antagonist WEB 2086, strongly indicating that both LTB_4 and PAF mediate neutrophil chemotaxis induced by $sPLA_2$s. Previous studies showed that type-II PLA_2s with preserved enzymatic activity attach to cell heparan sulfate, generating arachidonic acid and prostaglandin E_2 in a mammalian cell line [42,44]. However, our findings that enzymatically inactive Lys-49 PLA_2s such as piratoxin-I and bothropstoxin-I induced a marked neutrophil chemotaxis may suggest that cell chemotaxis is unrelated to the enzymatic activity of $sPLA_2$s. This is surprising since phospholipase activity of endogenous PLA_2s is recognized as crucial to the release of chemoattractant mediators from membrane-

bound lipid precursors. One may speculate therefore that following stimulation of heparin/heparan sulfate sites on the neutrophil surface by the $sPLA_2$s, an endogenous neutrophil PLA_2 is activated, causing the release of arachidonic acid and hence eicosanoid generation. Three distinct PLA_2s have been found in various mammalian cell types, namely, type II 14-kDa $sPLA_2$ that is associated with neutrophil granules [45], an 88-kDa calcium-independent PLA_2 ($iPLA_2$) [46], and an 85-kDa cytosolic PLA_2 ($cPLA_2$) [47]. The latter is apparently the most important PLA_2 isozyme involved in regulating the generation of the lipid mediator resulting from cell activation [47]. This PLA_2 requires a nanomolar Ca^{2+} concentration for activation and exhibits preferential hydrolysis of phospholipids bearing arachidonic acid [47, 48]. Nevertheless, the possibility that Asp-49 and Lys-49 PLA_2s display enzymatic activity in neutrophil membranes, and that this activity is required to induce neutrophil chemotaxis, may not be excluded in our study. However, this is unlikely since Lys-49 PLA_2s generally have a much lower enzymatic activity (or are completely devoid of this activity) than do Asp-49 PLA_2s (or other typical PLA_2s), irrespective of the substrate used to measure this activity [49]. In a skeletal muscle cell culture system, bothropstoxin-I has a very low enzymatic activity, and the contracture and blockade of the mouse-isolated diaphragm induced by this toxin were shown to be unrelated to this activity [50], reinforcing our proposal that enzymatic activity does not play an important role on *in vitro* neutrophil chemotaxis. The importance of enzymatic activity on this response could be assessed using *p*-bromophenacyl bromide, a commonly used PLA_2 inhibitor that acts by alkylating the His-48 residue located in the active site [51]. However, this compound has been shown to inhibit the inflammatory responses induced by Lys-49 and Asp-49 variants equally [14,15], suggesting it may not be a good pharmacological tool to advance our studies.

In conclusion, $sPLA_2$s cause LTB_4- and PAF-mediated human neutrophil chemotaxis, a phenomenon that is suggested to involve neutrophil glycosaminoglycan as the binding site. This induced neutrophil migration does not correlate with the level of enzymatic activity of these proteins.

References

[1] Murakami M, Nakatani Y, Atsumi G, Inoue K. Regulatory functions of phospholipase A_2. Crit Rev Immunol 1997;17:225–83.

[2] Gutierrez JM, Lomonte B, Phospholipase A_2 myotoxins from Bothrops snake venoms. Toxicon 1995;33:1405–24.

[3] van den Bergh CJ, Slotboom AJ, Verheij HM, de Haas GH. The role of aspartic acid-49 in the active site of phospholipase A_2. A site-specific mutagenesis study of porcine pancreatic phospholipase A_2 and the rationale of the enzymatic activity of [lysine^{49}] phospholipase A_2 from *Agkistrodon piscivorus piscivorus* venom. Eur J Biochem 1988;176:353–7.

[4] Ownby CL, Selistre-de-Araujo HS, White SP, Fletcher JE, Lysine 49 phospholipase A_2 proteins. Toxicon 1999;37:411–45.

[5] Lomonte B, Tarkowski A, Hanson LA. Host response to *Bothrops asper* snake venom. Analysis of edema formation, inflammatory cells and cytokine release in a mouse model. Inflammation 1993;17:93–105.

[6] Lloret S, Moreno JJ. Oedema formation and degranulation of mast cells by phospholipase A_2 purified from porcine pancreas and snake venoms. Toxicon 1993;31:949–56.

[7] Chiu HF, Chen IJ, Teng CM. Edema formation and degranulation of mast cells by a basic phospholipase A_2 purified from *Trimeresurus mucrosquamatus* snake venom. Toxicon 1989;27:115–25.

[8] Cirino G, Peers SH, Wallace JL, Flower RJ. A study of phospholipase A_2-induced oedema in rat paw. Eur J Pharmacol 1989;166:505–10.

[9] Chaves F, Leon G, Alvarado VH, Gutierrez JM. Pharmacological modulation of edema induced by Lys-49 and Asp-49 myotoxic phospholipases A_2 isolated from the venom of the snake *Bothrops asper* (terciopelo). Toxicon 1998;36:1861–9.

[10] Homsi-Brandemburgo MI, Queiroz LS, Santo-Neto H, Rodrigues-Simioni L, Giglio JR. Fractionation of *Bothrops jararacussu* snake venom: partial chemical characterization and biological activity of bothropstoxin. Toxicon 1988;26:615–27.

[11] Cintra ACO, Marangoni S, Oliveira B, Giglio JR. Bothropstoxin-I: amino acid sequence, and function. J Protein Chem 1993;12:57–64.

[12] Pereira MF, Novello JC, Cintra AC, Giglio JR, Landucci ET, Oliveira B, Marangoni S. The amino acid sequence of bothropstoxin-II, an Asp-49 myotoxin from *Bothrops jararacussu* (Jararacucu) venom with low phospholipase A_2 activity. J Protein Chem 1998;17:381–6.

[13] Mancuso LC, Correa MM, Vieira CA, Cunha OAB, Lachat JJ, Selistre de Araujo HS, Ownby CL, Giglio JR. Fractionation of *Bothrops pirajai* snake venom: isolation and characterization of piratoxin-I, a new myotoxic protein. Toxicon 1995;33:615–26.

[14] Landucci ECT, Castro RC, Pereira MF, Cintra ACO, Giglio JR, Marangoni S, Oliveira B, Cirino G, Antunes E, De Nucci G. Mast cell degranulation induced by two phospholipase A_2 homologues: dissociation between enzymatic and biological activities. Eur J Pharmacol 1998;343:257–63.

[15] Landucci ECT, Castro RC, Toyama M, Giglio JR, Marangoni S, De Nucci G, Antunes E. 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. Biochem Pharmacol 2000;59:1289–94.

[16] Castro RC, Landucci ECT, Toyama MH, Giglio JR, Marangoni S, De Nucci G, Antunes E. Leucocyte recruitment induced by type II phospholipases A_2 into the rat pleural cavity. Toxicon 2000;38:1773–85.

[17] Holzer M, Mackessy SP. An aqueous endpoint assay of snake venom phospholipase A_2. Toxicon 1996;34:1149–55.

[18] Nader HB, Porcinatto MA, Tersariol ILS, Pinhal MAS, Oliveira FW, Moraes CT, Dietrich CP. Purification and substrate specificity of heparitinase I and heparitinase II from *Flavobacterium heparinum*. J Biol Chem 1990;265:16807–13.

[19] Nader HB, Kobayashi EY, Chavante SF, Tersariol ILS, Castro RAB, Shinjo SK, Torri G, Casu B, Dietrich CP. New insights on the specificity of heparin and heparan sulfate lyases from *Flavobacterium heparinum* revealed by the use of synthetic derivatives of K5 polysaccharide from *E. coli* and 2-O-desulfated heparin. Glycoconj J 1999;16:265–70.

[20] Dietrich CP, Tersariol ILS, Toma L, Moraes CT, Porcionatto MA, Oliveira FW, Nader HB. Sequencing of heparan sulfate: identification of variable and constant oligosaccharide regions in eight heparan sulfates from different origins. Cell Mol Biol 1998;44:417–29.

[21] Richards KI, McCullough JM. A modified microchamber method for chemotaxis, and chemokinesis. Immunol Commun 1984;13:49–62.

[22] Hook M, Kjellen L, Johansson S, Robinson J. Cell surface glycosaminoglycans. Annu Rev Biochem 1984;53:8469–78.

[23] Kolset SO, Gallagher JT. Proteoglycans in haemopoietic cells. Biochim Biophys Acta 1990;1032:191–211.

[24] Ohhashi Y, Hasumí F, Mori Y. Comparative study on glycosaminoglycans synthesized in peripheral and peritoneal polymorphonuclear leucocytes from guinea pigs. Biochem J 1984;217:199–207.
[25] Bartold PM, Harkin DG, Bignold LP, Proteoglycans synthesized by human polymorphonuclear leucocytes *in vitro*. Immunol Cell Biol 1989;67:9–17.
[26] Needham L, Hellewell PG, Williams TJ, Gordon JL. Endothelial functional responses and increased vascular permeability induced by polycations. Lab Invest 1988;59:538–48.
[27] Antunes E, Mariano M, Cirino G, Levi S, De Nucci G. Pharmacological characterization of polycation-induced rat hind-paw oedema. Br J Pharmacol 1990;101:986–90.
[28] Rosengren S, Arfors KE. Polycations induce microvascular leakage of macromolecules in hamster cheek pouch. Inflammation 1991;15: 159–72.
[29] Santana A, Hyslop S, Antunes E, Mariano M, Bakhle YS, De Nucci G. Inflammatory responses induced by poly-L-arginine in rat lungs in vivo. Agents Actions 1993;39:104–10.
[30] Marcondes S, Grassi-Kassisse DM, Hyslop S, De Nucci G. Modulation of polycation-induced human platelet aggregation. Thromb Haemorrh Disorders 1993;7:47–50.
[31] Efferink GR, Deierkauf M. Protamine sulfate-induced enzyme secretion from rabbit neutrophils. Inflammation 1986;10:413–23.
[32] Melo PA, Homsi-Brandeburgo MI, Giglio JR, Suarez-Kurtz G. Antagonism of the myotoxic effects of *Bothrops jararacussu* venom and bothropstoxin by polyanions. Toxicon 1993;31:285–91.
[33] Lomonte B, Moreno E, Tarkowski A, Hanson LA, Maccarana M. Neutralizing interaction between heparin and myotoxin II, a lysine 49 phospholipase A_2 from *Bothrops asper* snake venom. J Biol Chem 1994;269:29867–73.
[34] Lomonte B, Tarkowski A, Bagge U, Hanson LÅ. Neutralization of the cytolytic and myotoxic activities of phospholipases A_2 from *Bothrops asper* snake venom by glycosaminoglycans of the heparin/heparan sulfate family. Biochem Pharmacol 1994;47:1509–18.
[35] Diccianni MB, Lilly-Stauderman M, McLean LR, Balasubramaniam A, Harmony JAK. Heparin prevents the binding of phospholipase A_2 to phospholipid micelles: importance of the amino-terminus. Biochemistry 1991;30:9090–7.
[36] Dua R, Cho W. Inhibition of human secretory class II phospholipase A_2. Eur J Biochem 1994;221:481–90.
[37] Lin Y-H, Lee S-C, Chang PY, Rajan PK, Sue S-C, Wu WG. Heparin binding to cobra basic phospholipase A_2 depends on heparin chain length and amino acid specificity. FEBS Lett 1999;453:395–9.
[38] Hanasaki K, Arita H. Characterization of a high affinity binding site for pancreatic-type phospholipase in the rat. Its cellular and tissue distribution. J Biol Chem 1992;267:6414–20.
[39] Kanemasa T, Hanasaki K, Arita H. Migration of vascular smooth muscle cells by phospholipase A_2 via specific binding sites. Biochim Biophys Acta 1992;1125:210–4.
[40] Murakami M, Hara N, Kudo I, Inoue K. Triggering of degranulation in mast cells by exogenous type II phospholipase A_2. J Immunol 1993;151:5675–84.
[41] Gill PJ, Silbert CK, Silbert JE. Effects of heparan sulfate removal on attachment and reattachment of fibroblasts and endothelial cells. Biochemistry 1986;25:405–10.
[42] Suga H, Murakami M, Kudo I, Inoue K. Participation in cellular prostaglandin synthesis of type-II phospholipase A_2 secreted and anchored on cell-surface heparan sulfate proteoglycan. Eur J Biochem 1993;218:807–13.
[43] Petersen F, Bock L, Hans-Dieter F, Brandt E. A chondroitin sulfate proteoglycan on human neutrophils specifically binds platelet factor 4, and is involved in cell activation. J Immunol 1998;161:4347–55.
[44] Murakami M, Nakatani Y, Kudo I. Type II secretory phospholipase A_2 associated with cell surfaces via C-terminal heparin-binding lysine residues augments stimulus-initiated delayed prostaglandin generation. J Biol Chem 1996;271:30041–51.
[45] Rosenthal MD, Gordon MN, Buescher ES, Slusser JH, Harris JK, Franson RC. Human neutrophils store type II 14-kDa phospholipase A_2 in granules and secrete active enzyme in response to soluble stimuli. Biochem Biophys Res Commun 1995;208:650–6.
[46] Portilla D, Shah SV, Lehman PA, Creer MH. Role of cytosolic calcium-independent plasmogen-selective phospholipase A_2 in hypoxic injury to rabbit proximal tubules. J Clin Invest 1994;93:1609–15.
[47] Clark JD, Lin L, Kriz RW, Ramesha CS, Sultman LA, Lin AY, Milona N, Knopf JL. A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC, and GAP. Cell 1991;65:1043–51.
[48] Sharp JD, White DL, Chiou XG, Goodson T, Gamboa GC, McClure D, Burgett S, Hoskins J, Skatrud PL, Sportsman JR, Becker GW, Kang LH, Roberts EF, Kramer RM. Molecular cloning and expression of human Ca^{2+}-sensitive cytosolic phospholipase A_2. J Biol Chem 1991;266:14850–3.
[49] Fletcher JE, Jiang M-S. Lys49 phospholipase A_2 myotoxins lyse cell cultures by two distinct mechanisms. Toxicon 1998;36:1549–55.
[50] Rodrigues-Simioni L, Prado-Franceschi J, Cintra AC, Giglio JR, Jiang MS, Fletcher JE. No role for enzymatic activity or dantrolene-sensitive Ca^{2+} stores in the muscular effects of Bothropstoxin, a Lys-^{49} phospholipase A_2 myotoxin. Toxicon 1995;33:1479–89.
[51] Volwerk JJ, Pieterson WA, Haas GH. Histidine at the active site of phospholipase A_2. Biochemistry 1974;13:1446–54.