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ANTIFLAMMINS

ANTI-INFLAMMATORY ACTIVITY AND EFFECT ON HUMAN PHOSPHOLIPASE A_2

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Abstract—Two anti-inflammatory peptides (antiflammins) corresponding to a high amino acid similarity region between lipocortin I and uteroglobin were tested for their ability to inhibit purified human synovial fluid phospholipase A_2 (HSF-PLA_2). No inhibitory activity was observed, even at such high concentrations of peptides as 50 μM. When antiflammins were preincubated with the enzyme and/or the substrate, no HSF-PLA_2 inhibition was detected. *In vivo* anti-inflammatory activity of these peptides was evaluated in several experimental models of inflammation induced by carrageenan, croton-oil, oxazolone and *Naja naja naja* venom phospholipase A_2 (PLA_2). In contrast to the *in vitro* results, anti-inflammatory activity was observed in all tests, except when inflammation was induced by snake venom PLA_2. Taken together, our results do not support the hypothesis that the *in vivo* anti-inflammatory effect of antiflammins is directly related to inhibition of PLA_2 activity.

Glucocorticoids are among the most effective drugs for the treatment of inflammatory diseases. Several studies indicate that their anti-inflammatory effects are mediated, at least in part, by the induction of regulatory proteins. Some of these proteins, called lipocortins [1] or uteroglobins [2], have been shown to inhibit phospholipase A_2 (PLA_2,† EC 3.1.1.4), thereby preventing the mobilization of precursors for the synthesis of pro-inflammatory mediators [3]. One of the most significant pro-inflammatory PLA_2s has been isolated from human synovial fluid [4]. High levels of this enzyme are associated with inflammatory processes in humans and animals, and the catalytic activity of the isolated enzyme is directly correlated to its pro-inflammatory effect when administered to animals [4, 5].

Two basic synthesis nonapeptides with amino acid sequences derived from regions of high similarity in uteroglobin and lipocortin I have been described as inhibitors of porcine pancreatic PLA_2 [6, 7]. The nonapeptide MQMKKVLDS, called P1 or AF-1, is equivalent to the nine amino acid C-terminal portion of α-helix 3 in uteroglobin, whereas nonapeptide P2 or AF-2, HDMNKVLDL, corresponds to the 246–254 sequence of lipocortin. These peptides, named "antiflammins", showed anti-inflammatory effect in carrageenan-induced rat paw edema [6, 7].

The ability of antiflammins to inhibit pancreatic and *Naja naja naja* PLA_2 *in vitro* as well as their anti-inflammatory activity *in vivo* has been questioned by several authors [8–12]. However, it has been suggested that these controversial results could be related to the chemical instability of antiflammins. Thus, failure in maintaining the conditions for preparation and storage of the peptides and for PLA_2 assay has been recently proposed as a possible explanation of the lack of reproducibility [7].

In order to investigate whether the anti-inflammatory activity of P1 and P2 is a consequence of PLA_2 inhibition, we studied the effect of commercially available antiflammins on purified PLA_2 from human synovial fluid (HSF-PLA_2), as well as their activity in several models of experimental inflammation. Reported PLA_2 inhibitors such as retinoids [13] and oleyloxyethylphosphorylcholine (OPL) [14] were tested as reference compounds *in vitro* using the same enzyme preparation and under the same experimental conditions. We also tested the effect of P1 and P2 on porcine pancreatic PLA_2.

MATERIALS AND METHODS

Animals

Sprague–Dawley rats (weighing 120–150 g) and Swiss Webster mice (18–22 g) were purchased from Interfauna Iberica (Barcelona, Spain) and were maintained with free access to laboratory food (UAR 03) and water.

Peptides

P1 and P2 were purchased from Bachem Feinchemikalien AG (Switzerland) with a purity by HPLC >98%. They were stored under argon in sealed glass vials and desiccated at −20° until use. Prior to opening the flask they were warmed at room temperature to avoid water condensation, and then dissolved before each assay in Tris–HCl 10 mM pH 8.0 buffer containing 1 mM dithiothreitol to

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† Abbreviations: DMSO, dimethyl sulfoxide; DTT, dithiothreitol; HSF-PLA_2, human synovial fluid phospholipase A_2; OPC, oleyloxyethylphosphorylcholine; PAF, platelet activating factor; PLA_2, phospholipase A_2; PLPi, total cell phospholipid phosphorous; ZAP, zymosan-activated plasma.

prepare stock solution 0.1 mM. Peptides were never stored in solution.

PLA_2 inhibitors

Retinol, retinal and retinoic acid from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and OPC from Biomol were dissolved in DMSO or ethanol.

Reagents

Zymosan A, carrageenan (λ, type IV), arachidonic acid, croton oil, 4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone), histamine, platelet activating factor (PAF) and snake venom (*N. naja naja*) PLA_2 were obtained from Sigma. WEB 2086 was from Boehringer-Ingelheim. Porcine pancreatic PLA_2 (700 U/mg) was from Boehringer Mannheim (Germany). All other reagents and chemicals used in the experiments were of analytical grade. Radiochemicals 1-stearoyl-2-[1-^{14}C]arachidonyl-phosphatidylcholine (52 mCi/mmol) and 1-palmitoyl-2-[1-^{14}C]arachidonyl-phosphatidylethanolamine (52 mCi/mmol) were purchased from NEN (Boston, MA, U.S.A.).

Zymosan-activated plasma (ZAP)

ZAP was used as a source of C_{5a}. ZAP was prepared by mixing a part of rat plasma with a part of boiled saline-washed zymosan A (50 mg/mL) for 60 min at 37°. The zymosan particles were removed by centrifugation prior to injection of ZAP.

Human synovial fluid PLA_2 preparation

Human synovial fluid was obtained from arthritic patients. Fluid was centrifuged at 3000 *g* for 20 min to sediment cells. HSF-PLA_2 was purified 30,000-fold from the supernatant fraction by acid extraction, ion-exchange and gel-filtration chromatography, as described by Fawzy and Franson [15].

Assay for PLA_2 activity

Using biomembranes. Reaction mixtures in a total volume of 0.5 mL consisted of 10 nmol of total cell phospholipid phosphorus (PLPi) and [1-^{14}C]oleate-labelled autoclaved *Escherichia coli* to give 10,000 cpm, $CaCl_2$ 5 mM and NaCl 150 mM in 50 mM of HEPES buffer pH 7 for HSF-PLA_2 or 50 mM of Tris buffer pH 8.0 for pancreatic PLA_2. Reaction was started by addition of 20–50 ng of enzyme and stopped after 5 min at 37° with 3 mL of $CHCl_3$:CH_3OH (1:2 v/v). Lipids were extracted by the method of Bligh and Dyer [16] and separated by TLC. Radioactivity was quantitated by liquid scintillation.

Using substrate-detergent mixed micelles

The procedure described by Miele *et al.* [6] was used in this case. 1-Stearoyl-2-[1-^{14}C]arachidonoyl-phosphatidylcholine as substrate of pancreatic PLA_2 and 1-palmitoyl-2-[1^{14}C]arachidonoylphosphatidylethanolamine as substrate of HSF-PLA_2 were previously dispersed with 5 mM sodium deoxycholate. Reaction mixtures in a total volume of 500 μL consisted of 10 μM of the substrate phospholipid (20,000 cpm), 100 mM NaCl, 2 mM $CaCl_2$ and 1 mM sodium deoxycholate in 100 mM Tris pH 8.0 or 100 mM HEPES pH 7.0. The reaction was started by addition of 20–50 ng of PLA_2 and stopped after 4 min at 37°. Products were separated and quantitated as above. To determine the inhibitory activity of peptides, PLA_2 was preincubated to 10 min with the putative inhibitors at 37° and reaction was started by addition of substrate.

Experimental inflammation models

Rat paw oedema. Paw oedema was induced by intraplantar injection of 0.1 mL of carrageenan (10 mg/mL), arachidonic acid (5 mg/mL) or PLA_2 (25 μg/mL) solution. Oedema was measured plethysmometrically at different times after injection of the phlogogens. Selected times were 0, 15, 30 and 60 min, except for carrageenan oedema, which was measured at 0, 30, 60, 180 and 360 min.

Murine ear oedema. Mice received arachidonic acid or croton oil on the right ear, dissolved in acetone at concentrations of 100 and 20 μg/mL, respectively. The phlogogens were applied in 10 μL volume by means of an automatic pipet. The left ear received acetone, delivered in the same manner. Treatments were given topically 5 min after phlogogen application. Mice were killed by CO_2 inhalation and a 7-mm diameter section, measured from the apex, was cut from the right and left auditory pinna of each animal and the samples were weighed. The measurements were taken 1 hr after arachidonic acid-induced inflammation and 6 hr after croton oil application.

Vascular permeability increase measurement. After shaving of the backs of the rats, 0.1-mL aliquots of *N. naja naja* PLA_2 or another phlogogen compound were injected intradermally into the dorsal surface. Antiflammins and standard anti-inflammatory drugs were coinjected with the phlogogens. An application of the latter contained a similar concentration of albumin to control for a possible unspecific effect of antiflammins on the agonist. Thirty minutes before, the animals received an i.v. injection of 1 mL/kg of 0.9% saline solution of Evans blue 1%. The inflammatory compounds left a circular blue spot, the intensity of which is proportional to the dye diffused into the tissue surrounding the injection site. Thirty minutes after administration the rats were killed by CO_2 inhalation, the skin of the wheals was removed and the Evans blue was extracted by the method of Harada *et al.* [17]. The optical density of the dye extracted was read at 620 nm, being the amount (μg) of Evans blue removed proportional to the vascular permeability increase and oedema formation.

Contact hypersensitivity. Oxazalone-induced contact hypersensitivity was developed in the mouse ear as reported by Asherson and Ptak [18]. Compounds were prepared in saline solution and administered topically to the right ear immediately after challenge. After 6 hr, ear oedema was measured as described above for the murine ear oedema test.

RESULTS

The effect of P1 and P2 on HSF-PLA_2, using [1-^{14}C]oleate labelled autoclaved *E. coli* as substrate,

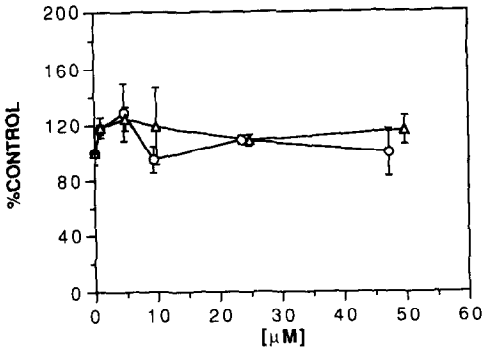

Fig. 1. Effect of antiflammins P1 (○) and P2 (△) on HSF-PLA$_2$ activity, using biomembranous substrate [1-^{14}C]-oleate-labelled autoclaved *E. coli*. Peptides were dissolved in Tris–HCl 10 mM pH 8.0 buffer. Reaction was initiated by addition of 80 ng of enzyme and performed for 5 min at 37°. Values are expressed as means ± SD (N = 2).

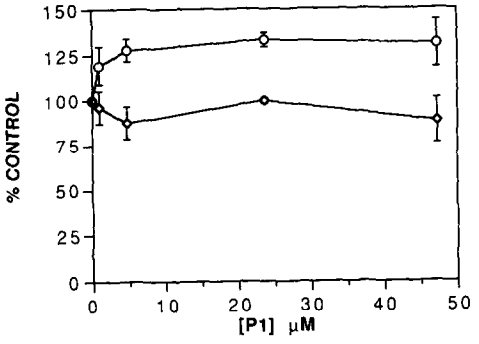

Fig. 2. Effect of antiflammin P1 preincubations on HSF-PLA$_2$ activity, using [1-^{14}C]oleate-labelled autoclaved *E. coli* (○). The enzyme (80 ng) was preincubated with P1 for 10 min at 37° and reaction was started by addition of substrate (10 nmol PLPi). (◇) The substrate (10 nmol PLPi) was preincubated with P1 for 10 min at 37° and reaction was started by addition of enzyme (80 ng). Enzymatic reaction was performed for 5 min at 37°. In both cases peptides were dissolved with Tris–HCl 10 mM pH 8.0 buffer. Values are expressed as means ± SD (N = 2).

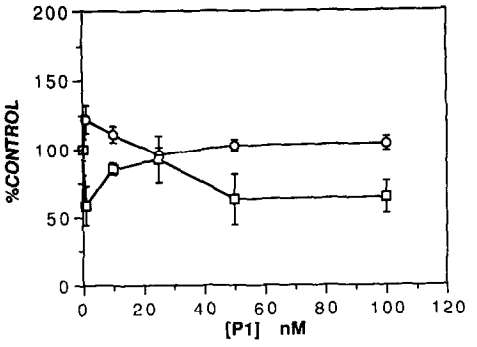

Fig. 3. Effect of antiflammin P1 at nanomolar concentrations on HSF-PLA$_2$ activity, using different substrates. [1-^{14}C]-Oleate-labelled autoclaved *E. coli* (10 nmol of PLPi) (○): reaction was started by addition of 80 ng of enzyme and stopped after 5 min at 37°. Phosphatidylethanolamine-deoxycholate mixed micellar substrate (10 μM 1-stearoyl-2-[1-^{14}C]arachidonoyl phosphatidylcholine) (□): peptide was preincubated with 80 ng of enzyme for 10 min at 37° and then reaction was initiated by addition of substrate and performed for 15 min at 37°. In both cases P1 was dissolved in Tris–HCl 10 mM pH 8.0 buffer containing 1 mM DTT and the same molarity of DTT solution was added to controls. Values are expressed as means ± SD (N = 2).

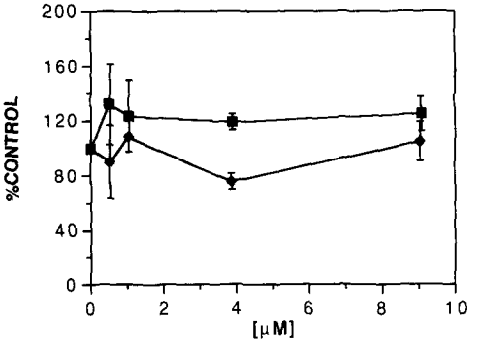

Fig. 4. Effect of antiflammins P1 (■) and P2 (◆) on porcine pancreatic PLA$_2$ activity, using phosphatidylcholine-deoxycholate mixed micellar substrate (10 μM 1-stearoyl-2-[1-^{14}C]arachidonoyl phosphatidylcholine) and pre-incubating the peptide with the enzyme (25 ng) for 10 min at 37°. Reaction was stopped after 4 min at 37°. Values are expressed as means ± SD (N = 2).

is plotted in Fig. 1. When P1 or P2 was added to the reaction mixture just before addition of enzyme, no inhibition was observed at peptide concentrations as high as 50 μM. Possible irreversible interactions between peptides and substrate or enzyme were ruled out by performing incubation experiments using the peptides with either the enzyme or the substrate. When the enzyme was previously incubated with P1 for 10 min at 37°, a slight stimulation of activity was observed (Fig. 2). When the substrate was incubated with peptide P1 for 10 min at 37°, no significant change was detected (Fig. 2). Inhibition of HLF-PLA$_2$ was not observed under any of the assayed experimental conditions. The effect of antiflammin P1 on HSF-PLA$_2$ was also investigated at nanomolar concentrations in order to rule out a loss of activity due to a possible aggregation of the peptides [7]. In addition, the complexity of the supramolecular structure of the autoclaved *E. coli* could affect the interaction between PLA$_2$ and antiflammins. Therefore, we also tested the effect of nanomolar concentrations of P1 on a different substrate, namely phosphatidylethanolamine-deoxycholate mixed micelles (Fig. 3). In both experiments peptide P1 was pretreated with DTT as described by Camussi *et al.* [19] and the same concentration of DTT was added to the

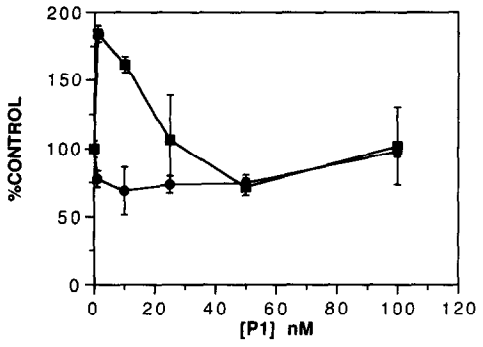

Fig. 5. Effect of antiflammin P1 at nanomolar concentrations on porcine pancreatic PLA_2 activity, using different substrates [1-^{14}C]Oleate-labelled autoclaved *E. coli* (10 nmol of PLPi) (●): reaction was started by addition of 80 ng of enzyme and stopped after 5 min at 37°. Phosphatidylethanolsamine-deoxycholate mixed micellar substrate (10 μM 1-stearoyl-2-[1-^{14}C]arachidonyl phosphatidylcholine)(■): peptide was preincubated with 80 ng of enzyme for 10 min at 37° and then reaction was initiated by addition of substrate and performed for 15 min at 37°. In both cases P1 was dissolved in Tris–HCl 10 mM pH 8.0 buffer containing 1 mM DTT and the same molarity of DTT solution was added to controls. Values are expressed as means ± SD (N = 2).

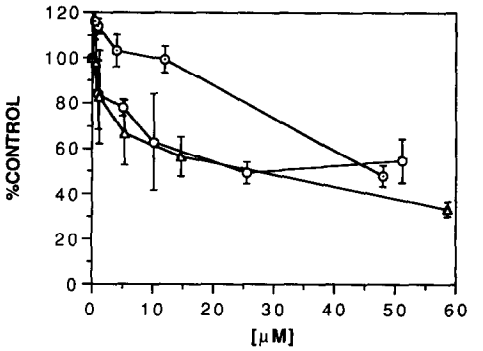

Fig. 6. Effect of retinal (△), retinol (⊙) and retinoic acid (○) on HSF-PLA_2 activity, using [1-^{14}C]oleate-labelled autoclaved *E. coli* (10 nmol PLPi). Retinoids were dissolved in DMSO and added to reaction mixture before enzyme (80 ng) addition. In all cases the final amount of DMSO was less than 5%. Reaction was stopped after 5 min at 37°. Values are expressed as means ± SD (N = 2).

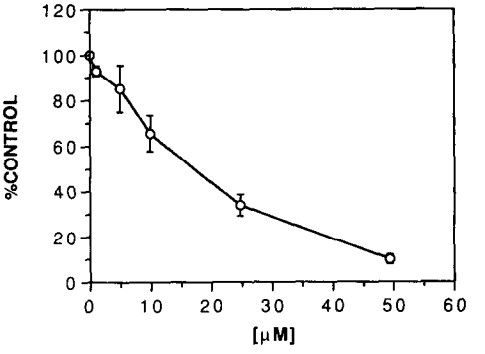

Fig. 7. Effect of OPC on HSF-PLA_2 activity using [1-^{14}C]-oleate-labelled autoclaved *E. coli* (10 nmol PLPi). Compound was dissolved in ethanol and added to reaction mixture before enzyme (80 ng) addition. The final amount of ethanol was less than 5%. Reaction was stopped after 5 min at 37°. Values are expressed as means ± SD (N = 2).

controls. No significant inhibition was found using biomembranous substrate, whereas a maximum of 35% inhibition was observed using mixed micelles.

Since HSF enzyme is a Type II PLA_2 and many reported data with antiflammins were obtained with Type I enzymes, we performed another set of experiments using porcine pancreatic PLA_2 and the same two substrates used above (Figs 4 and 5). PLA_2 was preincubated for 10 min with either P1 or P2 at 37°, as described by Miele *et al.* [6]. No significant inhibitory effect was observed at either micromolar concentrations of peptide (Fig. 4) or using P1 in the 1–100 nM range (Fig. 5). In this latter case, when mixed micelles were used as substrate enzyme activity increased at very low concentrations of peptide.

Several reported PLA_2 inhibitors such as retinoids and OPC were used to test the efficiency of the procedure for the inhibition of HSF-PLA_2. The results obtained were in good agreement with those reported by other authors [11, 12]; thus, IC_{50} = 10–20 μM was found for retinoic acid and retinal, whereas retinol and OPC showed weaker inhibition, with IC_{50} values of 50 and 45 μM, respectively (Figs 6 and 7).

Figure 8 shows the effect of dexamethasone and indomethacin treatment on carrageenan-induced rat paw oedema. This inflammation was inhibited dose dependently also by local injections of P1 and P2 (Fig. 8). The effects of steroid and antiflammins were evident 3 hr after carrageenan injection. The antiflammins lost their inhibitory activity when arachidonic acid or PLA_2 was used as an inducer of oedema (Fig. 9).

To confirm these results in a different animal model, we assayed the effect of antiflammins on croton oil- or arachidonic acid-induced ear oedema in mice. Both peptides significantly inhibited croton oil-induced oedema and, in agreement with results obtained with paw oedema, there was no significant difference between P1 and P2 (Table 1). On the other hand, the two antiflammins were without any effect on the arachidonic acid-induced oedema.

To explore the inhibitory effect on other inflammatory processes, we determined the action of these peptides on the vascular permeability increase produced by PLA_2, arachidonic acid, histamine, PAF and zymosan-activated plasma. P1 and P2 were without effect in all these tests, in contrast with the significant inhibition observed with specific antagonists, such as aristolochic acid, indomethacin, chlorpheniramine and WEB 2086 (Table 2).

Finally, the effect of antiflammins was tested on

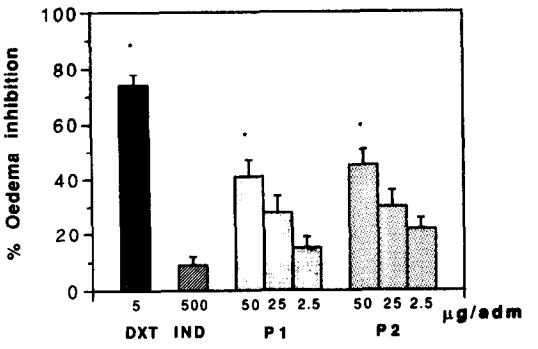

Fig. 8. Anti-inflammatory effect of peptides P1 and P2, dexamethasone (DXT) and indomethacin (IND) on carrageenan-induced rat paw oedema. Inhibitor, control substance, or vehicle alone was co-injected with carrageenan. Per cent oedema inhibition was measured 13 hr after treatment. The mean values ± SEM are of at least six determinations. Results were analysed by a Student's *t*-test for groups of unpaired observations. Significance was taken at $^*P < 0.05$.

Table 1. Per cent inhibition mouse ear oedema induced by croton oil or arachidonic acid

Compound	Dose (µg)	Croton oil	Arachidonic acid
Dexamethasone	5	63 ± 11†	ND
	1	53 ± 6†	ND
	0.1	33 ± 4*	ND
Indomethacin	1000	78 ± 12†	81 ± 11†
	500	40 ± 8*	51 ± 5†
	50	30 ± 7	22 ± 5
P1	100	65 ± 9†	8 ± 3
	50	58 ± 7†	10 ± 5
	25	33 ± 5	7 ± 3
P2	100	71 ± 12†	16 ± 7
	50	61 ± 11†	10 ± 3
	25	49 ± 3*	8 ± 5

The mean values ± SE are of 8–10 determinations; $^*P < 0.05$, †$P < 0.01$, Student's *t*-test.
ND, not determined.

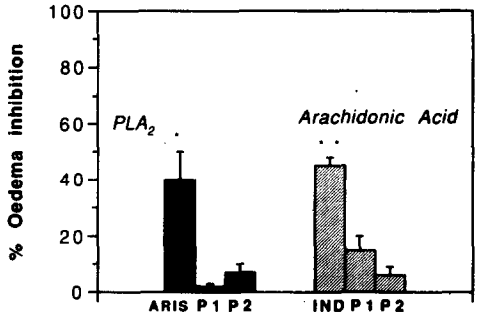

Fig. 9. Anti-inflammatory effect of peptides P1 and P2 on PLA_2 or arachidonic acid-induced rat paw oedema. Inhibitor (P1 and P2, 50 µg), control substance [aristolochic acid (ARIS) 50 µg or indomethacin (IND) 150 µg] or vehicle alone was injected 30 sec after the agonist. Per cent oedema inhibition was measured 1 hr after treatment. The mean values ± SEM are of at least six determinations. Results were analysed by a Student's *t*-test for groups of unpaired observations. Significance was taken at $^*P < 0.05$*, $^{**}P < 0.01$.

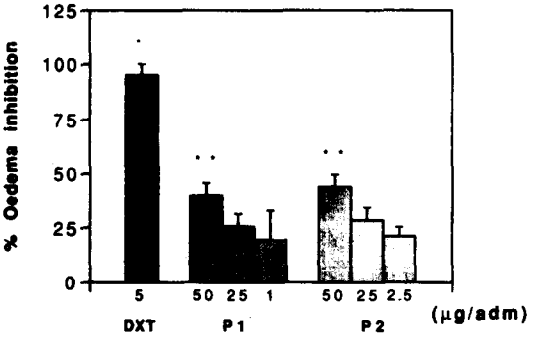

Fig. 10. Dose-dependent inhibition of oxazolone-induced hypersensitivity induced by P1, P2 or dexamethasone (DXT). The mean values ± SE are of at least six determinations. All values were significantly different from controls, $^*P < 0.05$, $^{**}P < 0.01$.

an experimental model of hypersensitivity induced by oxazolone, which has been described as being inhibited mainly by corticosteroids [20]. Both peptides had a significant dose-dependent effect on this experimental model. However, as shown in Fig. 10, topically applied dexamethasone (5 µg/ear) could almost completely suppress the oedema, whereas antiflammins (50 µg/ear) only achieved a 40% inhibition.

DISCUSSION

In the last 2 years there has been great controversy about the biological activity of antiflammins [6–12, 21]. However, all *in vitro* PLA_2 inhibitory studies have been performed with non-human extracellular enzymes. In this work, we selected a pro-inflammatory enzyme such as human synovial fluid PLA_2 and a natural source of phospholipid, [1-^{14}C]oleate-labelled autoclaved *E. coli*, as the substrate, in order to investigate a possible correlation between *in vitro* PLA_2 inhibition and the *in vivo* anti-inflammatory activity of antiflammins P1 and P2.

In a first series of experiments we did not observe any inhibitory activity even at concentrations of peptides as high as 50 µM. Our results are in agreement with those of several authors [8, 10, 11], who tested the two antiflammins on porcine pancreatic and *N. naja naja* PLA_2 and were unable to detect inhibition under different assay conditions. Results from preincubation experiments suggest that there is no interaction of the peptide P1 with either

Table 2. Per cent inhibition of cutaneous vascular permeability increase in rat skin induced by several pro-inflammatory agents

Compound	Dose (μg)	PLA_2 (2.5 μg)	Arachidonic acid (0.5 mg)	Histamine (20 μg)	PAF (5 μg)	ZAP (0.1 mL)
Aristolochic acid	100	35 ± 3*	ND	ND	ND	ND
Indomethacin	100	ND	53 ± 4†	ND	ND	ND
Chlorpheniramine	20	ND	ND	57 ± 6†	ND	ND
WEB 2086	5	ND	ND	ND	37 ± 6†	ND
P1	50	11 ± 2	5 ± 3	9 ± 3	11 ± 3	6 ± 2
P2	50	9 ± 4	2 ± 4	7 ± 5	5 ± 6	8 ± 3

The mean values ± SE are based on 8–10 determinations; *$P < 0.05$, †$P < 0.01$, Student's *t*-test. ND, means not determined.

the enzyme or the substrate, in good agreement with the results obtained by van Binsbergen *et al.* [8]. These authors showed that there was neither a significant inhibitory influence of antiflammins on the hydrolytic and penetrating properties of PLA_2 nor a clear interaction with several phospholipids used as substrates. However, it has been suggested [7] that these peptides could aggregate at micromolar concentrations losing their anti-PLA_2 activity. In addition, Camussi *et al.* [19] observed that P2 inhibits PAF release in human PMN and rat macrophages activated by different stimuli, but only in the presence of reducing agents. We therefore performed a second series of assays with HSF and porcine pancreatic PLA_2s using nanomolar concentrations of P1 and adding DTT to the reaction mixture. The experiments were performed with two different substrates, *E. coli* biomembranes and phospholipid-deoxycholate mixed micelles. No significant inhibitory effect was observed, with the possible exception of the hydrolysis of mixed micelles by HSF-PLA_2. In this case, a maximum of 35% inhibition was found for P1 at nanomolar concentrations (Fig. 3). These findings suggest that the inability of P1 to inhibit PLA_2 is not a consequence of peptide aggregation or oxidation, in good agreement with results previously reported using antiflammin P2 in similar systems and under similar assay conditions [22]. Also, the physical form of the substrate, i.e. biomembranes or mixed micelles, does not seem to affect dramatically the effect of antiflammins, in accordance with van Binsbergen *et al.* [8].

In contrast with the *in vitro* results, subplantar administration of P1 and P2 on carrageenan-induced rat paw oedema produced a significant inhibition of inflammation during the early and late phases of the process (Fig. 8). These data are in agreement with those of Miele *et al.* [6], but only partially in accordance with those of Di Rosa *et al.* [1], who found that P2 inhibited carrageenan paw oedema after 3 hr, while being ineffective in the early phase (1 hr). These authors found that only vasocortin inhibited the early phase of carrageenan oedema, while our results show that antiflammins also can inhibit this phase of inflammation, which is known to develop mainly through histamine and serotonin release.

When experimental paw oedema was induced with PLA_2 (Fig. 9), antiflammins did not display any anti-inflammatory activity, confirming the data obtained in the *in vitro* tests of PLA_2 inhibition using several substrates. Thus, the reduction of inflammation caused by the antiflammins does not seem to involve a direct interaction between these peptides and the enzyme.

Topical administration of antiflammins inhibited murine ear oedema induced by croton oil (Table 1). In this test the anti-inflammatory potency of P1 and P2 was significantly greater than that of indomethacin and only 10-fold lower than that of dexamethasone. In both models, croton oil ear oedema and carrageenan paw oedema, antiflammins showed more anti-oedematous activity than classical non-steroidal anti-inflammatory drugs. However, the antiflammins were unable to inhibit the inflammation caused by arachidonic acid (Table 1 and Fig. 9), suggesting that these peptides have no activity on cyclooxygenase or lipoxygenase enzymes.

We also investigated whether antiflammins can inhibit the *in vivo* action of some mediators of inflammation such as histamine, bradikinin, PAF and C_{5a}. Utilizing the vascular permeability test in the rat skin, we observed that antiflammins did not modify significantly the biological effects of these mediators (Table 2). Thus, a direct antihistaminic effect on the early phase of carrageenan paw oedema seems unlikely, although an indirect action of antiflammins through inhibition of mast cell degranulation cannot be ruled out.

Topical application of oxazolone to presensitized mice produces an immunological inflammatory process that is reported to be suppressed only by corticosteroids [20]. In this model, topical application of P1 and P2 (50 μg) inhibited about 50% of the oedematous reaction.

On the basis of the *in vivo* results, we can conclude that antiflammins show a significant anti-inflammatory activity, although their mechanism of action is still unclear. Our experimental data and the results reported by several other authors do not support the hypothesis that the *in vivo* anti-inflammatory effect of antiflammins P1 and P2 is directly related to inhibition of PLA_2 activity.

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