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IN VITRO AND *IN VIVO* EFFECTS OF THE ANTI-INFLAMMATORY PEPTIDES, ANTIFLAMMINS

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Abstract—The anti-inflammatory synthetic polypeptides referred to as antiflammins are thought to be inhibitors of phospholipase A_2 (PLA_2) (EC 3.1.1.4). These peptides are from the sequence of amino acids of greatest similarity between uteroglobin and lipocortin I. The effect of these peptides was studied on PLA_2 activation in rat platelets and on acute inflammatory models after local or parenteral administration of drug. We found that antiflammins decreased collagen-induced platelet activation, but had no effect when arachidonic acid was used as activator. The peptides were able to inhibit acute inflammatory processes induced by carrageenan or phorbol esters when administered locally or parenterally. However, antiflammins had no effect on inflammation induced by exogenous PLA_2 administration. These results indicate that the antiflammins may have a direct inhibitory effect on PLA_2 activation but not on the enzyme or enzyme–substrate interaction.

The anti-inflammatory activity of glucocorticoids is the result of several modulating actions on cells involved in the inflammatory process [1]. Inhibition of biosynthesis and release of eicosanoids is an important mechanism of steroid action. It has been proposed that this effect could be the result of the synthesis and/or release of proteins with anti-phospholipase A_2 (PLA_2†) activity such as lipocortins [2, 3]. On the other hand, Levin *et al.* [4] reported that uteroglobin, a progesterone-induced protein, also inhibited PLA_2 (EC 3.1.1.4) activity. In the sequence of amino acids of greatest similarity between uteroglobin and lipocortin 1, two nine-amino acid peptides have been identified. Antiflammin 1 corresponds to residues 39–47 (MQMKKVLDS) of uteroglobin and antiflammin 2 corresponds to residues 247–255 (HDMNKVLDL) of lipocortin 1. Miele *et al.* [5] proposed that these peptides had anti-PLA_2 activity *in vitro* against porcine pancreatic PLA_2 and were potent anti-inflammatory agents without any of the known side-effects of corticosteroids and/or non-steroidal anti-inflammatory agents. However, several authors have reported recently that these peptides do not have anti-PLA_2 or anti-inflammatory activity [6–8]. The aim of the present paper was to examine the effects of these nonapeptides on platelet aggregation *in vitro* and *in vivo* when they were administered locally and parenterally in animals with acute inflammatory processes.

MATERIALS AND METHODS

Male Sprague–Dawley rats (120–150 g) and male Swiss albino mice CD1 (18–22 g) were obtained from Charles River (Spain) and maintained on a standard pellet diet and water *ad lib*. Housing conditions were also standard.

Peptides were purchased from Bachem Feinchemikalien AG (Budendorf, Switzerland). Purity was always >95% as determined by HPLC; amino acid composition and molecular mass were confirmed by mass spectrometry (data from the manufacturer). Peptides were stored as lyophilized powders at 4° and dissolved in either phosphate buffer or saline solution prior to use. Ketoprofen was a gift from Menarini S.A. (Spain). All other chemicals and drugs were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Platelet-rich plasma was obtained from rat blood anticoagulated with one-tenth volume of EDTA 1% followed by centrifugation at 230 *g* for 10 min. Platelet aggregation was monitored continuously as the change in light transmission in an aggregometer (NKK Hema Tracer 1, Niko Bioscience Co., Japan). Platelet aggregation was induced by 5 μg/mL collagen of 100 μM arachidonic acid. The light transmission of the platelets after 5 min stimulation in the absence of drugs was taken as 100%. Peptides were dissolved in phosphate-buffered saline, and ketoprofen was dissolved in dimethyl sulphoxide in aggregometer cuvettes with a maximal concentration of 0.1%.

Aggregation was stopped by the addition of 1 mL of 1% formic acid, and the amount of thromboxane B_2 (TxB_2) formed was determined by extraction and quantification by radioimmunoassay. Extraction was carried out in ethyl acetate (5 mL) and, after discarding the aqueous phase, the organic phase was evaporated using a stream of nitrogen gas. TxB_2 levels were determined by radioimmunoassay with a kit from New England Nuclear (Boston, MA, U.S.A.).

Rat paw oedema was induced by intraplantar injection of 0.1 mL of a solution of 10 mg/mL carrageenan or 25 μg/mL snake venom PLA_2 (*Naja naja*). Oedema was measured plethysmometrically at different times (15, 30 and 60 min for PLA_2, and

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† Abbreviations: PLA_2, phospholipase A_2; TxB_2, thromboxane B_2; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

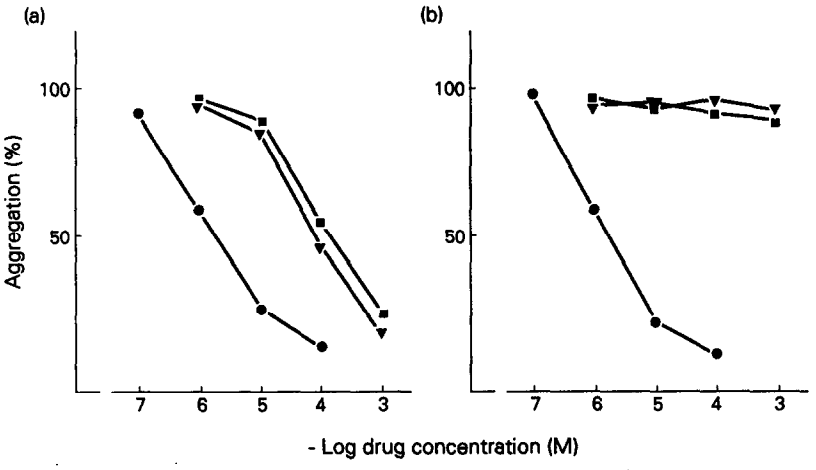

Fig. 1. Inhibition by antiflammins of platelet aggregation induced by 5 μg/mL collagen (a) or 100 μM arachidonic acid (b). Platelet-rich plasma was incubated with various concentrations of antiflammins 1 (■) or 2 (▼), or ketoprofen (●) at 37° for 2 min, and then stimulated with each agonist. The light transmission of the platelets after 5 min stimulation in the absence of antagonists was taken as 100%. Each point represents the average of four experiments.

30, 60, 180 and 360 min for carrageenan) after injection of phlogogens. Test compounds were coinjected with the phlogogens or given 5 min after carrageenan or PLA_2 application, except for dexamethasone, which was administered 2 hr before inflammation was induced. Control animals received 0.1 mL of the vehicle with albumin at the same concentration as of antiflammins.

Ear oedema was induced in mice that received phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA; 10 μg) on the surface of the right ear. The left ear received vehicle delivered in the same manner. Test compounds were given locally, intraperitoneally or subcutaneously 5 min after TPA application, except for dexamethasone, which was administered 2 hr before inflammation was induced. Six hours after induced inflammation a 7-mm section was cut from the right and left auditory pinna of each animal, as measured from the apex, and the samples were weighed. Oedema was measured as the difference between the weights of the two ears.

Statistical significance was assessed by one-tailed Student's *t*-test for unpaired samples with $P < 0.05$ regarded as significant.

RESULTS

The effect of peptides on platelet aggregation induced by collagen or arachidonic acid is shown in

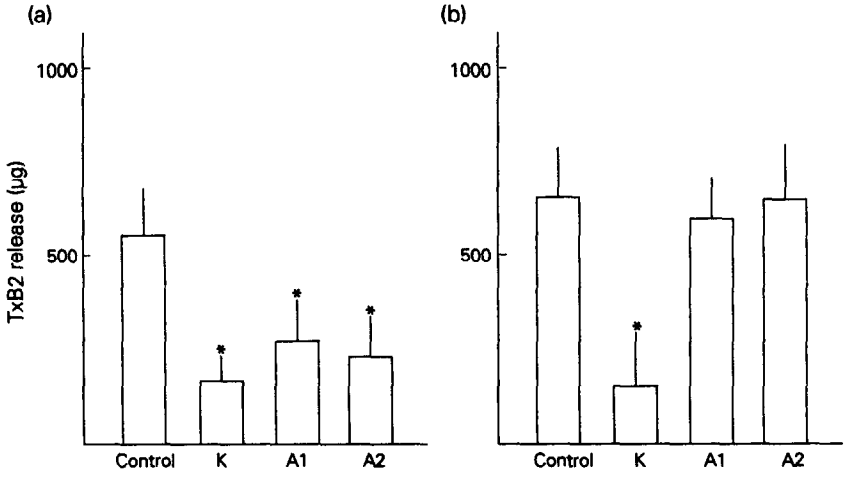

Fig. 2. Effect of antiflammins 1 (A1, 10^{-4} M) and 2 (A2, 10^{-4} M), and ketoprofen (K, 10^{-6} M) on TxB_2 release induced by 5 μg/mL collagen (a) or 100 μM arachidonic acid (b). TxB_2 was determined as described in Materials and Methods. Each point represents the mean ± SEM of four separate experiments. Difference with respect to control group: * $P < 0.01$.

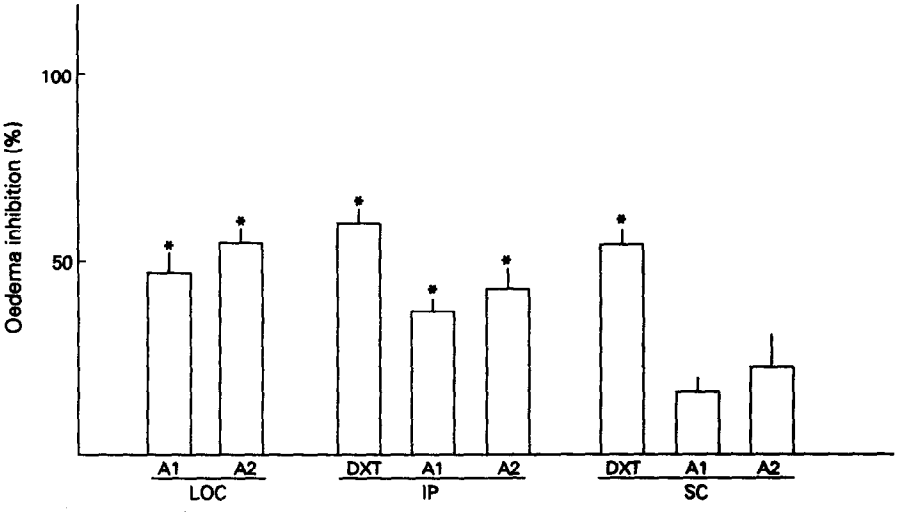

Fig. 3. Anti-inflammatory activity of dexamethasone (DXT, 2 mg/kg), and antiflammins 1 (A1, 2 mg/kg) and 2 (A2, 2 mg/kg) injected locally (LOC), intraperitoneally (IP) or subcutaneously (SC) on carrageenan-induced rat paw oedema. Data represent the means of area under curve of time-course of oedema inhibition ± SEM from six determinations. Difference with respect to control group: * $P < 0.01$.

Fig. 1. Collagen and arachidonic acid-induced aggregation was dose-dependently blocked by ketoprofen. The responses induced by these agonists were seen to be dependent on TxB_2 generation, as shown in Fig. 2. Antiflammins inhibited the collagen-induced aggregation dose dependently; however, these peptides did not have any effect on arachidonic acid-induced aggregation, even at 1 mM concentration. Therefore, no suppressive effect of these peptides was observed on arachidonic acid-induced TxB_2 release (Fig. 2). However, antiflammins prevented the increase in TxB_2 in response to platelet stimulation with collagen. Figure 3 shows the effect of dexamethasone treatment (2 mg/kg, i.p. or s.c.) on carrageenan-induced rat paw oedema. This oedema was also significantly inhibited by local or intraperitoneal injection of antiflammins 1 and 2, but not by subcutaneous administration. The steroid and antiflammin effects were evident 1 hr after carrageenan injection (data not shown). Peptide 1 was always less active than peptide 2. However, the antiflammins lost their inhibitory activity when the inducer of paw oedema was PLA_2 (Fig. 4). To confirm these findings in a different animal model, we assayed the effect of antiflammins on TPA ear oedema induced in mice. In these assays, local

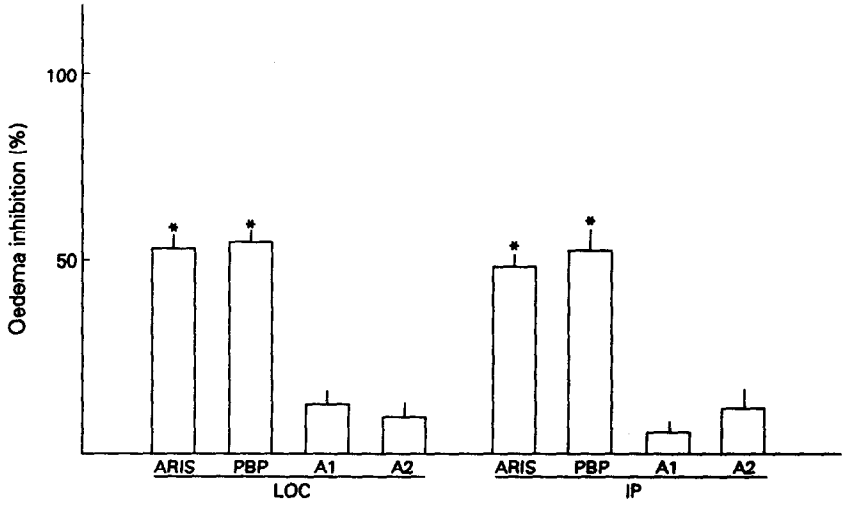

Fig. 4. Anti-inflammatory activity of aristolochic acid (ARIS, 2 mg/kg), *p*-bromo phenacyl bromide (PBP, 2 mg/kg), and antiflammins 1 (A1, 2 mg/kg) and 2 (A2, 2 mg/kg) injected locally (LOC) or intraperitoneally (IP) on PLA_2-induced rat paw oedema. Data represent the means of area under curve of time-course of oedema inhibition ± SEM from six determinations. Difference with respect to control group: * $P < 0.01$.

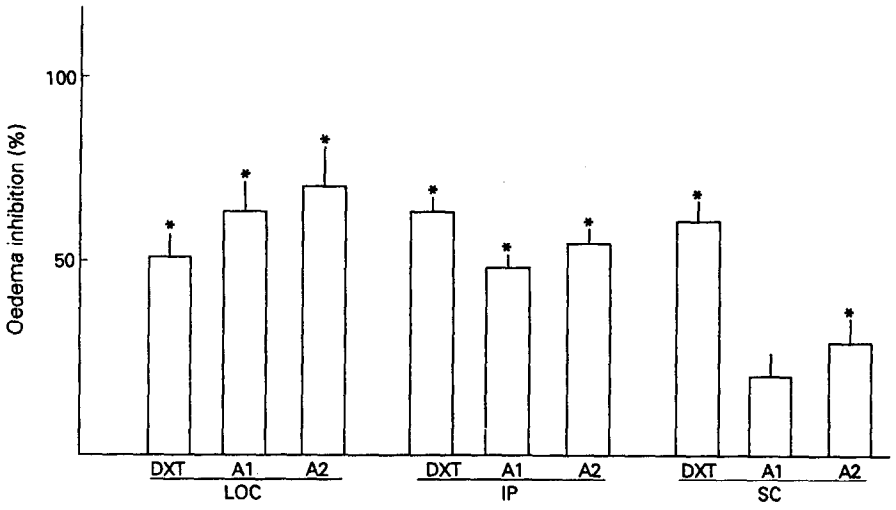

Fig. 5. Anti-inflammatory activity of dexamethasone (DXT, 2 mg/kg), and antiflammins 1 (A1, 2 mg/kg) and 2 (A2, 2 mg/kg) injected locally (LOC), intraperitoneally (IP) or subcutaneously (SC) on TPA-induced mice ear oedema. Data represent the means of oedema inhibition ± SEM from six determinations. Differences with respect to control group: * $P < 0.01$.

and intraperitoneal administration of peptides significantly inhibited oedema (Fig. 5). As shown in previous experiments, peptide 1 was less active than peptide 2, which significantly inhibited TPA ear oedema when it was administered subcutaneously.

DISCUSSION

PLA_2 activation is the essential first step in collagen-induced aggregation, but not in arachidonic acid-induced aggregation. Therefore, arachidonic acid liberation in response to collagen is reported to occur mainly through the action of PLA_2 on membrane phospholipids [9, 10]. Arachidonic acid is the substrate in the synthesis of various eicosanoids, some of which may mediate platelet aggregation [11]. The present study revealed that antiflammins inhibited the aggregation induced by collagen but not by arachidonic acid, whereas ketoprofen, a cyclooxygenase inhibitor, reduced the aggregation induced by arachidonic acid and collagen. On the other hand, the peptides tested only inhibited TxB_2 formation when platelets were stimulated with collagen. This result suggests that these nonapeptides have no suppressive effects on cyclooxygenase, whereas they have a specific action to inhibit platelet PLA_2 activation, with an IC_{50} of 10^{-4} M. These results confirm the recent findings of Perretti *et al.* [12], who observed that antiflammins inhibited the contractions of isolated rat stomach strips when elicited by porcine pancreatic PLA_2, although contractions caused by arachidonic acid or prostaglandin E_2 were not affected. In addition, Tetta *et al.* [13] recently described two antiflammin derivatives which appeared to interfere with the activation of PLA_2. Platelet aggregation was less sensitive to inhibition by antiflammins 1 and 2 than the activity of purified PLA_2 [5] and platelet-activating factor synthesis by rat peritoneal macrophages or human neutrophil migration [14]. We have found that antiflammins are weak inhibitors of collagen-induced platelet aggregation compared with ketoprofen (IC_{50} 10^{-6} M). These results agree with those of Vostal *et al.* [14], who observed the effect of antiflammins on thrombin-induced platelet aggregation.

Results obtained *in vitro* were in agreement with the *in vivo* results, where antiflammins inhibited carrageenan-induced paw oedema in the rat and TPA-induced mouse ear oedema, two classical models of experimental inflammation. Subplantar administration of antiflammins in rat paw oedema produced a significant inhibition of inflammation, as described by Miele *et al.* [5]. When experimental paw oedema was directly induced by PLA_2 (*N. naja*), aristolochic acid and *p*-bromo phenacyl bromide, two PLA_2 inhibitors [15, 16], significantly decreased the inflammation, whereas antiflammins did not have any antioedematous activity. In our experiments, these peptides also had significant anti-inflammatory effects when administered systemically by intraperitoneal injection. However, they were ineffective when given subcutaneously. Similar results were obtained when peptides were used to inhibit ear oedema induced by TPA.

In conclusion, we have demonstrated that antiflammins have the capacity to inhibit PLA_2 activation. This inhibition is probably due to a mechanism other than direct inhibition of the enzyme or interference with the enzyme–substrate interaction. Moreover, it has been reported by Miele *et al.* [5] that antiflammin peptides inhibit monomeric PLA_2s such as porcine pancreatic enzymes. However, in our study these peptides did not inhibit the pro-inflammatory effects of dimeric enzymes such as those obtained from *N. naja* venoms. An alternative hypothesis to explain the mechanism of action of

these peptides is that antiflammins may inhibit the dimerization of the monomeric PLA_2 and thereby prevent the interfacial activation of this enzyme.

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