British Journal of Pharmacology (1996) 117, 1773-1779

Involvement of secretory phospholipase A_2 activity in the zymosan rat air pouch model of inflammation

Miguel Payá, M. Carmen Terencio, M. Luisa Ferrándiz & [1]M. José Alcaraz

Departamento de Farmacología, Universidad de Valencia, Facultad de Farmacia, Avda; Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain

1 In the zymosan rat air pouch model of inflammation we have assessed the time dependence of phospholipase A_2 (PLA_2) accumulation in the inflammatory exudates as well as cell migration, myeloperoxidase activity, prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) levels.

2 A significant increase in PLA_2 activity was detected in 1,200 *g* supernatants of exudates 8 h after injection of zymosan into rat air pouch. This event coincided with peaks in cell accumulation (mainly neutrophils) and myeloperoxidase activity in exudates and was preceded by a rise in eicosanoid levels.

3 This enzyme (without further purification) behaved as a secretory type II PLA_2 with an optimum pH at 7-8 units, lack of selectivity for arachidonate release and dependence on mM calcium concentrations for maximal activity.

4 The PLA_2 inhibitors manoalide and scalaradial inhibited this enzyme activity *in vitro* in a concentration-dependent manner. Scalaradial also inhibited zymosan stimulated myeloperoxidase release *in vitro*.

5 Injection of the marine PLA_2 inhibitor scalaradial together with zymosan into the pouch at doses of 0.5, 1 and 5 μmol per pouch resulted in a dose-dependent inhibition of PLA_2 activity in exudates collected 8 h later. Myeloperoxidase levels and cell migration were also decreased, while eicosanoid levels were not modified.

6 Colchicine administration to rats prevented infiltration and decreased PLA_2 levels in the 8 h zymosan-injected air pouch.

7 These results indicate that during inflammatory response to zymosan in the rat air pouch a secretory PLA_2 activity is released into the exudates. The source of this activity is mainly the neutrophil which migrates into the pouch.

8 Scalaradial exerts anti-inflammatory effects in the zymosan air pouch.

Keywords: Zymosan; rat air pouch; phospholipase A_2; myeloperoxidase; scalaradial; manoalide

Introduction

The predominant pathway for the release of arachidonic acid from phospholipids is the phospholipase A_2 (PLA_2) activity, which hydrolyzes the *sn*-2 ester bonds in phosphoglyceride molecules. PLA_2s can be classified into secretory and intracellular forms. Secretory PLA_2s have a molecular mass of about 14 kDa and can be divided into groups I (mammalian pancreas, *Elapidae* and *Hydrophidae* snake venoms) and II (*Crotalidae* and *Viperidae* snake venoms, as well as in mammalian platelets, neutrophils, peritoneum, synovial fluids...). Other low molecular weight PLA_2s can be found in the venoms of bees and wasps and are included in a different group (III), for they are not closely related to the enzymes in groups I and II (Glaser *et al.*, 1993; Mukherjee *et al.*, 1994). High molecular weight PLA_2s (about 85 kDa) are present in various mammalian cells such as platelets, macrophage cell lines and neutrophils (Clarke *et al.*, 1990; Takayama *et al.*, 1991; Ramesha & Ives, 1993).

Mammalian group II PLA_2s are synthesized and secreted from many cell types and are found at inflammatory sites, suggesting a role in inflammatory processes. In fact, some of these enzymes have demonstrated proinflammatory effects when injected into animals (Vadas & Pruzanski, 1986; Vishwanath *et al.*, 1988; Neves *et al.*, 1993). Nevertheless, evaluation of the precise physiological role of PLA_2 enzymes has been limited by the difficulties in characterizing PLA_2 and the lack of suitable inhibitors. A number of natural products inhibit PLA_2 activity, mainly of the extracellular type and also show anti-inflammatory activity in animals, probably due to PLA_2 inhibition (Mayer *et al.*, 1988; Vishwanath *et al.*, 1988; Potts *et al.*, 1992; Miyake *et al.*, 1993; Marshall *et al.*, 1994; Tramposch *et al.*, 1994; Gil *et al.*, 1994), although there is no direct evidence for PLA_2 participation in experimental models of inflammation and its pharmacological modulation. Scalaradial, a marine natural product exhibits the *in vitro* pharmacological profile of a potent PLA_2 inhibitor acting on extracellular and intracellular enzymes (de Carvalho Jacobs, 1991; Marshall *et al.*, 1994; Farina *et al.*, 1994); however, to date no data on its possible inhibitory effects on PLA_2 *in vivo* have been reported.

The air pouch is a cavity lined with macrophages comparable to the macrophage colonization of the synovial surface during inflammatory states (Edwards *et al.*, 1981) and represents a suitable model for studying responses induced by inflammatory agents like zymosan. The aim of the present study was to determine if PLA_2 activity is involved in the inflammatory response induced by zymosan in the rat air pouch. For this purpose, the time-dependence of parameters like leukocyte migration, myeloperoxidase release, eicosanoid levels and secretory PLA_2 activity have been assessed. In addition, we have investigated *in vivo* the modulation of this response by the PLA_2 inhibitor scalaradial.

Methods

Rat air pouch

Male Wistar rats (120-150 g) were used. Air pouches were formed as previously described (Edwards *et al.*, 1981). The

[1] Author for correspondence.

animals were anaesthetized with ethyl ether and given a 20 ml injection of sterile air in the subcutaneous tissue of the back, and three days later 10 ml of sterile air was injected into the same cavity. After three days 1 ml of saline or 1 ml of 1% (w/v) zymosan in saline was administered into the air pouch. To test the effect of scalaradial we administered 1 ml of 1% (w/v) zymosan in saline (zymosan control group) or 1 ml of 1% (w/v) zymosan in saline + scalaradial at the doses of 0.5, 1 and 5 μmol per pouch. In another set of experiments, animals received colchicine (1.5 mg kg^{-1}, i.p.) 15 min before the injection of zymosan. At different time intervals after the injection of saline or zymosan into the air pouch, rats were killed and the exudate was collected in 1 ml of saline. Leukocytes in exudate fluids were counted by Coulter counter. Exudate smears were stained with Wright's stain for differential counts. After centrifugation of the exudate at 1,200 *g* at 4°C for 10 min, the supernatants were used to measure myeloperoxidase activity, phospholipase A_2 activity, as well as leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) levels by radioimmunoassay (Moroney *et al.*, 1988). Protein was quantified by the Bradford technique (Bradford, 1976) using bovine serum albumin as standard.

Myeloperoxidase assay

The reaction mixture contained 10 μl supernatant, 190 μl phosphate buffered saline, 20 μl 0.22 M NaH_2PO_4 (pH 5.4), 20 μl 0.026% (v/v) H_2O_2 and 20 μl 18 mM tetramethylbenzidine in 8% (v/v) dimethylformamide. After a 10 min reaction at 37°C, 30 μl 1.46 M sodium acetate, pH 3.0, was added and absorbance at 620 nm was read using a microtiter plate reader (Suzuki *et al.*, 1983; De Young *et al.*, 1989). Activity was determined by comparison with the standard curve using horseradish peroxidase. The possible direct influence of scalaradial on myeloperoxidase activity was also assessed *in vitro*. Supernatant (10 μl) from the exudates obtained in 8 h zymosan-injected rat air pouches (zymosan control group) in the same buffer as above was preincubated at 37°C for 10 min with 2.5 μl of test compound solution or its vehicle (methanol) before incubation proceeded.

Phospholipase A_2 assay

Phospholipase A_2 was assayed using [^{3}H]-oleate labelled membranes of *Escherichia coli*, following a modification of the method of Franson *et al.* (1974) (Ferrándiz *et al.*, 1994). *E. coli* strain CECT 101 was seeded in medium containing 1% tryptone, 0.5% (w/v) NaCl and 0.6% (w/v) sodium dihydrogen orthophosphate, pH 5.0, and grown for 6–8 h at 37°C in the presence of 5 μCi ml^{-1} [^{3}H]-oleic acid (sp. act. 10 Ci $mmol^{-1}$) until growth approached the end of the logarithmic phase. After centrifugation at 2,500 *g* for 10 min, the membranes were washed in buffer (0.7 M Tris-HCl, 10 mM $CaCl_2$, 0.1% bovine serum albumin, pH 8.0), resuspended in saline and autoclaved for 30–45 min. The membranes were then washed, centrifuged again and frozen at −70°C. At least 95% of the radioactivity was incorporated into the phospholipid fraction. Supernatants from the exudates obtained in the rat air pouch (10 μl) and 250 μl of 100 mM Tris-HCl, 1 mM $CaCl_2$ buffer, pH 7.5, were incubated for 10 min in the presence of 10 μl autoclaved oleate labelled membranes (1 μg protein and 3.2 pmol [^{3}H]-oleic acid incorporated into membranes) and was terminated by addition of 100 μl ice-cold solution of 0.25% bovine serum albumin in saline to a final concentration of 0.07% (w/v). After centrifugation at 2,500 *g* for 10 min at 4°C, the radioactivity in the supernatants was determined by liquid scintillation counting. For *in vitro* assessment of the influence of scalaradial or manoalide on secretory PLA_2, supernatant (10 μl) from the exudates obtained in 8 h zymosan-injected rat air pouches (zymosan control group) and 250 μl of 100 mM Tris-HCl, 1 mM $CaCl_2$ buffer pH 7.5 were preincubated at 37°C for 10 min with 2.5 μl of test compound solution or its vehicle (methanol) before incubation was started by addition of oleate labelled membranes as above. The inhibitory effect of scalaradial or manoalide was measured in relation to control enzyme activity in tubes containing enzyme and the inhibitor vehicle. To determine the influence of protein, calcium or pH on PLA_2 activity, different sets of experiments were performed in the presence of increasing volumes of supernatant, with or without 10 mM EDTA and 1 mM $CaCl_2$ or with different pH values, as indicated in the Results section.

Rat peritoneal leukocyte degranulation

Rat peritoneal leukocytes were collected 6 h after i.p. injection of glycogen (10 ml, 1%) and prepared by centrifugation and hypotonic lysis of contaminating red cells (Moroney *et al.*, 1988). The cell suspension contained 90% neutrophils; cell viability was assessed by trypan blue exclusion and was greater than 95%. Boiled zymosan was incubated at 37°C for 30 min with supernatant from 8 h zymosan air pouches to opsonize it (20 mg ml^{-1}), washed three times with saline and resuspended in Hank's solution. Leukocytes (5×10^6 cells ml^{-1}) were stimulated with zymosan (3 mg ml^{-1}) for 20 min at 37°C. Scalaradial was preincubated for 5 min with the cells before the addition of zymosan. The cell suspensions were then placed on ice and centrifuged at 1,200 *g* for 10 min at 4°C. Myeloperoxidase levels in supernatants were determined as above.

Materials

Scalaradial was a gift from Dr S. De Rosa (Istituto per la Chimica di Molecole di Interesse Biologico, C.N.R., Arco Felice, Napoli, Italy) and antibody against LTB_4 was kindly provided by Dr R.M. McMillan and Dr S.J. Foster (Zeneca Pharmaceuticals, Macclesfield, Cheshire). [9,10-^{3}H]-oleic acid was purchased from Du Pont, (Itisa, Madrid, Spain); [5,6,8,11,12,14,15(n)-^{3}H]-PGE_2 and [5,6,8,9,11,12,14,15(n)-^{3}H]-LTB_4 were from Amersham Iberica (Madrid, Spain). Manoalide and the rest of the reagents from Sigma Chem. (MO, U.S.A.). *E coli* strain CECT 101 was a gift from Prof. Uruburu (Department of Microbiology, University of Valencia, Spain).

Statistical analysis

The results are presented as mean ± s.e.mean. The level of statistical significance was determined by Student's unpaired *t* test (two tailed) or by analysis of variance (ANOVA) followed by Dunnett's *t* test for multiple comparisons.

Results

Cell influx into the rat air pouch

As indicated in Table 1 , zymosan stimulated an increase in the total number of cells present in exudates. The number of cells present within zymosan-stimulated pouches remained significantly greater than the number present in saline-injected pouches for at least 24 h, and this increase in cell influx was maximal at 8 h. The majority of the cells present in exudates at any time were neutrophils, especially in zymosan-stimulated pouches.

Eicosanoid levels

Exudates from saline-injected pouches contained barely detectable levels of PGE_2 and LTB_4 (Figure 1). 4 h after injection of zymosan, 7.2 ± 0.7 ng PGE_2 mg^{-1} protein and 7.8 ± 1.3 ng LTB_4 mg^{-1} protein were detected in exudates. These levels fell at 8 h (6.7 ± 1.5 ng PGE_2 mg^{-1} protein and 0.7 ± 0.2 ng LTB_4 mg^{-1} protein) and almost disappeared at 24 h.

Table 1 Accumulation of inflammatory cells in air pouches after saline or zymosan injection

	Total cells ($\times 10^6$ ml^{-1})	*White blood cells present in pouch fluid* % *neutrophils*	% *monocytes*	% *lymphocytes*
Saline 4 h	4.7 ± 1.0	77	6	7
Zymosan 4 h	59.7 ± 7.5**	92	3	5
Saline 8 h	19.3 ± 3.3	76	5	19
Zymosan 8 h	109.7 ± 11.6**	95	1	4
Saline 24 h	3.1 ± 0.6	72	7	21
Zymosan 24 h	89.5 ± 7.4**	97	1	2

The values are the mean ± s.e.mean ($n = 12$). **$P < 0.01$ with respect to the corresponding saline-injected group.

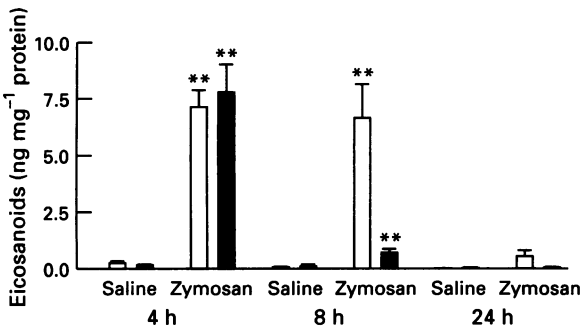

Figure 1 Eicosanoid levels in the exudates of rat air pouches injected with saline or zymosan. Open columns, prostaglandin E_2; solid columns, leukotriene B_4. Eicosanoids were measured in the inflammatory exudates of saline and zymosan groups in the rat air pouch 4 h, 8 h and 24 h after injection. The values are the mean ± s.e.mean ($n = 12$). **$P < 0.01$ with respect to the corresponding saline-injected group.

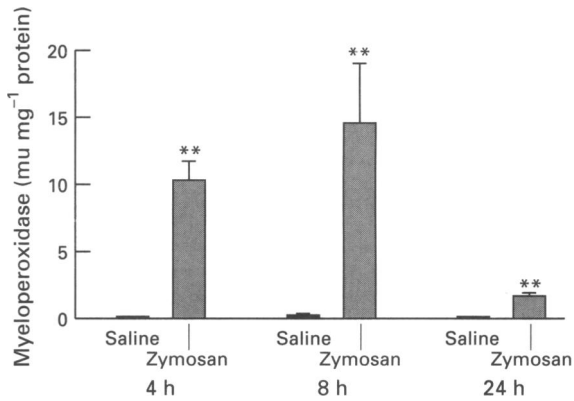

Figure 2 Myeloperoxidase levels in the exudates of rat air pouches injected with saline or zymosan. Myeloperoxidase activity was determined in the inflammatory exudates of saline and zymosan groups in the rat air pouch 4 h, 8 h and 24 h after injection. The values are the mean ± s.e.mean ($n = 12$). **$P < 0.01$ with respect to the corresponding saline group.

Myeloperoxidase activity

At all time points detectable levels of this enzyme activity were found in saline-injected pouches (Figure 2). In contrast, high levels of myeloperoxidase were measured in exudate samples from zymosan-stimulated pouches at 4 h (10.3 ± 1.3 mu mg^{-1} protein) and reached a peak at 8 h (13.0 ± 1.0 mu mg^{-1} protein). By 24 h the level of activity was reduced to 1.7 ± 0.2 mu mg^{-1} protein.

Phospholipase A_2 activity

At 4 h, the levels of PLA_2 in exudates from zymosan-activated pouches were not significantly different from those found in saline-injected pouches. Nevertheless, a significant increase in PLA_2 activity was detected in inflammatory exudates obtained 8 h after zymosan injection into the rat air pouch (Figure 3), followed by a decrease in activity in samples taken at 24 h. This PLA_2 is able to release 1.9 ± 0.2 nmol [^{3}H]-oleic acid mg^{-1} protein per 10 min incubation ($n = 12$) from labelled *E coli* membranes, and it was also dependent on the pH, with an optimum activity at 7–8 units (Figure 4). As seen in Figure 5, activity gradually increased with the amount of protein present in the incubation medium and it showed a requirement for calcium. At the concentration used (1 mM Ca^{2+}) maximal activity was reached.

In vivo *effect of scalaradial on the inflammatory response produced 8 h after injection of zymosan*

Since significant PLA_2 activity was measured in exudates 8 h after injection of zymosan, this time point was chosen to assess the influence of scalaradial on this enzyme activity and the rest of the parameters that characterize this inflammatory response. Scalaradial injected into the air pouch at doses of 0.5, 1 and 5 μmol per pouch, dose-dependently inhibited PLA_2 activity in exudates (Table 2). In addition, this PLA_2 inhibitor decreased leukocyte influx at the highest dose used, 5 μmol per pouch (without modification of differential counts with respect to the zymosan control group, data not shown), and myeloperoxidase levels at 1 and 5 μmol per pouch, whereas PGE_2 and LTB_4 levels did not show any significant modification. A direct inhibition of myeloperoxidase activity *in vitro* by scalaradial at concentrations between 1 and 100 μM was not observed (data not shown).

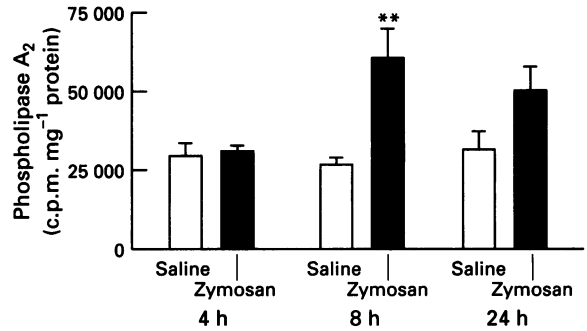

Figure 3 Phospholipase A_2 (PLA_2) activity present in the exudates of the rat airpouch. Activity was expressed as c.p.m. of [^{3}H]-oleic acid released per mg protein after 10 min incubation, and it was measured in the inflammatory exudates of saline and zymosan groups in the rat air pouch 4 h, 8 h and 24 h after injection. The values are the mean ± s.e.mean ($n = 12$). **$P < 0.01$ with respect to the corresponding saline group.

In vitro *effect of scalaradial and manoalide on PLA_2 from 8 h zymosan-injected rat air pouch*

Incubation of scalaradial or manoalide with 10 μl of supernatant of exudates obtained from rat air pouches 8 h after injection of zymosan resulted in the inhibition of PLA_2 activity present in these inflammatory exudates. The concentration-dependence of this effect appears in Figure 6. Scalaradial and manoalide showed IC_{50} values with 95% confidence limits of 3.2 (2.1–4.8) μM and 2.6 (1.6–3.1) μM, respectively.

Effect of colchicine on the inflammatory response produced 8 h after injection of zymosan

Since the elevated PLA_2 levels in the pouch fluid collected 8 h after injection of zymosan could be dependent upon leukocyte migration, colchicine was used to prevent cellular infiltration. Colchicine (1.5 mg kg^{-1}, i.p.) was given 15 min before zymosan administration, and the animals were killed 8 h later. Colchicine inhibited cellular infiltration into the air pouch by 96.4%, myeloperoxidase levels by 93.7% and PLA_2 activity by 89.1% (Table 3).

In vitro *effect of scalaradial on rat peritoneal leukocyte degranulation*

As shown in Table 4, pretreatment of neutrophils with scalaradial caused a concentration-dependent inhibition of zymosan-induced degranulation measured as myeloperoxidase release.

Discussion

It is known that zymosan particles are phagocytosed by activated macrophages in the air pouch (Dawson *et al.*, 1991) and also that zymosan activates the alternative pathway of complement (Schorlemmer *et al.*, 1977; Wedmore & Williams, 1981; Jose, 1987). In the rat air pouch zymosan causes the generation of tumour necrosis factor-α (TNF-α), PGE_2, LTB_4 and lower amounts of LTC_4 (Ferrándiz & Foster, 1991). In our experiments, as in mice (Dawson *et al.*, 1991), zymosan induced an exudate containing mainly neutrophils, at the three observations made (4, 8 and 24 h). The peak of PLA_2 activity in 8 h exudates correlated with the maximum rate of neutrophil influx, which suggests that neutrophils are the source of PLA_2. These cells have been described as a source of PLA_2 activity in inflammatory fluids due to the homology of the enzyme present in their granules and that found in ascitic fluids (Wright *et al.*, 1990), although other cells like macrophages could also contribute to the PLA_2 levels present in inflammatory exudates. Systemic treatment of rats with colchicine markedly reduced cell migration and this was accompanied by a significant reduction in PLA_2 levels, which confirms that infiltrating neutrophils are the main source of this enzyme activity in the zymosan-injected air pouch exudates. It is interesting to note that zymosan induces *in vitro* the release of lysosomal enzymes and PLA_2 activity in rabbit neutrophils (Traynor & Authi, 1981) and mouse peritoneal macrophages (Wightman *et al.*, 1981).

In the rat air pouch stimulated by zymosan, activated cells synthesized eicosanoids at an early stage (4 h) and released myeloperoxidase mainly at 8 h. At this time there was also secretion of PLA_2 activity into the inflammatory exudate. The similarity in the time-response dependence of these last two events suggests that there is a relationship between them,

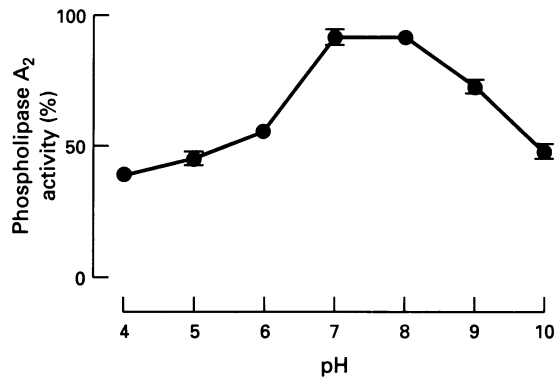

Figure 4 Effect of pH on phospholipase A_2 (PLA_2) activity. Data are expressed as percentages of [^{3}H]-oleic acid release (mean ± s.e.mean from $n=8$). Supernatant from 8 h zymosan-injected rat air pouches (10 μl, 200 μg of protein) was incubated for 10 min with 3.2 pmol [^{3}H]-oleic acid incorporated in *E. coli.* membranes, in the presence of 1 mM $CaCl_2$.

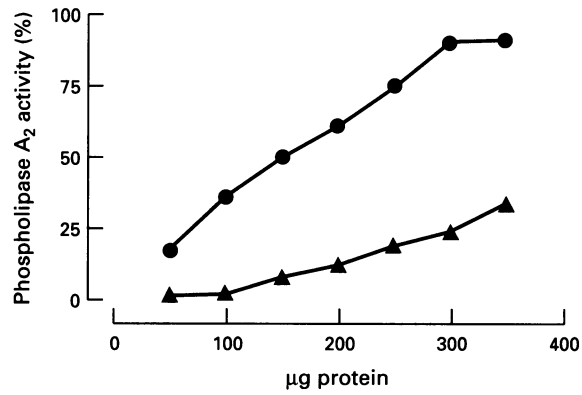

Figure 5 Protein dependence of phospholipase A_2 (PLA_2) activity and influence of calcium. Data are expressed as percentages of [^{3}H]-oleic acid release (means from $n=3$). Standard errors (not shown) were less than 5% of the respective means. Supernatant from 8 h zymosan-injected rat air pouches was incubated for 10 min with 3.2 pmol of [^{3}H]-oleic acid incorporated in *E. coli.* membranes. (●) Reaction in the presence of 1 mM $CaCl_2$. (▲) Reaction in the presence of 1 mM $CaCl_2$ + 10 mM EDTA. All activity values in the absence of EDTA are significantly different from their corresponding values in the presence of EDTA ($P<0.01$).

Table 2 Effect of scalaradial on the inflammatory response produced 8 h after injection of zymosan

		% inhibition			
	PLA_2	Leukocyte influx	Myeloperoxidase	PGE_2	LTB_4
Scalaradial (μmol/pouch)					
5	53.4 ± 4.3**	65.6 ± 6.1**	47.0 ± 5.5*	30.9 ± 9.0	17.5 ± 13.4
1	44.1 ± 5.2**	27.7 ± 9.5	26.9 ± 2.5*	31.4 ± 5.8	12.0 ± 8.3
0.5	34.4 ± 8.0*	6.7 ± 1.8	4.2 ± 0.8	20.2 ± 5.3	8.1 ± 8.1

The values are the mean ± s.e.mean ($n=6-12$). *$P<0.05$, **$P<0.01$ with respect to the zymosan control group.

whereas the eicosanoid increase in exudates occurs prior to PLA_2 secretion and thus does not seem to depend on the secretory PLA_2 activity present in inflammatory exudates.

Myeloperoxidase and PLA_2 levels in exudates were decreased by scalaradial, which could reflect the inhibition of the degranulation process in this experimental model. This compound inhibits degranulation of human neutrophils induced by receptor-activation and non-receptor-mediated stimuli (Barnette *et al.*, 1994). In fact, our experiments in rat peritoneal leukocytes stimulated with zymosan demonstrate that scalaradial inhibits myeloperoxidase release, and our results also exclude the participation of a direct inhibitory effect of scalaradial on myeloperoxidase activity.

On the other hand, scalaradial administered into the air pouch partially inhibits cell migration and, in addition, it was more potent as an inhibitor of PLA_2 than of myeloperoxidase levels in exudates. Its effect in the zymosan-injected rat air pouch probably results from a decrease in cell accumulation at the highest dose administered, although partial inhibition of degranulation can occur at lower doses of scalaradial, which are unable to affect cell migration.

We have demonstrated the presence of PLA_2 activity in the inflammatory exudates of the rat air pouch 8 h after injection of zymosan. Established PLA_2 inhibitors like manoalide (Jacobson *et al.*, 1990) and scalaradial (de Carvalho & Jacobs, 1991) inhibit this activity *in vitro* in a concentration-dependent manner. This enzyme presents the general characteristics of a secretory type II PLA_2 with an optimum pH in the neutral-basic range, an absolute requirement for calcium ions for expression of full activity in the mM range and a lack of selectivity for arachidonic acid release, for it caused the release of [^{3}H]-oleic acid from the 2-position of *E. coli* membrane phospholipids (Reynolds *et al.*, 1993; Kudo *et al.*, 1993).

Low molecular weight PLA_2s have no sequence homology with high molecular weight cytosolic PLA_2s, which translocate to membrane in response to changes in free calcium and would selectively cleave arachidonic acid (Clarke *et al.*, 1991). Cytosolic and secretory PLA_2s have been described in a number of cells, including neutrophils (Traynor & Authi, 1981; Balsinde *et al.*, 1988; Ramesha & Ives, 1993), monocytes (Wightman *et al.*, 1981; Ulevitch *et al.*, 1988; Clark *et al.*, 1990) and platelets (Matsumoto *et al.*, 1988; Kramer *et al.*, 1989; Takayama *et al.*, 1991). PLA_2 enzymes may be involved in cell proliferation and signal transduction as well as in the pathogenesis of disease processes such as inflammation (Mukherjee *et al.*, 1994). Secretory PLA_2s with the biochemical properties of group II enzymes can be induced and secreted from cytokine-stimulated cells (Daniels *et al.*, 1992; Glaser *et al.*, 1993; Angel *et al.*, 1993), and they are present at high levels in synovial fluid, peripheral blood, neutrophils and monocytes of patients with rheumatoid arthritis (Kramer *et al.*, 1989; Bomalaski & Clark, 1993). In this experimental model the synthesis and secretion of PLA_2 can be induced by TNFα, for this cytokine enhances the expression of group II PLA_2 and may also facilitate membrane phospholipid hydrolysis by this enzyme (Kudo *et al.*, 1993). Nevertheless, the involvement of secretory PLA_2 activity in the selective release of arachidonic acid is controversial, and it has been suggested that cytosolic enzymes play a role in human neutrophils (Ramesha & Ives, 1993). Thus, ligand-induced activation of a calcium-responsive cytosolic PLA_2 would, through the participation of G proteins and protein kinases, lead to the release of arachidonic acid, thus initiating the biosynthesis of inflammatory mediators (Clark *et al.*, 1990). However, in some cell types like rat mesangial cells, the involvement of a secretory group II PLA_2 in arachidonic acid release and PGE_2 synthesis in response to cytokine stimulation has been demonstrated (Pfeilschifter *et al.* 1993).

Since several forms of PLA_2 are present in the same cells, multiple pathways for arachidonic acid release may coexist. For instance, in P388D$_1$ cells a cytosolic PLA_2 is involved in the rise in intracellular arachidonic acid levels during the early steps of cell activation, while a secretory PLA_2 is related to the accumulation of arachidonic acid in the incubation medium, thus suggesting co-operations between the two types of PLA_2 in arachidonic acid release (Balsinde *et al.*, 1994). Apart from the release of arachidonic acid for the synthesis of mediators like eicosanoids and platelet activating factor (PAF), other suggested roles for secretory PLA_2 in inflammation can be the destruction of micro-organisms and cell damage by hydrolysis of membrane phospholipids in activated cells (Wright *et al.*,

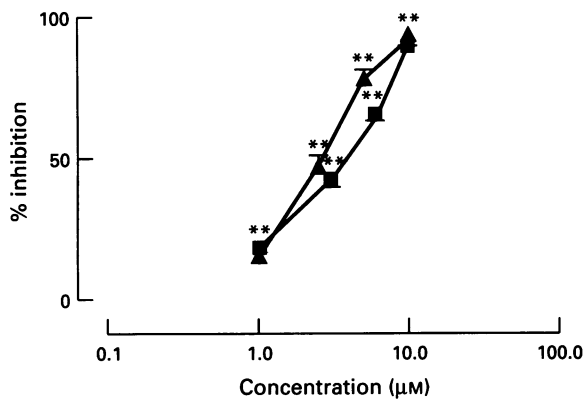

Figure 6 Concentration-dependence of the *in vitro* inhibitory effect of scalaradial and manoalide on phospholipase A_2 (PLA_2) from the 8 h zymosan-injected rat air pouch. (■) Scalaradial; (▲) Manoalide. The values are the means ± s.e.mean ($n=6$). $^{**}P<0.01$.

Table 4 Effect of scalaradial on the release of myeloperoxidase activity induced by zymosan in rat peritoneal leukocytes

	Myeloperoxidase (mu released by 10^6 cells)
Control	7.9 ± 0.2
Scalaradial (0.5 μM)	3.4 ± 0.2**
Scalaradial (1 μM)	2.9 ± 0.1**
Scalaradial (2.5 μM)	1.3 ± 0.2**
Scalaradial (5 μM)	0.7 ± 0.1**

The values are the mean ± s.e.mean ($n=6$). $^{**}P<0.01$.

Table 3 Effect of colchicine on cellular accumulation, myeloperoxidase and PLA_2 in the 8 h zymosan-injected rat air pouch

	Total cells ($\times 10^6$ ml^{-1})	*Myeloperoxidase* (mu mg^{-1} protein)	*PLA_2* ($\times 10^3$ c.p.m. mg^{-1} protein)
Saline	4.5 ± 0.5	0.0 ± 0.0	21.6 ± 6.4
Zymosan	64.3 ± 0.9	9.6 ± 0.9	73.6 ± 4.9
Zymosan + colchicine	2.3 ± 0.6**	0.6 ± 0.3**	8.0 ± 1.1**

The values are the means ± s.e.mean ($n=6$). $^{**}P<0.01$.

1990; Kudo *et al.*, 1993; Weiss *et al.*, 1994), or participation in a proliferative response and regulation of cytokine synthesis (Bomalaski & Clark, 1993).

The marine natural product scalaradial inhibits *in vitro* recombinant human synovial and bee venom PLA_2 in an irreversible way (de Carvalho & Jacobs, 1991; Marshall *et al.*, 1994), whereas it shows a lower potency on cytosolic PLA_2 preparations (Marshall *et al.*, 1994). Scalaradial also inhibits arachidonic acid release and consequently LTB_4 and PAF synthesis in human neutrophils stimulated *in vitro* by A23187 (Marshall *et al.*, 1994) as well as degranulation (Barnette *et al.*, 1994). *In vivo*, the 12-epimer of scalaradial inhibits the mouse ear oedema induced by 12-*O*-tetradecanoylphorbol 13-acetate after topical administration (Marshall *et al.*, 1994). Our results indicate that scalaradial is an inhibitor of secretory PLA_2 activity in the inflammatory response induced by zymosan in the rat air pouch and exerts anti-inflammatory effects *in vivo*, with inhibition of leukocyte migration into the pouch and myeloperoxidase levels. The fact that scalaradial does not decrease eicosanoid levels in the exudates suggests that the production of these mediators is dependent on the activity of a different PLA_2 or other enzymes like phospholipase C, which are not inhibited by scalaradial. Indeed, it is known that phagocytosis of zymosan particles initiates an increase in cytoplasmic calcium (Meagher *et al.*, 1991) and results in stimulation of phospholipase C with formation of inositol phosphates in macrophages (Emilsson & Sundler, 1984). The secretory PLA_2 detected in the rat air pouch stimulated by zymosan may contribute to the amplification of the inflammatory response, although other proinflammatory factors may be induced or activated by zymosan administration and they may act in conjunction with PLA_2.

This study was supported by grant PM92-0145, C.I.C.Y. T., Spanish Ministerio de Educación y Ciencia. We wish to thank Dr R.M. McMillan and Dr S.J. Foster, (Zeneca Pharmaceuticals, Macclesfield, Cheshire, U.K.) for the kind gift of antibody against LTB_4 and Dr S. De Rosa (Istituto per la Chimica de Molecole di Interesse Biologico, C.N.R., Arco Felice, Napoli, Italy) for providing scalaradial.

References

ANGEL, J., COLARD, O., CHEVY, F. & FOURNIER, C. (1993). Interleukin-1-mediated phospholipid breakdown and arachidonic acid release in human synovial cells. *Arthritis Rheum.*, **36**, 158–167.

BALSINDE, J., BARBOUR, S.E., BIANCO, I.D. & DENNIS, E.A. (1994). Arachidonic acid mobilization in P388D_1 macrophages is controlled by two distinct Ca^{2+}-dependent phospholipase A_2 enzymes. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 11060–11064.

BALSINDE, J., DIEZ, E., SCHÜLLER, A. & MOLLINEDO, F. (1988). Phospholipase A_2 activity in resting and activated human neutrophils. Substrate specificity, pH dependence, and subcellular localization. *J. Biol. Chem.*, **263**, 1929–1936.

BARNETTE, M.S., RUSH, J., MARSHALL, L.A., FOLEY, J.J., SCHMIDT, D.B. & SARAU, H.M. (1994). Effects of scalaradial, a novel inhibitor of 14kDa phospholipase A_2, on human neutrophil function. *Biochem. Pharmacol.*, **47**, 1661–1668.

BOMALASKI, J.S & CLARK, M.A. (1993). Phospholipase A_2 and arthritis. *Arthritis Rheum.*, **36**, 190–198.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

CLARK, J.D., LIN, L.-L., KRIZ, R.W., RAMESHA, C.S., SULTZMAN, L.A., LIN, A.Y., MILONA, N. & KNOPF, J.L. (1991). A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology by PKC and GAP. *Cell*, **65**, 1043–1051.

CLARK, J.D., MILONA, N. & KNOPF, J.L. (1990). Purification of a 110-kilodalton cytosolic phospholipase A_2 from the human monocytic cell line U937. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 7708–7712.

DANIELS, R.H., FINNEN, M.J., HILL, M.E. & LACKIE, J.M. (1992). Recombinant human monocyte IL-8 primes NADPH-oxidase and phospholipase A_2 activation in human neutrophils. *Immunology*, **75**, 157–163.

DAWSON, J., SEDGWICK, A.D., EDWARDS, J.C. & LEES, P. (1991). A comparative study of the cellular, exudative and histological responses to carrageenan, dextran and zymosan in the mouse. *Int. J. Tissue React.*, **13**, 171–185.

DE CARVALHO, M.S. & JACOBS, R.S. (1991). Two-step inactivation of bee venom phospholipase A_2 by scalaradial. *Biochem. Pharmacol.*, **42**, 1621–1626.

DE YOUNG, L.M., KHEIFETS, J.B., BALLARON, S.J. & YOUNG, J.M. (1989). Edema and cell infiltration in the phorbol ester-treated mouse ear are temporally separate and can be differentially modulated by pharmacologic agents. *Agents Actions*, **26**, 335–341.

EDWARDS, J.C.W., SEDGWICK, A.D. & WILLOUGHBY, D.A. (1981). The formation of a structure with the features of synovial lining by subcutaneous injection of air: an *in vivo* tissue culture system. *J. Pathol.*, **134**, 147–156.

EMILSSON, A. & SUNDLER, R. (1984). Differential activation of phosphatidylinositol deacylation and a pathway via diphosphoinositide in macrophages responding to zymosan and ionophore A23187. *J. Biol. Chem.*, **259**, 3111–3116.

FARINA, P.R., GRAHAM, A.G., HOFFMAN, A.F., WATROUS, J.M., BORGEAT, P., NADEAU, M., HANSEN, G., DINALLO, R., ADAMS, J., MIAO, C.K., LAZER, E.S., PARKS, T.P. & HOMON, C.A. (1994). BIRM 270: A novel inhibitor of arachidonate release that blocks leukotriene B_4 and platelet-activating factor biosynthesis in human neutrophils. *J. Pharmacol. Exp. Ther.*, **271**, 1418–1426.

FERRANDIZ, M.L. & FOSTER, S.J. (1991). Tumour necrosis factor production in a rat airpouch model of inflammation: role of eicosanoids. *Agents Actions*, **32**, 289–294.

FERRANDIZ, M.L., SANZ, M.J., BUSTOS, G., PAYA, M., ALCARAZ, M.J. & DE ROSA, S. (1994). Avarol and avarone, two new anti-inflammatory agents of marine origin. *Eur. J. Pharmacol.*, **253**, 75–82.

FRANSON, R., PATRIARCA, P. & ELSBACH, P. (1974). Phospholipid metabolism by phagocytic cells. Phospholipases A_2 associated with rabbit polymorphonuclear leukocyte granules. *J. Lipid Res.*, **15**, 380–388.

GIL, B., SANZ, M.J., TERENCIO, M.C., FERRANDIZ, M.L., BUSTOS, G., PAYA, M., GUNASEGARAN, R. & ALCARAZ, M.J. (1994). Effects of flavonoids on *Naja naja* and human recombinant synovial phospholipases A_2 and inflammatory responses in mice. *Life Sci.*, **54**, 333–338.

GLASER, K.B., MOBILIO, D., CHANGE, J.Y. & SENKO, N. (1993). Phospholipase A_2 enzymes: regulation and inhibition. *Trends Pharmacol. Sci.*, **14**, 92–98.

JACOBSON, P.B., MARSHALL, L.A., SUNG, A & JACOBS, R.S. (1990). Inactivation of human synovial fluid phospholipase A_2 by the marine natural product, manoalide. *Biochem. Pharmacol.*, **39**, 1557–1564.

JOSE, P.J. (1987). Complement-derived peptide mediators of inflammation. *Br. Med. Bull.*, **43**, 336–349.

KRAMER, R.M., HESSION, C., JOHANSEN, B., HAYES, G., MCGRAY, P., CHOW, E.P. & PEPINSKY, R.B. (1989). Structure and properties of a human non-pancreatic phospholipase A_2. *J. Biol. Chem.* **264**, 5768–5775.

KUDO, I., MURAKAMI, M., HARA, S. & INOUE, K. (1993). Mammalian non-pancreatic phospholipases A_2. *Biochim. Biophys. Acta.*, **117**, 217–231.

MARSHALL, L.A., WINKLER, J.D., GRISWOLD, D.E., BOLOGNESE, B., ROSHAK, A., SUNG, C.-M., WEBB, E.F. & JACOBS, R.S. (1994). Effects of scalaradial, a type II phospholipase A_2 inhibitor, on human neutrophil arachidonic acid mobilization and lipid mediator formation. *J. Pharmacol. Exp. Ther.*, **268**, 709–717.

MATSUMOTO, T., TAO, W. & SHA'AFI, R.I. (1988). Demonstration of calcium-dependent phospholipase A_2 activity in membrane preparation of rabbit neutrophils. Absence of activation by fMet-Leu-Phe, phorbol 12-myristate 13-acetate and A-kinase. *Biochem. J.*, **250**, 343–348.

MAYER, A.M.S., GLASER, K.B. & JACOBS, R.S. (1988). Regulation of eicosanoid biosynthesis *in vitro* and *in vivo* by the marine natural product manoalide: a protein inactivator of venom phospholipases. *J. Pharmacol. Exp. Ther.*, **244**, 871–878.

MEACHER, L.C., MOONGA, B.S., HASLETT, C., HUANG, C.L.-H. & ZAIDI, M. (1991). Single pulses of cytoplasmic calcium associated with phagocytosis of individual zymosan particles by macrophages. *Biochem. Biophys. Res. Commun.*, **177**, 460–465.

MIYAKE, A., YAMAMOTO, H., KUBOTA, E., HAMAGUCHI, K., KOUDA, A., HONDA, K. & KAWASHIMA, H. (1993). Suppression of inflammatory responses to 12-*O*-tetradecanoyl-phorbol-13-acetate and carrageenin by YM-26734, a selective inhibitor of extracellular group II phospholipase A_2. *Br. J. Pharmacol.*, **110**, 447–453.

MORONEY, M.A., ALCARAZ, M.J., FORDER, R.A., CAREY, F. & HOULT, J.R.S. (1988). Selectivity of neutrophil 5-lipoxygenase and cyclo-oxygenase inhibition by an anti-inflammatory flavonoid glycoside and related aglycone flavonoids. *J. Pharm. Pharmacol.*, **40**, 787–792.

MUKHERJEE, A.B., MIELE, L. & PATTABIRAMAN, N. (1994). Phospholipase A_2 enzymes: regulation and physiological role. *Biochem. Pharmacol.*, **48**, 1–10.

NEVES, P.C.A., NEVES, M.C.A., BELLA CRUZ, A., SANT'ANA, A.E.G., YUNES, R.A & CALIXTO, J.B. (1993). Differential effects of *Mandevilla velutina* compounds on paw oedema induced by phospholipase A_2 and phospholipase C. *Eur. J. Pharmacol.*, **243**, 213–219.

PFEILSCHIFTER, J., SCHALKWIJK, C., BRINER, V.A. & VAN DEN BOSCH, H. (1993). Cytokine-stimulated secretion of group II phospholipase A_2 by rat mesangial cells. Its contribution to arachidonic acid release and prostaglandin synthesis by cultured rat glomerular cells. *J. Clin. Invest.*, **92**, 2516–2523.

POTTS, B.C.M., FAULKNER, D.J. & JACOBS, R.S. (1992). Phospholipase A_2 inhibitors from marine organisms. *J. Nat. Prod.*, **55**, 1701–1717.

RAMESHA, C.S. & IVES, D.L. (1993). Detection of arachidonoyl-selective phospholipase A_2 in human neutrophil cytosol. *Biochim. Biophys. Acta.*, **1168**, 37–44.

REYNOLDS, L.J., HUGHES, L.L., LOUIS, A.I., KRAMER, R.M. & DENNIS, E.A. (1993). Metal ion and salt effects on the phospholipase A_2 lysophospholipase, and transacylase activities of human cytosolic phospholipase A_2. *Biochim. Biophys. Acta.*, **1167**, 272–280.

SCHORLEMMER, H.U., EDWARDS, J.H., DAVIES, P. & ALLISON, A.C. (1977). Macrophage responses to mouldy hay dust, *Micropholyspora faeni* and zymosan, activators of complement by the alternative pathway. *Clin. Exp. Immunol.* **27**, 198–203.

SUZUKI, K., OTA, H., SASAGAWA, S., SAKARANI, T. & FUJIKURA, T. (1983). Assay method for myeloperoxidase in human polymorphonuclear leukocytes. *Anal. Biochem.*, **132**, 345–352.

TAKAYAMA, K., KUDO, I., KUDO, D.K., NAGATA, K., NOZAWA, Y. & INOUE, K. (1991). Purification and characterization of human platelet phospholipase A_2 which preferentially hydrolyzes an arachidonoyl residue. *FEBS Lett.*, **282**, 326–330.

TRAMPOSCH, K.M., CHILTON, F.H., STANLEY, P.L., FRANSON, R.C., HAVENS, M.B., NETTLETON, D.O., DAVERN, L.B., DARLING, I.M. & BONNEY, R.J. (1994). Inhibitor of phospholipase A_2 blocks eicosanoid and platelet activating factor biosynthesis and has topical anti-inflammatory activity. *J. Pharmacol. Exp. Ther.*, **271**, 852–859.

TRAYNOR, J.R. & AUTHI, K.S. (1981). Phospholipase A_2 activity of lysosomal origin secreted by polymorphonuclear leucocytes during phagocytosis or on treatment with calcium. *Biochim. Biophys. Acta.*, **665**, 571–577.

ULEVITCH, R.J., WATANABE, Y., SANO, M., LISTER, M.D., DEEMS, R.A. & DENNIS, E.A. (1988). Solubilization, purification and characterization of a membrane-bound phospholipase A_2 from the $P388D_1$ macrophage-like cell line. *J. Biol. Chem.*, **263**, 3079–3085.

VADAS, P. & PRUZANSKI, W. (1986). Biology of disease. Role of secretory phospholipases A_2 in the pathobiology of disease. *Lab. Invest.*, **55**, 391–404.

VISHWANATH, B.S., FAWZY, A.A. & FRANSON, R.C. (1988). Edema-inducing activity of phospholipase A_2 purified from human synovial fluid and inhibition by aristolochic acid. *Inflammation*, **12**, 549–561.

WEDMORE, C.V. & WILLIAMS, T.J. (1981). Control of vascular permeability by polymorphonuclear leukocytes in inflammation. *Nature*, **289**, 646–650.

WEISS, J., INADA, M., ELSBACH, P. & CROWL, R.M. (1994). Structural determinants of the action against *Escherichia coli* of a human inflammatory fluid phospholipase A_2 in concert with polymorphonuclear leukocytes. *J. Biol. Chem.*, **269**, 26331–26337.

WIGHTMAN, P.D., DAHLGREN, M.E., DAVIES, P. & BONNEY, R.J. (1981). The selective release of phospholipase A_2 by resident mouse peritoneal macrophages. *Biochem. J.*, **200**, 441–444.

WRIGHT, G.W., OOI, C.E., WEISS, J. & ELSBACH, P. (1990). Purification of a cellular (granulocyte) and an extracellular (serum) phospholipase A_2 that participate in the destruction of *Escherichia coli* in a rabbit inflammatory exudate. *J. Biol. Chem.*, **265**, 6675–6681.

(Received November 30, 1995
Revised December 21, 1995
Accepted January 3, 1996)