View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by CiteSeerX

0022-3565/97/2821-0123$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 282, No. 1
Copyright © 1997 by The American Society for Pharmacology and Experimental Therapeutics
Printed in U.S.A.
JPET 282:123–131, 1997

Variabilin: A Dual Inhibitor of Human Secretory and Cytosolic Phospholipase A_2 With Anti-inflammatory Activity[1]

V. ESCRIG, A. UBEDA, M. L. FERRANDIZ, J. DARIAS, J. M. SANCHEZ, M. J. ALCARAZ and M. PAYA

Department of Pharmacology, University of Valencia and Institute of Natural Products and Agrobiology, Tenerife, Spain

Accepted for publication March 17, 1997

ABSTRACT

The marine product variabilin was identified as a novel inhibitor of phospholipase A_2 (PLA_2), which exhibited IC_{50} values of 6.9 μM and 7.9 μM for human synovial secretory PLA_2 and U937 cells cytosolic PLA_2 activities, respectively. This compound was less potent on bee venom or zymosan-injected rat air pouch enzymes and failed to affect *Naja naja* venom PLA_2. The production of leukotriene B_4 by human neutrophils stimulated with calcium ionophore A23187 was also inhibited by variabilin, which was without effect on 5-lipoxygenase, cyclo-oxygenase 1 and cyclo-oxygenase 2 activities in cell-free assays. Other functions of human neutrophils, such as degranulation and superoxide generation, were also significantly reduced *in vitro*. Variabilin administered topically suppressed the mouse ear edema induced by 12-*O*-tetradecanoylphorbol 13-acetate, whereas the ear edema induced by arachidonic acid was unaffected; this suggests an action previous to arachidonic acid metabolism. This compound administered p.o. at 30 mg/kg and 45 mg/kg significantly inhibited mouse paw edema induced by carrageenan and, at 0.01 to 1.0 μmol/pouch in the mouse air pouch injected with zymosan, exerted a marked inhibition on PGE_2 and leukotriene B_4 levels in exudates (ID_{50} values of approximately 0.028–0.029 μmol/pouch), without affecting cell migration. Our results indicate that variabilin is an inhibitor of human secretory and cytosolic PLA_2 activities that controls eicosanoid production *in vitro* and *in vivo*, inhibits neutrophil degranulation and superoxide generation *in vitro* and shows anti-inflammatory activity after topical or p.o. administration to mice.

Hydrolysis of arachidonic acid at the *sn*-2 position of the glycerol in membrane phospholipids is the rate-limiting step for eicosanoid production. Mammalian cells contain diverse types of PLA_2 that can play a key role in the release of arachidonic acid, leading to the generation of inflammatory mediators and the activation of signal transduction pathways (for review, see Glaser *et al.*, 1993; Kudo *et al.*, 1993; Dennis, 1994). Most cells contain at least two forms of PLA_2: a 14-kDa secretory enzyme, $sPLA_2$, and an 85-kDa cytosolic enzyme, $cPLA_2$, (Glaser *et al.*, 1993; Kramer *et al.*, 1989). The secretory enzymes can be classified mainly into group I and group II (Heinrikson *et al.*, 1977), although $sPLA_2$ from bee venom is sometimes included in a separate group III (Glaser *et al.*, 1993).

$sPLA_2$ has been found to induce release of arachidonic acid and eicosanoid production in several cell types (Pfeilschifter *et al.*, 1993; Fonteh *et al.*, 1994; Miyake *et al.*, 1994). On the other hand, $cPLA_2$ exhibits a more selective preference for arachidonyl-containing phospholipids and plays an important role in arachidonic acid release (Clark *et al.*, 1990; Ramesha and Ives, 1993). In fact, $cPLA_2$ is the main mediator in the hormonally regulated production of eicosanoids (Lin *et al.*, 1992).

$sPLA_2$s can induce an inflammatory response in animals (Vadas and Pruzanski, 1986; Vishwanath *et al.*, 1988; Neves *et al.*, 1993), whereas in humans, group II $sPLA_2$ is present at high levels in synovial fluids, articular cartilage and blood from patients with rheumatic diseases (Pruzanski *et al.*, 1987; Bomalaski and Clark, 1993), which suggests the participation of these types of enzymes in the inflammatory process.

Thus the inhibition of PLA_2 could result in the inhibition of inflammatory responses acting at an early step in the biosynthesis of inflammatory mediators such as prostaglandins, leukotrienes and platelet-activating factor. Marine organisms are an important source of PLA_2 inhibitors; some of them can be of interest either as pharmacological tools to establish the role of the different PLA_2 activities in disease or as anti-inflammatory agents (for review, see Potts *et al.*, 1992). We have shown recently that a number of terpenoids

Received for publication November 12, 1996.
[1] This work was supported by grant SAF95-1046 from CICYT, Spanish Ministerio de Educación y Ciencia.

ABBREVIATIONS: PLA_2, phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PTK, palmityl trifluoromethyl ketone; BSA, bovine serum albumin; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase; FMLP, formyl-L-methionil-L-leucyl-L-phenylalanine; PAF, platelet-activating factor; IC_{50}, inhibitory concentration 50%; ID_{50}, inhibitory dose 50%.

present in sponges inhibit PLA_2 with some differences in potency and selectivity (Gil *et al.*, 1995; Cholbi *et al.*, 1996). The purpose of our studies was to examine the influence of variabilin (fig. 1), a sesterterpene isolated for the first time from the marine sponge *Ircinia variabilis* (Faulkner, 1973), on $sPLA_2$ activity of types I, II and III, as well as on $cPLA_2$ activity from U937 cells. We have also studied its influence on human neutrophil responses *in vitro* and its effect on inflammatory responses in mice and eicosanoid synthesis *in vivo*. Recently, a different compound has been named variabilin, a protein that is isolated from the hard tick *Dermacentor variabilis* and inhibits platelet aggregation (Wang *et al.*, 1996).

Materials and Methods

Reagents. The variabilin utilized in this work was isolated from *Hemimycale columela* following known procedures (Faulkner, 1973). Antibody against LTB_4, the 5-lipoxygenase inhibitor ZM230,487 and human synovial recombinant PLA_2 were kindly provided by Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK. [9,10-^{3}H]oleic acid and L-3-phosphatidylcholine 1-palmitoyl-2-arachidonyl [arachidonyl-1-^{14}C] were purchased from Du Pont (Itisa, Madrid, Spain); ([5,6,8,11,12,14,15(n)-^{3}H]PGE_2, [5,6,8,9,11,12,14,15(n)-^{3}H]LTB_4 and [5,6,8,9,11,12,14,15(n)-^{3}H]LTC_4 were from Amersham Iberica, (Madrid, Spain). PTK was purchased from Universal Biologicals Ltd. (London, UK). The rest of the reagents were from Sigma Chemical Co., St. Louis, MO. *E. coli* strain CECT 101 was a gift from Prof. Uruburu, Department of Microbiology, University of Valencia, Spain.

Assay of $sPLA_2$. $sPLA_2$ was assayed by using a modification of the method of Franson *et al.* (1974). *E. coli* strain CECT 101 were seeded in medium containing 1% tryptone, 0.5% NaCl and 0.6% sodium dihydrogen orthophosphate, pH 5.0, and grown for 6 to 8 hr at 37°C in the presence of 5 μCi/ml [^{3}H]oleic acid (sp. act. 10 Ci/mmol). After centrifugation at 2,500 $\times$ *g* for 10 min, the cells were washed in buffer (0.7 M Tris-HCl, 10 mM $CaCl_2$, 0.1% BSA, pH 8.0), resuspended in saline and autoclaved for 30 to 45 min. At least 95% of the radioactivity was incorporated into phospholipids. *Naja naja* venom enzyme, bee venom enzyme and human recombinant synovial enzyme were diluted in 10 μl of 100 mM Tris-HCl, 1 mM $CaCl_2$ buffer, pH 7.5. Supernatants (10 μl) of exudates from zymosan-injected rat air pouch (Payá *et al.*, 1996) were also used as a source of $sPLA_2$. Enzymes were preincubated at 37°C for 5 min with 2.5 μl of test compound solution or its vehicle in a final volume of 250 μl. Incubation proceeded for 15 min in the presence of 10 μl of autoclaved oleate-labeled membranes and was terminated by addition of 100 of μl ice-cold solution of 0.25% BSA in saline to a final concentration of 0.07% w/v. After centrifugation at 2,500 $\times$ *g* for 10 min at 4°C, the radioactivity in the supernatants was determined by liquid scintillation counting.

Preparation of human leukocytes. The citrated blood of healthy volunteers was centrifuged at 200 $\times$ *g* for 15 min at room temperature. The platelet-rich plasma was removed, and the leukocytes contained in the residual blood were isolated by sedimentation with 2% (w/v) dextran in 0.9% NaCl at room temperature. The supernatant was centrifuged at 1,200 $\times$ *g* for 10 min at 4°C. Contaminating erythrocytes were lysed by hypotonic treatment. The pellet was resuspended in PBS, and Ficoll-hypaque was layered under the cell mixture. The cell gradient mixture was centrifuged at 400 $\times$ *g* for 40 min at 20°C. Neutrophils were separated and resuspended in PBS containing 1.26 mM Ca^{++} and 0.9 mM Mg^{++} (Bustos *et al.*, 1995). Viability was greater than 95% by the trypan blue exclusion test. The monocyte and lymphocyte layer was removed and pelleted by centrifugation. The cell pellet was resuspended in RPMI-1640 media, pH 7.4, with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin and was incubated at a cell density of 10^7/ml in 60/15-mm tissue culture dishes. The cells were allowed to adhere for 2 hr at 37°C in a 5% CO_2 atmosphere incubator. The nonadherent cells were removed by vacuum suction of media followed by two washes with 1 ml of RPMI-1640. The adherent cells resulted in a greater than 90% pure monocyte population as assessed by differential staining.

Cell viability assays. LDH was determined by measuring the rate of oxidation of NADH (Bergmeyer and Bernt, 1974). Tubes containing 0.5% Triton X-100 were used for measurement of the total cellular content of LDH.

Assay of $cPLA_2$. $cPLA_2$ was prepared from human monocytic U937 cells (Cell Collection, Department of Animal Cell Culture, C.S.I.C:, Madrid, Spain) grown in the above medium which were disrupted by sonication in 10 mM HEPES buffer, pH 7.4, containing 0.32 M sucrose, 100 μM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulphonylfluoride and 100 μM leupeptin. The homogenated cells were centrifuged at 2,000 $\times$ *g* for 10 min at 4°C, and the resulting supernatant was further centrifuged at 100,000 $\times$ *g* for 100 min at 4°C to obtain the cytosolic fraction. $cPLA_2$ activity was measured as the release of radiolabeled arachidonic acid according to the method of Clark *et al.* (1990). 1-Palmitoyl-2-[^{14}C] arachidonyl-*sn*-glycero-3-phosphocholine (57.0 mCi/mmol, 2 $\times$ 10^6 cpm) was dried under nitrogen and suspended in 1 ml of 100 mM glycine buffer, pH 9.0, containing 200 μM Triton X-100, 10 mM $CaCl_2$, 0.25 mg/ml BSA and 40% v/v glycerol. The suspension was then sonicated to form mixed micelles of phospholipid and Triton X-100. The reaction was started by adding the enzyme solution (approximately 24 μg of protein of cytosolic fraction from human monocytes) to a final volume of 100 μl of the assay mixture, which contained 1 mM $CaCl_2$, 2 mM 2-mercaptoethanol, 150 mM NaCl, 40% glycerol, 1 mg/ml BSA and 50 mM HEPES, pH 9.0. The substrate consisted of 5 μl of micelles (10^4 cpm) containing dioleoyl glycerol at the molar ratio 2:1 (Kramer *et al.*, 1987). Test compounds were dissolved in methanol and added to the reaction mixture just before the addition of the enzyme solution. The final concentration of methanol in the reaction mixture was less than 1%, which showed no effect on the enzyme activity. The reaction was stopped after a 60-min incubation period at 37°C by mixing with 0.5 ml of isopropyl alcohol/heptane/0.5 M H_2SO_4 (10:5:1). Heptane (0.7 ml) and water (0.2 ml) were then added, and the solution was vigorously mixed for 15 sec. The heptane phase was mixed with 100 mg silica gel 60 (Merck, 70–230 mesh) and centrifuged, and the radioactivity in each supernatant was measured (Zhang *et al.*, 1991).

Elastase release by human neutrophils. 2.5 $\times$ 10^6 neutrophils/ml were preincubated with test compound or vehicle for 5 min and then stimulated with cytochalasin B (10 μM) and FMLP (10 nM or 10 μM), or PAF (0.5 μM) for 10 min at 37°C. In other experiments, calcium ionophore A23187 (1 μM) was used as stimulus. After centrifugation at 1,200 $\times$ *g* at 4°C, supernatants were incubated with N-tert-butoxy-carbonyl-L-alanine p-nitrophenyl ester (200 μM) for 20 min at 37°C (Barrett, 1981). The extent of p-nitrophenol release was measured at 414 nm in a microtiter plate reader. Possible direct inhibitory effects on elastase activity were assessed by preincubating variabilin for 5 min with supernatants of cytochalasin B + FMLP-stimulated human neutrophils, followed by addition of substrate and a 20-min incubation at 37°C. Direct effects on myeloperoxidase were also tested using aliquots of supernatants of cytochalasin B + FMLP-stimulated human neutrophils following published procedures (Suzuki *et al.*, 1983; De Young *et al.*, 1989).

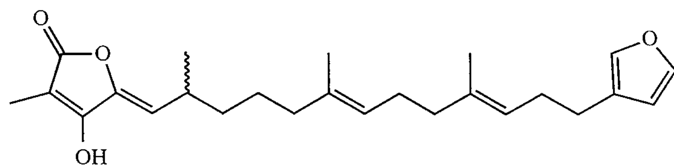

Fig. 1. Structural formula of variabilin.

Superoxide generation by human neutrophils. A neutrophil suspension (0.5 ml) containing 2.5×10^6 cells/ml was preincubated for 5 min at 37°C with test compounds or vehicle (methanol, 1% final concentration) and TPA (1 μM) was added to induce superoxide generation, which was estimated as the superoxide dismutase-inhibitable reduction of cytochrome c at 550 nm (Payá *et al.*, 1993).

Synthesis and release of LTB_4 by human neutrophils. A suspension of human neutrophils (5×10^6/ml) in PBS was preincubated with test compounds or vehicle and then stimulated with 1 μM A23187 for 10 min at 37°C. After centrifugation at 1,200 × *g* for 10 min at 4°C, the supernatants were frozen at −80°C until the radioimmunoassay for LTB_4 was performed (Moroney *et al.*, 1988).

Synthesis of LTB_4 by high-speed supernatants from human neutrophils. High-speed (100,000 × *g*) supernatants from sonicated human neutrophils were obtained as previously described (Tateson *et al.*, 1988). Aliquots (50 μg of protein/tube) in PBS containing 2 mM $CaCl_2$ were incubated with 5 μM arachidonic acid at 37°C for 5 min, in the presence of test compounds or vehicle. The samples were then heated at 90°C for 5 min and centrifuged at 10,000 × *g* at 4°C for 30 min. The LTB_4 levels in supernatants were measured by radioimmunoassay (Moroney *et al.*, 1988).

Cyclo-oxygenase-1. J774 cells (Cell Collection, Department of Animal Cell Culture, C.S.I.C:, Madrid, Spain) were sonicated at 4°C in an ultrasonicator at maximum potency. Microsomes were prepared by centrifugation at 2,000 × *g* for 5 min at 4°C followed by centrifugation of the supernatant at 100,000 × *g* for 100 min at 4°C. Microsomes (20 μg of protein/tube) were incubated for 30 min at 37°C in 50 mM Tris-HCl, pH 7.4, with 5 μM arachidonic acid and test compound or vehicle in the presence of 2 μM hematin and 1 mM L-tryptophan. The reaction was terminated by boiling the samples for 5 min, and PGE_2 levels were determined by radioimmunoassay (Moroney *et al.*, 1988).

Cyclo-oxygenase-2. Human monocytes or J774 cells were resuspended in RPMI1640 culture medium containing aspirin (300 μM) and incubated at 37°C for 2 hr. The cells were washed twice, resuspended in RPMI1640 with 10% fetal bovine serum and incubated with *E. coli* lipopolysaccharide (10 μg/ml) at 37°C for 24 hr (Grossman *et al.*, 1995). After centrifugation the cells were sonicated at 4°C in an ultrasonicator at maximum potency, and microsomes were prepared as above. Microsomes (40 μg of protein/tube) were used as a source of cyclo-oxygenase-2, and reactions were carried out in the same conditions as above. PGE_2 synthesis was determined by radioimmunoassay (Moroney *et al.*, 1988).

Release of PGE_2 by rat cecum. Male Wistar rats weighing between 140 and 180 g were fasted overnight but allowed water *ad libitum*. The animals were sacrificed, and the intestine was removed. Cecum was cleaned with saline and cut with scissors. Fragments of rat cecum weighing 37.8 ± 1.4 mg (mean ± S.E.M., n = 47) were placed in 0.4 ml of 50 mM Tris-HCl buffer, pH 7.5. Drugs were dissolved in the medium at appropriate concentrations. After preincubation for 60 min at 4°C, the fragments were transferred to tubes containing fresh medium with the same concentrations of drugs and incubated for 15 min at 37°C. Aliquots of the solution were used for PGE_2 radioimmunoassay as above.

Mouse ear edema. The protocols were approved by the institutional Animal Care and Use Committee. All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals. TPA (5 μg) or arachidonic acid (2.0 mg) dissolved in 20 μl of acetone was applied in 10-μl volumes to both inner and outer surfaces of the right ear of Swiss mice (20–25 g). Test compounds were applied topically in acetone before TPA administration or 20 min before arachidonic acid. The left ear (control) received only acetone. The animals were killed by cervical dislocation after 4 hr (TPA) or 1 hr (arachidonic acid), and equal sections of both ears were punched out and weighed. The increase in the weight of the right ear punch over that of the left indicated the edema (Carlson *et al.*, 1985). The ear sections were homogenized in 750 μl of saline, and after centrifugation at 10,000 × *g* for 15 min at 4°C, the PGE_2 and LTC_4 content in supernatants was determined by radioimmunoassay (arachidonic acid edema). Direct inhibitory effects on myeloperoxidase activity were assessed by preincubating variabilin for 5 min with supernatants of homogenized control TPA-treated ears (Suzuki *et al.*, 1983; De Young *et al.*, 1989).

Mouse paw edema. Swelling was induced by a modification of the technique of Sugishita *et al.* (1981). Female Swiss mice (20–25 g) were fasted for 12 hr with free access to water. Drug or vehicle (ethanol/Tween 80/distilled water, 5:5:90, v/v/v) was administered p.o. (0.5 ml) 1 hr before the injection of carrageenan (0.05 ml; 3% w/v in saline) into the subplantar area of the right hind paws of groups of six animals. The volumes of injected and contralateral paws were measured at 1, 3 and 5 hr after induction of edema by using a plethysmometer (Ugo Basile, Comerio, Italy). The volume of edema was expressed for each animal as the difference between the carrageenan-injected and contralateral paws.

Mouse air pouch. Male Swiss mice (25–30 g) were anesthetized with ethyl ether, and 10 ml of sterile air was injected into the s.c. tissue of the back, and 3 days later, 5 ml of sterile air was injected into the same cavity. Another 3 days later, mice were administered, into the air pouch, 1 ml of 1% w/v zymosan in saline + vehicle (10 μl of ethanol: control group) or 1 ml of 1% w/v zymosan in saline + test drug (dissolved in 10 μl of ethanol) at the concentrations indicated in the results (treated groups). Another group received only 1 ml of saline + vehicle (saline group). Four hours after administration, the animals were killed by cervical dislocation, and the exudate in the pouch was collected with 1 ml of saline (Edwards *et al.*, 1981). Leukocytes present in exudates were measured using a Coulter counter. After centrifugation of exudates at 1,200 × *g* at 4°C for 10 min, the supernatants were used to measure LTB_4 and PGE_2 levels by radioimmunoassay (Moroney *et al.*, 1988).

Statistical analysis. The results are presented as mean ± S.E.M. IC_{50} values and their 95% CL were calculated from at least four significant concentrations (n = 6). The approximate ID_{50} value was estimated from three significant doses (n = 6). The level of statistical significance was determined by analysis of variance followed by Dunnett's t test for multiple comparisons.

Results

Inhibition of PLA_2 activities. Variabilin significantly inactivated $sPLA_2$ and $cPLA_2$ in a concentration-dependent manner (fig. 2). As shown in table 1, this marine product was

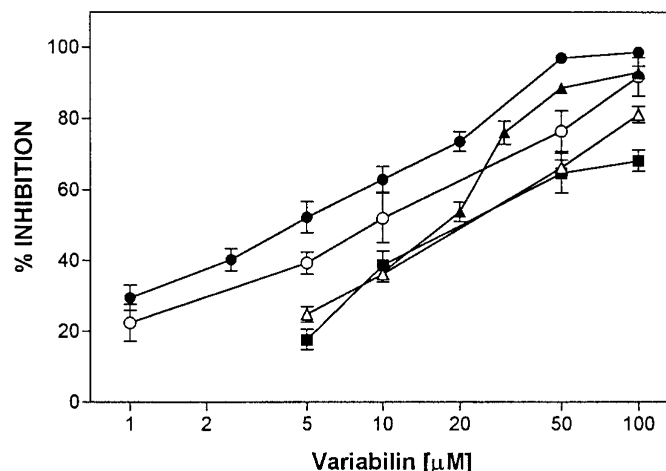

Fig. 2. Dose-response curves showing the effect of variabilin on several PLA_2 activities. Data are mean ± S.E.M. (n = 6). (■) Bee venom $sPLA_2$. (▲) RAP + zymosan $sPLA_2$. (●) Human recombinant synovial $sPLA_2$. (△) Porcine pancreatic $sPLA_2$. (○) U937 $cPLA_2$. The inhibitory effect of variabilin was measured in relation to control enzyme activity in tubes containing enzyme and the inhibitor vehicle (methanol).

TABLE 1
Effect of variabilin on PLA_2 activities
Data represent IC_{50} values (μM) with 95% CL

	IC_{50} (μM)	
	Variabilin	Scalaradial
Naja naja venom $sPLA_2$	Inactive at 50 μM	3.9 (2.2–7.6)
Human synovial $sPLA_2$	6.9 (4.0–9.4)	0.5 (0.3–0.7)
Bee venom $sPLA_2$	26.4 (10.2–89.0)	3.6 (1.5–10.0)
Rat air pouch $sPLA_2$	15.1 (5.6–22.4)	3.2 (2.1–4.8)
Porcine pancreatic $sPLA_2$	20.0 (16.6–23.4)	2.4 (0.9–6.5)
U937 $cPLA_2$	7.9 (5.2–11.4)	>50 μM

somewhat less potent on human synovial PLA_2 than the reference $sPLA_2$ inhibitor, scalaradial. Inhibition of $cPLA_2$ gave an IC_{50} value and 95% CL of 84.2 (47.0–170.0) nM for the selective inhibitor PTK. Variabilin inhibited *in vitro* human synovial $sPLA_2$ and U937 $cPLA_2$ with IC_{50} values in the μM range and showed less inhibition of nonhuman $sPLA_2$ activities from bee venom, porcine pancreas or zymosan-injected rat air pouch. This last activity, which does not show selectivity for arachidonyl phospholipids, has recently been reported by us (Payá *et al.*, 1996). In contrast, variabilin was inactive on *Naja naja* venom $sPLA_2$. To determine whether the effects on PLA_2 were reversible, we used the dilution method (Lister *et al.*, 1989) and bee venom and human synovial enzymes. No significant difference in the degree of inactivation was observed; variabilin (100 μM) inhibited bee venom $sPLA_2$ by 81.6 ± 1.0% (mean ± S.E.M, n = 6) and after a 25-fold dilution, the observed inhibition was 73.0 ± 1.1% (mean ± S.E.M, n = 6). On human synovial $sPLA_2$, variabilin (10 μM) exhibited 92.6 ± 0.6% inhibition (mean ± S.E.M, n = 6), and after a 25-fold dilution, the value was 89.5 ± 0.2% inhibition (mean ± S.E.M, n = 6). An analysis of drug influence on enzyme activity *vs.* enzyme concentration was also performed. Figures 3 and 4 illustrate the concentration-response relationship for bee venom and human synovial $sPLA_2$, respectively, as a function of enzyme concentration. The results were similar with both enzymes. The regression line for variabilin-treated samples was shifted to the right of control values at a given velocity, and there was no significant difference between the slopes of these lines,

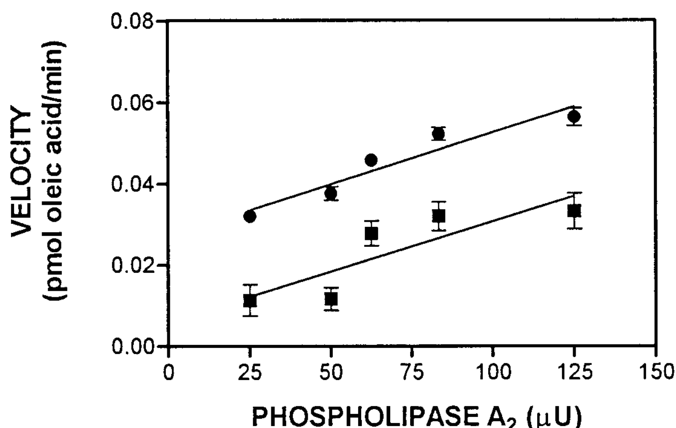

Fig. 3. Activity of bee venom $sPLA_2$ as a function of enzyme concentration in the absence and presence of variabilin. (●) Control. (■) 10 μM variabilin. Data are mean ± S.E.M. (n = 6). Different enzyme concentrations were preincubated with vehicle (control) or variabilin (10 μM) for 5 min at 37°C, and after addition of substrate, incubation proceeded for 15 min.

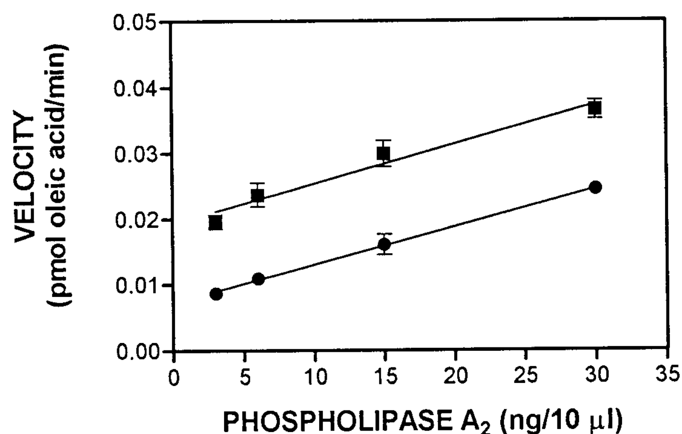

Fig. 4. Activity of human synovial $sPLA_2$ as a function of enzyme concentration in the absence and presence of variabilin. (●) Control. (■) 1 μM variabilin. Data are mean ± S.E.M. (n = 6). Different enzyme concentrations were preincubated with vehicle (control) or variabilin (1 μM) for 5 min at 37°C, and after addition of substrate, incubation proceeded for 15 min.

which suggests an irreversible inhibition (Segel, 1975). The loss of enzyme activity was progressive with time and linear on a semilogarithmic plot (fig. 5). The rate of inactivation increased as the concentration of variabilin increased from 5 to 50 μM, with appreciable inhibitory activity at $t = 0$ (without preincubation of variabilin with the enzyme), a result that suggests an initial quick binding to the enzyme. Time-dependent bee venom PLA_2 inactivation has been reported for irreversible inhibitors such as manoalide and scalaradial (Glaser and Jacobs, 1986; de Carvalho and Jacobs, 1991).

Effect on degranulation, superoxide and LTB_4 generation by human neutrophils. No cytotoxic effects of variabilin were observed at the concentrations used in our study, because only at the high concentration of 100 μM did we observe a release of 24.4 ± 4.1% (mean ± S.E.M, n = 9, P < .01) LDH. Variabilin exerted direct inhibitory effects on human neutrophil enzymes, showing an IC_{50} value of 1.2 (0.6–2.0) μM for myeloperoxidase and a lower effect on elastase with an inhibition of 36.5 ± 3.8% (mean ± S.E.M., n = 6, P < .05) at the highest concentration tested in human neutrophils (50 μM). Thus we chose elastase instead of myeloperoxidase for degranulation assays. The degranulation of neutrophils activated with FMLP or PAF in cytochalasin-pretreated neutrophils was blocked by variabilin in a concentration-dependent manner, as shown in figure 6. Variabilin was more potent against neutrophils treated with a submaximal concentration of FMLP, with IC_{50} values of 0.4 (0.1–0.9) μM and 9.6 (4.0–26.0) μM for 10 nM FMLP and 10 μM FMLP, respectively. Inhibition of degranulation induced by PAF or A23187 was also observed for variabilin, which exhibited IC_{50} values of 1.5 (1.2–1.9) μM and 7.4 (5.6–9.5) μM, respectively. Scalaradial was also an inhibitor of degranulation with IC_{50} values of 2.2 (0.8–5.3) μM and 1.3 (0.8–17.9) μM for the response induced by PAF and by 10 μM FMLP, respectively. Generation of superoxide anion by human neutrophils was inhibited by variabilin with a lower potency (IC_{50} = 33.1; 24.4–43.6 μM), whereas scalaradial showed an IC_{50} value of 3.1 (2.8–3.9) μM, a value similar to that observed for inhibition of degranulation (fig. 7). Variabilin caused a concentration-dependent suppression of neutrophil LTB_4 production induced by the calcium ionophore A23187

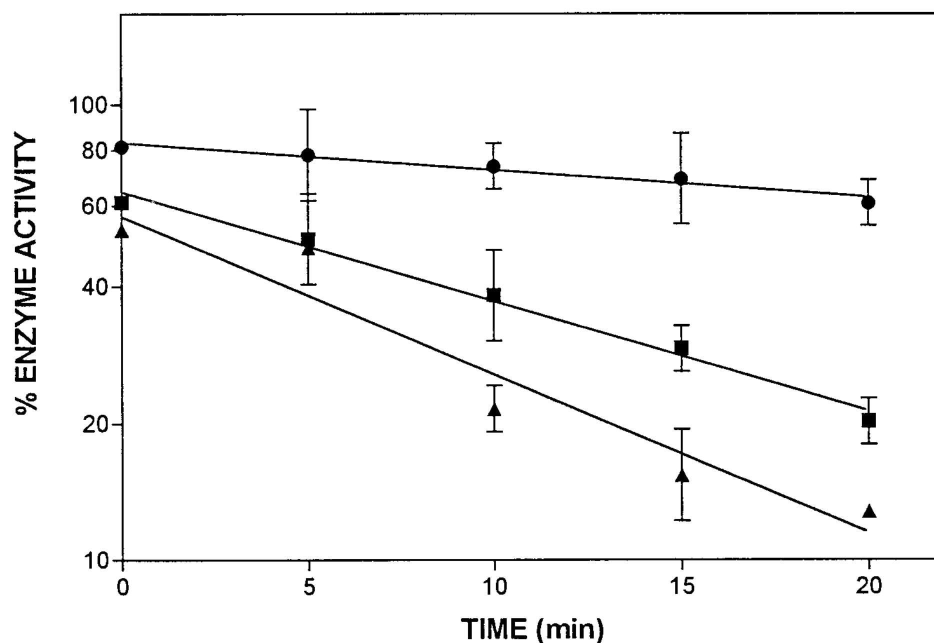

Fig. 5. Time dependence of PLA_2 inactivation for three concentrations of variabilin. Data represent mean ± S.E.M. (n = 3). (●) 5 μM variabilin. (■) 10 μM variabilin. (▲) 50 μM variabilin. Variabilin was preincubated with μU bee venom PLA_2 for different times, followed by a 15-min incubation with substrate.

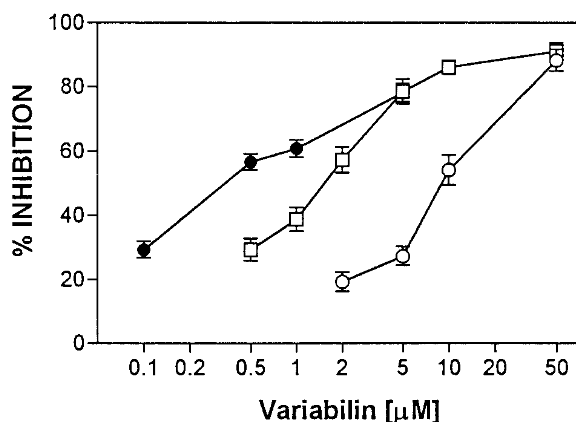

Fig. 6. Concentration dependence of the inhibitory effect of variabilin on elastase release by human neutrophils. Data represent mean ± S.E.M. (n = 6–10). (□) Cytochalasin B + 0.5 μM PAF. (●) Cytochalasin B + 10 nM FMLP. (○) Cytochalasin B + 10 μM FMLP.

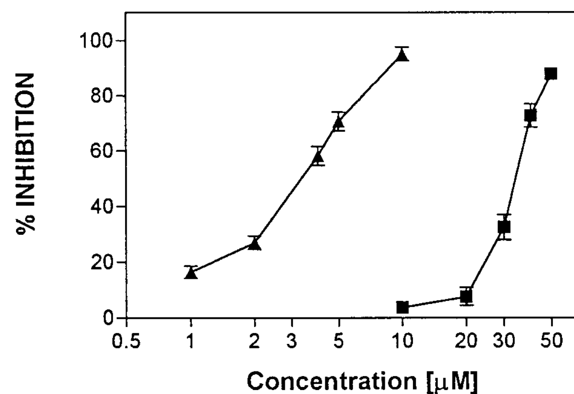

Fig. 7. Concentration dependence of the inhibitory effect of variabilin and scalaradial on superoxide generation by human neutrophils stimulated by TPA. Data are mean ± S.E.M. (n = 6). (■) Variabilin. (▲) Scalaradial.

(fig. 8), with an IC_{50} value of 1.4 (1.2–1.6) μM. Total inhibition of LTB_4 production was observed with 5 μM. Scalaradial potently inhibited this response with an IC_{50} value of 0.1 (0.1–0.3) μM. Because the rise in intracellular calcium concentration induced by A23187 activates PLA_2 and 5-lipoxygenase, leading to the release of arachidonic acid and the synthesis of LTB_4 in neutrophils, inhibition of one or both enzyme activities can result in a reduction in LTB_4 production. In order to distinguish between these possibilities, variabilin was incubated with a high-speed supernatant of neutrophils in the presence of substrate, arachidonic acid. In this case, variabilin at concentrations up to 10 μM failed to inhibit LTB_4 synthesis (table 2). Thus it appears that the effects of variabilin on LTB_4 production are not due to the inhibition of 5-lipoxygenase.

Effect of variabilin on cyclo-oxygenase 1, cyclo-oxygenase 2 and PGE_2 release by rat cecum. Variabilin at concentrations up to 10 μM did not affect the generation of PGE_2 by cyclo-oxygenase 1 or cyclo-oxygenase 2 present in microsomal fractions from human monocytes or J774 cells (data not shown). To determine the influence of variabilin on prostaglandin synthesis in the digestive tract, we performed experiments using rat cecum with indomethacin as reference. Tissue fragments released 470.0 ± 40.0 pg PGE_2/mg (mean ± S.E.M, n = 12) in control tubes. Incubation with 10 μM indomethacin significantly inhibited this release (75%, $n = 6$, $P < .01$), whereas variabilin at the same concentration showed no effect (4% inhibition, $n = 6$, $P > .05$).

Effect of variabilin on mouse ear edema. Both topical application of variabilin and that of indomethacin profoundly affected ear edema induced by TPA in mice, compared with control animals, inhibiting this inflammatory response at 200 μg/ear by 65% and 53%, respectively (fig. 9), with ID_{50} values of approximately 133.6 μg/ear (variabilin) and 183.5 μg/ear (indomethacin). A high level of myeloperoxidase was noted in TPA-treated ears 4 hr after induction of inflammation. Variabilin incubated *in vitro* with supernatants of ear homogenates from the control group inhibited myeloperoxidase activity, showing an IC_{50} value of 32.9 (26.0–44.3) μM. As expected in a PLA_2 inhibitor, variabilin at 250 or 500 μg/ear failed to modify arachidonic acid-induced ear edema

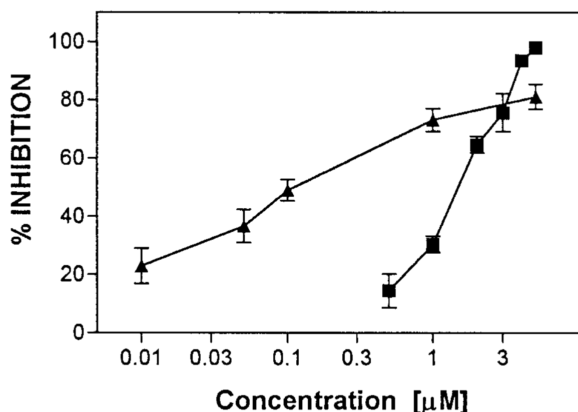

Fig. 8. Concentration dependence of the inhibitory effect of variabilin and scalaradial on LTB_4 biosynthesis by human neutrophils stimulated by A23187. Data are mean ± S.E.M. (n = 6). (■) Variabilin. (▲) Scalaradial.

TABLE 2

Effect of variabilin on LTB_4 synthesis by high-speed supernatants from human neutrophils

Data are mean ± S.E.M. (n = 6–12). ** P < .01 or IC_{50} value (μM) with 95% CL. N.D. = not determined.

	LTB_4 (ng/5 × 10^6 cells)	IC_{50} (μM)
Control	30.4 ± 1.5	
Variabilin		N.D.
1 μM	35.5 ± 3.2	
10 μM	21.1 ± 1.5	
Scalaradial		1.3(0.7–2.6)
1 μM	17.3 ± 1.0**	
10 μM	6.0 ± 1.1**	

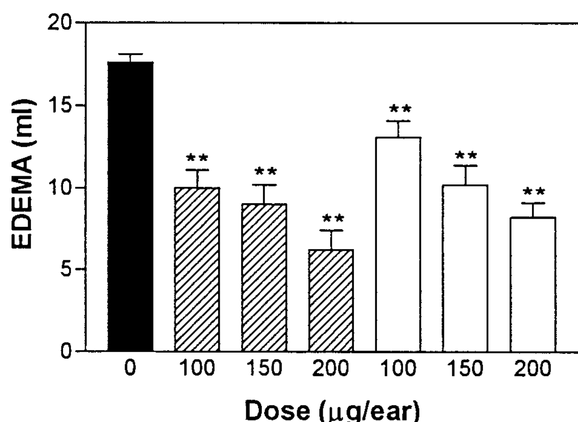

Fig. 9. Effect of variabilin and indomethacin on TPA-induced edema. Drugs were topically administered at the time of TPA application. Data are mean ± S.E.M. from six animals. ** P < .01. Control (solid bars); variabilin (hatched bars); indomethacin (open bars).

or eicosanoid levels in ear homogenates, whereas indomethacin caused 30% inhibition of edema at the dose of 500 μg/ear, accompanied by a marked reduction of PGE_2 levels in ear homogenates (table 3).

Effect of variabilin on mouse paw edema. Variabilin at p.o. doses of 30 or 45 mg/kg demonstrated the ability to inhibit swelling early (1 hr after carrageenan) and exhibited continuously significant suppression of hind paw swelling between 1 and 5 hr after carrageenan administration, achieving a maximal response at the determination at 3 hr (fig. 10). Indomethacin exerted a higher effect and decreased edema

TABLE 3

Effect of variabilin on arachidonic acid-induced mouse ear edema

Mice were treated topically with arachidonic acid according to the procedure described in "Materials and Methods." Drugs were applied topically 20 min before arachidonic acid administration. PGE_2 and LTC_4 were determined by radioimmunoassay in ear homogenates. Data are mean ± S.E.M. from 6 to 10 animals. * P < .05; ** P < .01.

	Edema (mg)	PGE_2 (ng/ear)	LTC_4 (ng/ear)
Control	15.3 ± 1.1	44.0 ± 10.8	38.4 ± 3.1
Variabilin			
250 μg/ear	12.9 ± 1.4	35.4 ± 5.8	49.4 ± 5.1
500 μg/ear	14.2 ± 0.6	23.9 ± 4.9	48.7 ± 8.7
Indomethacin			
250 μg/ear	11.7 ± 0.6	4.3 ± 0.8**	38.3 ± 2.0
500 μg/ear	10.7 ± 1.5*	4.8 ± 1.0**	49.7 ± 3.1

values to 36.0 ± 12.0 μl (1 hr), 36.0 ± 5.0 μl (3 hr) and 56.0 ± 2.5 μl (5 hr) at the dose of 10 mg/kg p.o.

Effect of variabilin on mouse air pouch. Inhibition of leukocyte migration into the zymosan-injected mouse pouch seems to be related to the inhibition of 5-lipoxygenase metabolites, because the selective inhibitor ZM230,487 was the only compound able to inhibit this parameter (table 4). Variabilin was less effective than indomethacin or ZM230,487 in decreasing the levels of PGE_2 or LTB_4, respectively, in mouse air pouch exudates. However, it is interesting to note that this marine product decreased the levels of both eicosanoids with the same potency (ID_{50} values of about 0.028 and 0.029 μmol/pouch for PGE_2 and LTB_4, respectively). This activity profile suggests the inhibition of a previous step common to cyclo-oxygenase and 5-lipoxygenase pathways.

Discussion

The results of the studies presented here demonstrate the inhibitory activity of variabilin on PLA_2 from different sources. This marine compound did not display *in vitro* preference toward a type of PLA_2, although is more active on human enzymes, with IC_{50} values in the μM range for $sPLA_2$ and $cPLA_2$.

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). $sPLA_2$ could induce the release of arachidonic acid and PGE_2 production in neutrophils, HL-60 granulocytes treated with calcium ionophore (Hara *et al.*, 1991), rat mesangial cells (Pfeilschifter *et al.*, 1993), mast cells (Fonteh *et al.*, 1994) and mouse peritoneal macrophages (Miyake *et al.*, 1994). Nevertheless, PLA_2 secreted by guinea pig peritoneal macrophages does not participate in the synthesis of PGE_2 accumulating in the media (Marshall *et al.*, 1994a). $sPLA_2$ could also play a role in cellular defense against infection, because this enzyme activity is bactericidal against *E. coli* (Weiss *et al.*, 1994), *L. monocytogenes* (Weiss *et al.*, 1994; Harwig *et al.*, 1995) and *S. aureus* (Weinrauch *et al.*, 1996). It has also been suggested that the roles $sPLA_2$ plays in inflammation may include production of cell damage by hydrolysis of membrane phospholipids in activated cells (Wright *et al.*, 1990; Kudo *et al.*, 1993; Weiss *et al.*, 1994) and participation in a proliferative response and regulation of cytokine synthesis (Bomalaski and Clark, 1993).

On the other hand, $cPLA_2$ but not $sPLA_2$ is involved in arachidonic acid release in thrombin-stimulated human

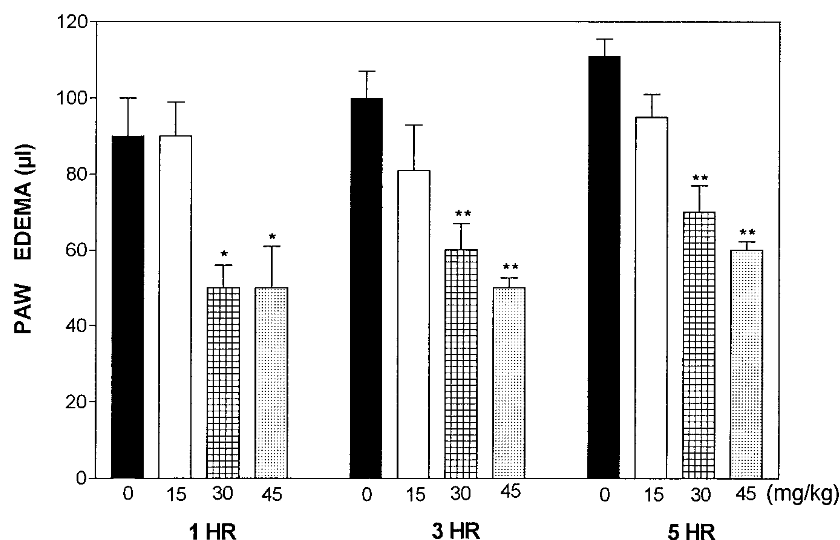

Fig. 10. Effect of variabilin on mouse paw edema induced by carrageenan. Data represent mean ± S.E.M. ($n = 6$). * $P < .05$, ** $P < .01$. Compounds were administered p.o. 1 hr before the injection of carrageenan.

TABLE 4
Effect of variabilin on zymosan-injected mouse air pouch
Data are mean ± S.E.M. from 6 to 12 animals. * $P < .05$; ** $P < .01$.

	Cell Accumulation ($\times 10^6$/ml)	PGE_2 (ng/ml)	LTB_4 (ng/ml)
Saline	1.1 ± 0.2	2.3 ± 0.7	1.4 ± 0.3
Zymosan	16.5 ± 1.3	63.6 ± 4.9	37.5 ± 4.1
Variabilin			
0.001 (µmol/pouch)	17.6 ± 2.6	46.3 ± 5.9	26.4 ± 5.3
0.01 (µmol/pouch)	15.3 ± 3.0	42.7 ± 5.9*	22.2 ± 3.7*
0.1 (µmol/pouch)	18.9 ± 3.1	31.5 ± 4.5**	16.7 ± 2.6**
1 (µmol/pouch)	11.7 ± 1.4	23.6 ± 1.9**	13.4 ± 1.3**
Indomethacin (0.1 µmol/pouch)	13.6 ± 1.0	2.0 ± 0.4**	44.7 ± 2.8
ZM230,487 (0.1 µmol/pouch)	11.0 ± 0.8*	59.2 ± 6.0	4.3 ± 1.2**

platelets (Bartoli *et al.*, 1994) and calcium ionophore-challenged platelets (Riendeau *et al.*, 1994) and is widely accepted as the main mediator of agonist-regulated production of eicosanoids (Lin *et al.*, 1992). There is evidence that arachidonic acid release in response to zymosan or TPA is dependent on cytosolic PLA_2 stimulation through protein kinase C and MAP kinase activation in mouse peritoneal macrophages (Qiu and Leslie, 1994).

Recent investigations suggest that both type II PLA_2 and $cPLA_2$ are responsible for eicosanoid synthesis. Thus arachidonic acid mobilization seems to be dependent on both types of PLA_2 in P388D1 macrophages (Balsinde *et al.*, 1994) and human umbilical vein endothelial cells (Murakami *et al.*, 1993). In addition, exocytosis of $sPLA_2$ could modulate the activity of the $cPLA_2$ by initiating the formation of LTB_4, which after release would stimulate its own receptor, resulting in activation of the $cPLA_2$ in neutrophils (Wijkander *et al.*, 1995). Assuming that both enzymes are involved in the production of inflammatory mediators, it is reasonable to expect that a compound with dual inhibitory activity could control inflammatory responses efficiently.

Increases in type II PLA_2 have been shown in various inflammatory processes, including rheumatoid arthritis, although whether this is the cause or the consequence of the disease has not been established (Glaser *et al.*, 1993). In fact, administration of $sPLA_2$ of different types can induce or amplify inflammatory responses in animals (Vadas and Pruzanski, 1986; Vishwanath *et al.*, 1988; Neves *et al.*, 1993; Cirino *et al.*, 1994). In contrast, there are no data on potent $cPLA_2$ inhibitors and their pharmacological effects *in vivo*, so variabilin appears to be a novel dual $sPLA_2$ and $cPLA_2$ inhibitor that shows anti-inflammatory activity after either topical or p.o. administration to laboratory animals. We have not determined the mechanism of PLA_2 inactivation by variabilin. Nevertheless, this marine compound shares some structural features with manoalide, and thus it may interact with $sPLA_2$ at the γ-hydroxybutenolide ring and hydrophobic region of the compound (Glaser *et al.*, 1989).

We have also demonstrated that variabilin inhibits cellular functions in human neutrophils *in vitro*. During inflammation, neutrophils stimulated by various agents release reactive oxygen species and granular enzymes that mediate tissue injury (Smith, 1994). Neutrophil proteases and specifically elastase mediate damage to endothelium (Westlin and Gimbrone, 1993). In addition, myeloperoxidase is necessary to form the strong oxidant HOCl, which by reaction with superoxide can in turn generate the reactive hydroxyl radical (Ramos *et al.*, 1992). Therefore, inhibition of cell-mediated responses could be considered an additional mechanism for attenuating inflammation. In this regard, variabilin potently inhibited *in vitro* some functions that contribute to tissue damage by the cellular component of

inflammatory processes, such as degranulation, and was less effective in suppressing the TPA-stimulated oxidative burst.

Variabilin has inhibited neutrophil responses triggered by structurally divergent agonists that induce neutrophil responsiveness through different pathways, which suggests that this marine product, apart from a possible influence on ligand-receptor interactions, may inhibit intracellular signal transduction pathways. In this respect, inhibition of $sPLA_2$ activity has been related to neutrophil exocytosis, because released arachidonic acid or lysophospholipids could act as fusogens (Barnette *et al.*, 1994), as well as to superoxide generation by human eosinophils (White *et al.*, 1993). The inhibition of myeloperoxidase activity could also participate in the control of neutrophil-mediated tissue injury by this marine compound.

LTB_4 biosynthesis by human neutrophils *in vitro* and PGE_2 and LTB_4 generation *in vivo* in the mouse air pouch were inhibited by variabilin. Because the measured endpoint of our assays was the generation of eicosanoids, compounds able to inhibit cyclo-oxygenase or 5-lipoxygenase enzymes could appear as PLA_2 inhibitors. In our experiments, variabilin had no effect on arachidonic acid metabolism directly in cell-free assays for 5-lipoxygenase, cyclo-oxygenase 1 or cyclo-oxygenase 2 activities. In addition, the data presented here demonstrate that variabilin does not inhibit *in vitro* prostaglandin generation in the digestive tract. In contrast, known inhibitors of $sPLA_2$ such as scalaradial (Marshall *et al.*, 1994b) are *in vitro* inhibitors of 5-lipoxygenase, which explains the high potency of this compound on LTB_4 generation by human neutrophils.

Furthermore, variabilin failed to inhibit the *in vivo* generation of eicosanoids in the presence of an excess of substrate (arachidonic acid-induced edema) but decreased with the same potency LTB_4 and PGE_2 levels generated from endogenous substrate in the mouse air pouch. This result indicates that variabilin affected arachidonate availability rather than metabolism. It is interesting to note that group II $sPLA_2$ has been related to inflammatory responses to TPA or carrageenan (Miyake *et al.*, 1993; Tramposch *et al.*, 1994), which have been inhibited by variabilin. Thus our results support an inhibitory action of variabilin on PLA_2 in human intact cells as well as in experimental models in mice.

Our data indicate that variabilin is a dual inhibitor of human $sPLA_2$ and $cPLA_2$ that is able to control the production of arachidonic acid metabolites *in vitro* and *in vivo*. This marine compound exerts anti-inflammatory effects after topical or p.o. administration to laboratory animals, probably because the reduction in arachidonic acid availability leads to inhibition of the biosynthesis of inflammatory mediators, with the partial contribution of inhibitory actions on neutrophil degranulation or lysosomal enzymes.

Acknowledgments

The authors thank Dr. S. J. Foster, Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK, for the gift of several reagents and Dr. B. Perez, C.S.I.C., Madrid, Spain, for technical support in cell culture.

References

Balsinde, J., Barbour, S. E., Bianco, I. D. and Dennis, E. A.: 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, 1994.

Barnette, M. S., Rush, J., Marshall, L. A., Foley, J. J., Schmidt, D. B. and Sarau, H. M.: Effects of scalaradial, a novel inhibitor of 14-kDa phospholipase A_2, on human neutrophil function. Biochem. Pharmacol. **47:** 1661–1668, 1994.

Barrett, A. J.: Leukocyte elastase. Meth. Enzymol. **80:** 581–588, 1981.

Bartoli, F., Lin, H.-K., Ghomashchi, F., Gelb, M. H., Jain, M. K. and Apitz-Castro, R.: Tight binding inhibitors of 85-kDa phospholipase A_2 but not 14-kDa phospholipase A_2 inhibit release of free arachidonate in thrombin-stimulated human platelets. J. Biol. Chem. **269:** 15625–15630, 1994.

Bergmeyer, H. U. and Bernt, E.: Lactate dehydrogenase: UV assay with pyruvate and NADH. *In* Methods of Enzymatic Analysis, ed. by H. U. Bergmeyer, pp. 574–577, Academic Press, New York, 1974.

Bomalaski, J. S. and Clark, M. A.: Phospholipase A_2 and arthritis. Arthritis Rheum. **36:** 190–198, 1993.

Bustos, G., Ferrandiz, M. L., Sanz, M. J., Paya, M. and Alcaraz, M. J.: A study of the novel anti-inflammatory agent florifenine. Topical anti-inflammatory activity and influence on arachidonic acid metabolism and neutrophil functions. Naunyn-Schmiedeberg's Arch. Pharmacol. **351:** 298–304, 1995.

Carlson, R. P., O'Neill-Davis, L., Chang, J. and Lewis, A. J.: Modulation of mouse ear edema by cyclooxygenase and lipoxygenase inhibitors and other pharmacologic agents. Agents Actions **17:** 197–204, 1985.

Cholbi, R., Ferrandiz, M. L., Terencio, M. C., De Rosa, S., Alcaraz, M. J. and Paya, M.: Inhibition of phospholipase A_2 and some inflammatory responses by the marine product ircinin. Naunyn-Schmiedeberg's Arch. Pharmacol. **354:** 1–7, 1996.

Cirino, G., Cicala, C., Sorrentino, L., Maiello, F. M. and Browning, J. L.: Recombinant secreted nonpancreatic phospholipase A_2 induces a synovitis-like inflammation in the rat air pouch. J. Rheumatol. **21:** 824–829, 1994.

Clark, J. D., Milona, N. and Knopf, J. L.: 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, 1990.

De Carvalho, M. S. and Jacobs, R. S.: Two-step inactivation of bee venom phospholipase A_2 by scalaradial. Biochem. Pharmacol. **42:** 1621–1626, 1991.

De Young, L. M., Kheifets, J. B., Ballaron, S. J. and Young, J. M.: 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, 1989.

Dennis, E. A.: Diversity of group types, regulation, and function of phospholipase A_2. J. Biol. Chem. **269:** 13057–13060, 1994.

Edwards, J. C. W., Sedgwick, A. D. and Willoughby, D. A.: 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, 1981.

Faulkner, D. J.: Variabilin, an antibiotic from the sponge *Ircinia variabilis*. Tetrahedron Lett. 3821–3822, 1973.

Fonteh, A. N., Bass, D. A., Marshall, L. A., Seeds, M., Samet, J. M. and Chilton, F. H.: Evidence that secretory phospholipase A_2 plays a role in arachidonic acid release and eicosanoid biosynthesis by mast cells. J. Immunol. **152:** 5438–5446, 1994.

Franson, R., Patriarca, P. and Elsbach, P.: Phospholipid metabolism by phagocytic cells. Phospholipases A_2 associated with rabbit polymorphonuclear leukocyte granules. J. Lipid Res. **15:** 380–388, 1974.

Gil, B., Sanz, M. J., Terencio, M. C., Giulio, A., De Rosa, S., Alcaraz, M. J. and Paya, M.: Effects of marine 2-polyprenyl-1,4-hydroquinones on phospholipase A_2 activity and some inflammatory responses. Eur. J. Pharmacol. **285:** 281–288, 1995.

Glaser, K. B., De Carvalho, M. S., Jacobs, R. S., Kernan, M. R. and Faulkner, D. J.: Manoalide: Structure-activity studies and definition of the pharmacophore for phospholipase A_2 inactivation. Mol. Pharmacol. **36:** 782–788, 1989.

Glaser, K. B. and Jacobs, R. S.: Molecular pharmacology of manoalide. Inactivation of bee venom phospholipase A_2. Biochem. Pharmacol. **35:** 449–453, 1986.

Glaser, K. B., Mobilio, D., Chang, J. Y. and Senko, N.: Phospholipase A_2 enzymes: Regulation and inhibition. Trends Pharmacol. Sci. **14:** 92–98, 1993.

Grossman, C. J., Wiseman, J., Lucas, F. S., Trevethick, M. A. and Birch, P. J.: Inhibition of constitutive and inducible cyclooxygenase activity in human platelets and mononuclear cells by NSAIDS and Cox 2 inhibitors. Inflamm. Res. **44:** 253–257, 1995.

Hara, S., Kudo, I. and Inoue, K.: Augmentation of prostaglandin E_2 production by mammalian phospholipase A_2 added exogenously. J. Biochem. (Tokyo) **110:** 163–165, 1991.

Harwig, S. S. L., Tan, L., Qu, X.-D., Cho, Y., Eisenhauer, P. B. and Lehrer, R. I.: Bactericidal properties of murine intestinal phospholipase A_2. J. Clin. Invest. **95:** 603–610, 1995.

Heinrikson, R. L., Krueger, E. T. and Keim, P. S.: Amino acid sequence of phospholipase A_2-α from the venom of *Crotalus adamanteus*. A new classification of phospholipases A_2 based upon structural determinants. J. Biol. Chem. **252:** 4913–4921, 1977.

Kramer, R. M., Checani, G. C. and Deykin, D.: Stimulation of Ca^{2+}-activated human platelet phospholipase-A_2 by diacylglycerol. Biochem. J. **248:** 779–783, 1987.

Kramer, R. M., Hession, C., Johansen, B., Hayes, G., Mcgray, P., Chow, E. P. and Pepinsky, R. B.: Structure and properties of a human nonpancreatic phospholipase A_2. J. Biol. Chem. **264:** 5768–5775, 1989.

Kudo, I., Murakami, M., Hara, S. and Inoue, K.: Mammalian nonpancreatic phospholipases A_2. Biochim. Biophys. Acta **117:** 217–231, 1993.

LIN, L.-L., LIN, A. Y. AND KNOPF, J. L.: Cytosolic phospholipase A_2 is coupled to hormonally regulated release of arachidonic acid. Proc. Natl. Acad. Sci. U.S.A. **89:** 6147–6151, 1992.

LISTER, M. D., GLASER, K. B., ULEVITCH, R. J. AND DENNIS, E. A.: Inhibition studies on the membrane-associated phospholipase A_2 *in vitro* and prostaglandin E_2 production *in vivo* of the macrophage-like P388D_1 cell. Effects of manoalide, 7,7-dimethyl-5,8-eicosadienoic acid, and *p*-bromophenacyl bromide. J. Biol. Chem. **264:** 8520–8528, 1989.

MARSHALL, L. A., BOLOGNESE, B. AND ROSHAK, A.: Characterization of phospholipase A_2 release by elicited-peritoneal macrophage and its relationship to eicosanoid production. J. Lipid Mediat. Cell Signal. **10:** 295–313, 1994a.

MARSHALL, L. A., WINKLER, J. D., GRISWOLD, D. E., BOLOGNESE, B., ROSHAK, A., SUNG, C.-M., WEBB, E. F. AND JACOBS, R. S.: 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, 1994b.

MIYAKE, A., YAMAMOTO, H., ENOMORI, T. AND KAWASHIMA, H.: Exogenous group II phospholipase A_2 induces prostaglandin E_2 production in mouse peritoneal macrophages. Eur. J. Pharmacol. **253:** 155–161, 1994.

MIYAKE, A., YAMAMOTO, H., KUBOTA, E., HAMAGUCHI, K., KOUDA, A., HONDA, K. AND KAWASHIMA, H.: 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, 1993.

MORONEY, M. A., ALCARAZ, M. J., FORDER, R. A., CAREY, F. AND HOULT, J. R. S.: 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, 1988.

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

MURAKAMI, M., KUDO, I. AND INOUE, K.: Molecular nature of phospholipases A_2 involved in prostaglandin I_2 synthesis in human umbilical vein endothelial cells. Possible participation of cytosolic and extracellular type II phospholipases A_2. J. Biol. Chem. **268:** 839–844, 1993.

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

PAYA, M., FERRANDIZ, M. L., MIRALLES, F., MONTESINOS, C., UBEDA, A. AND ALCARAZ, M. J.: Effects of coumarin derivatives on superoxide anion generation. Arzneim. Forsch. **43:** 655–658, 1993.

PAYA, M., TERENCIO, M. C., FERRANDIZ, M. L. AND ALCARAZ, M. J.: Involvement of secretory phospholipase A_2 activity in the zymosan rat air pouch model of inflammation. Br. J. Pharmacol. **117:** 1773–1779, 1996.

PFEILSCHIFTER, J., SCHALKWIJK, C., BRINER, V. A. AND VAN DEN BOSCH, H.: 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, 1993.

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

PRUZANSKI, W., KEYSTONE, E. C., BOMBARDIER, C., SNOW, K. M. AND VADAS, P.: Phospholipase A_2 correlates with disease activity in rheumatoid arthritis. Arthritis Rheum. **30:**(suppl.)-S-114, 1987.

QIU, Z.-H. AND LESLIE, C. C.: Protein kinase C-dependent and -independent pathways of mitogen-activated protein kinase activation in macrophages by stimuli that activate phospholipase A_2. J. Biol. Chem. **269:** 19480–19487, 1994.

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

RAMOS, C. L., POU, S., BRITIGAN, B. E., COHEN, M. S. AND ROSEN, G. M.: Spin trapping evidence for myeloperoxidase-dependent hydroxyl radical formation by human neutrophils and monocytes. J. Biol. Chem. **267:** 8307–8312, 1992.

RIENDEAU, D., GUAY, J., WEECH, P. K., LALIBERTÉ, F., YERGEY, J., LI, C., DESMARAIS, S., PERRIER, H., LIU, S., NICOLL-GRIFFITH, D. AND STREET, I. P.: Arachidonyl trifluoromethyl ketone, a potent inhibitor of 85-kDa phospholipase A_2, blocks production of arachidonate and 12-hydroxyeicosatetraenoic acid by calcium ionophore-challenged platelets. J. Biol. Chem. **269:** 15619–15624, 1994.

SEGEL, I. H.: Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-state Enzyme Systems, pp. 127–128, John Wiley & Sons, New York, 1975.

SMITH, J. A.: Neutrophils, host defense, and inflammation: A double-edged sword. J. Leukoc. Biol. **56:** 672–686, 1994.

SUGISHITA, E., AMAGAYA, S. AND OGIHARA, Y.: Anti-inflammatory testing methods: Comparative evaluation of mice and rats. J. Pharmacobio-Dynam. **4:** 565–575, 1981.

SUZUKI, K., OTA, H., SASAGAWA, S., SAKATANI, T. AND FUJIKURA, T.: Assay method for myeloperoxidase in human polymorphonuclear leukocytes. Anal. Biochem. **132:** 345–352, 1983.

TATESON, J. E., RANDALL, R. W., REYNOLDS, C. H., JACKSON, W. P., BHATTACHERJEE, P., SALMON, J. A. AND GARLAND, L. G.: Selective inhibition of arachidonate 5-lipoxygenase by novel acetohydroxamic acids: Biochemical assessment *in vitro* and *ex vivo*. Br. J. Pharmacol. **94:** 528–539, 1988.

TRAMPOSCH, K. M., CHILTON, F. H., STANLEY, P. L., FRANSON, R. C., HAVENS, M. B., NETTLETON, D. O., DAVERN, L. B., DARLING, I. M. AND BONNEY, R. J.: 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, 1994.

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

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

WANG, X., COONS, L. B., TAYLOR, D. B., STEVENS, S. E. AND GARTNER, T. K.: Variabilin, a novel RGD-containing antagonist of glycoprotein IIb-IIIa and platelet aggregation inhibitor from the hard tick *Dermacentor variabilis*. J. Biol. Chem. **271:** 17785–17790, 1996.

WEINRAUCH, Y., ELSBACH, P., MADSEN, L. M., FOREMAN, A. AND WEISS, J.: The potent anti-*Staphylococcus aureus* activity of a sterile rabbit inflammatory fluid is due to a 14-kDa phospholipase A_2. J. Clin. Invest. **97:** 250–257, 1996.

WEISS, J., INADA, M., ELSBACH, P. AND CROWL, R. M.: 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, 1994.

WESTLIN, W. F. AND GIMBRONE, M. A.: Neutrophil-mediated damage to human vascular endothelium. Role of cytokine activation. Am. J. Pathol. **142:** 117–128, 1993.

WHITE, S. R., STREK, M. E., KULP, G. V. P., SPAETHE, S. M., BURCH, R. A., NEELEY, S. P. AND LEFF, A. R.: Regulation of human eosinophil degranulation and activation by endogenous phospholipase A_2. J. Clin. Invest. **91:** 2118–2125, 1993.

WIJKANDER, J., O'FLAHERTY, J. T., NIXON, A. B. AND WYKLE, R. L.: 5-Lipoxygenase products modulate the activity of the 85-kDa phospholipase A_2 in human neutrophils. J. Biol. Chem. **270:** 26543–26549, 1995.

WRIGHT, G. W., OOI, C. E., WEISS, J. AND ELSBACH, P.: 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, 1990.

ZHANG, Y. Y., DEEMS, R. A. AND DENNIS, E. A.: Lysophospholipases I and II from P388D_1 macrophage-like cell line. Meth. Enzymol. **197:** 456–468, 1991.

Send reprint requests to: Dr. M. Payá, Department of Pharmacology. University of Valencia, Faculty of Pharmacy. Avda. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain.