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DIFFERENTIAL EFFECTS OF MANOALIDE ON SECRETED AND INTRACELLULAR PHOSPHOLIPASES

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Abstract—Manoalide, a novel nonsteroidal sesterterpenoid, is a potent inhibitor of phospholipase A_2 isolated from bee and cobra venoms. This report compares the inhibition by manoalide of phospholipase A_2 in crude cytosol fractions from four mammalian tissues with that of four purified extracellular phospholipase A_2's. Phospholipase A_2 isolated from bee venom (*Apis mellifera*) was the most sensitive to inactivation by manoalide ($IC_{50} \simeq 0.12\ \mu M$). Extracellular phospholipase A_2 from rattlesnake and cobra venom was intermediate in sensitivity to manoalide (IC_{50} values of 0.7 and 1.9 μM respectively). Porcine pancreatic phospholipase A_2 was relatively resistant to inactivation by manoalide ($IC_{50} \simeq 30\ \mu M$). The phospholipase A_2 assayed in crude cytosol fractions from four mammalian tissues exhibited IC_{50} values of 30 μM or greater. Cytosolic proteins as well as bovine serum albumin and poly-L-lysine (Mr = 57,000) protected purified bee venom phospholipase A_2 from inactivation by manoalide. In contrast, amino acids such as lysine and alanine failed to protect the purified enzyme from inactivation. Proteins and certain amino acids, such as lysine, formed a chromogenic product when incubated with manoalide. These data suggest that lysine is capable of reacting with manoalide, but only when it is present in macromolecules is it capable of protecting phospholipase A_2 from inactivation by manoalide. Because cellular proteins protect PLA_2 from inactivation by manoalide, high concentrations of manoalide must be applied topically to produce statistically significant inactivation of intracellular phospholipase A_2. Finally, a chemical model is presented which explains the formation of a chromogenic product when manoalide is incubated with proteins and amino acids.

Phospholipase A_2 (EC 3.1.1.4) catalyzes the hydrolysis of fatty acids from the sn-2 position of phospholipids [1]. Phospholipase A_2 (PLA_2)† is a ubiquitous enzyme found in extracellular fluids, such as the venoms of bees and snakes and mammalian pancreatic secretions, and, in addition, it is an intracellular enzyme present in virtually every mammalian tissue examined [2, 3]. Venom and pancreatic PLA_2's have been purified and well characterized both mechanistically and with regard to their primary sequence [2, 3]. Both venom and pancreatic enzymes are relatively stable, require Ca^{2+} for optimal activity, and have a molecular weight of approximately 14,000 [2]. In contrast to the extracellular forms of phospholipase A_2, little is known concerning the intracellular enzymes.

Given the proper substrate, both products of phospholipase A_2 (e.g. free fatty acid and lysophospholipid) can be metabolized into bioactive substances such as platelet activating factor [4] and the arachidonic acid metabolites prostaglandins, thromboxanes, and leukotrienes [5, 6]. These metabolites have been implicated in various pathological conditions [5–9]. In addition, lysophosphatidylcholine is thought to exert a direct cytotoxic effect on cells [10] and may contribute to ischemic injury in the heart [11]. Specific PLA_2 inhibitors may be useful in treating those pathological conditions resulting from the overproduction of eicosanoids and platelet activating factor. This hypothesis is supported by the observation that glucocorticoids are potent anti-inflammatory agents, which inhibit phospholipase A_2 by inducing the synthesis of lipocortin, the phospholipase A_2 inhibitory protein [12–14].

Manoalide is a non-steroidal sesterterpenoid isolated from the sponge *Luffariella variabilis* [15]. The pharmacological properties of manoalide include analgesic activity and inhibition of phorbol-ester-induced inflammation in the mouse ear [16, 17]. Preliminary studies concerning the mechanism of action of manoalide demonstrated potent inhibition of bee (IC_{50} = 0.05 μM) and cobra (IC_{50} = 2.0 μM) venom PLA_2 [18–20]. The inhibition of PLA_2 by manoalide was both time and pH dependent, and irreversible [19, 20], possibly forming a covalent adduct with lysine residues [20]. Manoalide inhibited the hydrolysis of phosphatidylcholine by cobra venom PLA_2, but failed to inhibit the hydrolysis of phosphatidylethanolamine [20]. Because of this finding it was proposed that manoalide does not inhibit PLA_2 by interacting at the active site of the enzyme, but may interact at the phospholipid binding domain of PLA_2 [20].

Because manoalide antagonized phorbol-ester-induced inflammation in the mouse ear [16, 17], it was of interest to determine if manoalide inhibited intracellular PLA_2 as effectively as it inhibited secreted PLA_2 [19, 20]. Using the same assay conditions, the sensitivities of four secreted PLA_2's to

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† Abbreviations: PLA_2, phospholipase A_2; BSA, bovine serum albumin; and PBS, phosphate-buffered saline (10 mM sodium phosphate/150 mM NaCl, pH 7.4).

inactivation by manoalide were compared to the sensitivity of cytosolic PLA_2 obtained from four mammalian tissues. The ability of macromolecules to protect purified PLA_2 from inactivation by manoalide was also demonstrated. Finally, a model describing the interaction of manoalide with nucleophiles is presented.

MATERIALS AND METHODS

Purified PLA_2 from bee venom (*Apis mellifera*), (sp. act. 1350 units/mg protein) cobra venom (*Naja naja*) (sp. act. 800 units/mg protein) and porcine pancreas (sp. act. 870 units/mg protein), deoxycholate, arachidonic acid, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, polylysine, lysine, and alanine were obtained from the Sigma Chemical Co. (St. Louis, MO). Purified PLA_2 from rattlesnake venom (*Crotalus durissus*) (sp. act. 200 units/mg protein) was purchased from Boehringer Mannheim (Indianapolis, IN). Phosphatidylcholine (L-α-1-palmitoyl-2-[1-^{14}C]arachidonyl, sp. act. 54.5 mCi/mmole) was purchased from New England Nuclear (Boston, MA). Fatty acid-free bovine serum albumin was obtained from Calbiochem (LaJolla, CA). Thin-layer chromatography silica gel G plates were purchased from Analtech (Newark, DE). Guinea pigs were from Hazelton Research. Manoalide was provided by George Chan (SKF Laboratories) and Dr. L. Wheeler (Allergan Co., Irvine, CA).

Preparation of cell extracts. Guinea pig uterus and lung were homogenized in 0.25 M sucrose/5.0 mM $MgCl_2$/1.0 mM EDTA/10.0 mM Tris·HCl, pH 7.4 (buffer A) with a polytron homogenizer (Kinematica, Switzerland). The following protease inhibitors were added to buffer A just prior to homogenization: phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (0.1 mM), and aprotinin (10 μg/ml). The homogenate was centrifuged for 10 min at 10,000 *g*; the supernatant fraction was then centrifuged at 100,000 *g* for 60 min. Greater than 90% of the total PLA_2 enzyme activity was found in the 100,000 *g* supernatant. Cells grown in culture were washed twice with PBS (10 mM sodium phosphate/150 mM NaCl, pH 7.4), homogenized in buffer A containing protease inhibitors, using a Dounce homogenizer, and then centrifuged as described above. Guinea pig uterus cytosolic PLA_2 was calcium dependent and exhibited a pH optimum of 8.5* [21], while PLA_2 in BC_3H_1 cell homogenates exhibited a pH optimum of 9.0 [22]. Protein concentrations were determined as described by Bradford [23].

Phospholipase assays. Phospholipase A_2 activity was quantitated by the release of [^{14}C]arachidonic acid from 1-palmitoyl-2-[1-^{14}C]arachidonyl phosphatidylcholine in a mixed micelle reaction. The phospholipid substrate was dried under nitrogen, dissolved in a solution containing 5 mg/ml deoxycholate, and sonicated for 10 min in a sonicating water bath. The reaction mixture (50 μl final volume) contained 100 mM Tris·HCl (pH 8.0), 5.0 mM $CaCl_2$, 50 mM KCl, 2.4 mM deoxycholate, 5% glycerol, 10 μM labeled substrate (60,000 dpm), and 1 to 5×10^{-6} units of the appropriate enzyme. One enzyme unit causes hydrolysis of 1 μmole substrate per min at 37°. Reactions were started by the addition of substrate and incubated for 30 min at 37°, unless stated otherwise. The reaction was stopped and lipids were extracted by sequential addition of 50 μl chloroform–methanol (2:1), 50 μl chloroform, and 50 μl of 4.0 M KCl. Samples were vortexed, and the organic and aqueous phases were separated by centrifugation at 10,000 *g* for 1 min. The organic layer was spotted onto TLC plates with unlabeled standards and chromatographed using a petroleum ether–diethyl ether–acetic acid (70:30:1) solvent system. The bands corresponding to arachidonic acid and diacylglycerol were visualized with iodine vapors, scraped into scintillation vials, and extracted with 0.5 ml methanol. Radioactivity was detected in a Beckman model 9800 liquid scintillation counter using 10 ml of Beckman HP/b scintillation mixture.

Assays were linear with respect to time and protein concentration if less than 15% of substrate was consumed. Enzyme concentrations were adjusted such that 5–10% of the substrate was utilized in the reactions. In tissue and cell cytosol, the formation of diacylglycerol was less than 10% of arachidonic acid liberated (data not shown). Therefore, the possible contribution of free arachidonic acid from the combined actions of phospholipase C and diacylglycerol lipase was negligible. The enzyme assays were further substantiated by measuring the formation of the other reaction product of the PLA_2, lysophosphatidylcholine, as described by Clark *et al.* [22]. Using the reaction conditions described above, the amount of lysophosphatidylcholine formed was equal to the amount of arachidonic acid formed (data not shown), ruling out lysophospholipase as a source of liberated arachidonic acid.

Manoalide, when used, was dissolved in ethylene glycol and preincubated with the enzymes for 20 min at 37°. The final concentration of ethylene glycol in the reaction mixture was 8.0% which had no effect on enzyme activity (data not shown). Following the preincubation period, substrate was added and the mixture was incubated for an additional 30 min at 37°.

Topical application of manoalide. Manoalide was dissolved in acetone to a final concentration of either 10 or 15 mM. In control mice, 20 μl of acetone was applied to both surfaces of each ear. Mice treated with manoalide were likewise treated with 20 μl of manoalide solutions. At the indicated times, the mice were killed, and the ears were removed and homogenized in 5 vol. of buffer A with protease inhibitors as described previously. The homogenate was centrifuged at 1000 *g* for 40 min, and the enzymes activity in the 1000 *g* supernatant fraction was determined (cytosolic and membrane bound enzyme).

Scanning spectrophotometry. Manoalide was dissolved in ethylene glycol to a final concentration of 5.0 mM. Amino acids and proteins were dissolved in deionized water. Samples were scanned with a Beckman model DU8 Scanning Spectrophotometer at 600 nm per min for 50 min at 25°. The pH was maintained at 8.5 with 100 mM sodium borate.

* C. F. Bennett and S. T. Crooke, manuscript in preparation.

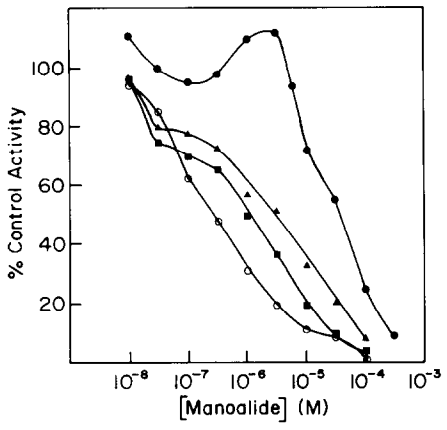

Fig. 1. Inhibition of purified extracellular PLA_2 by manoalide. Increasing concentrations of manoalide were incubated with the enzymes for 20 min at 37° prior to assay of PLA_2 activity as described in Materials and Methods. Data are expressed as the percentage of control activity remaining for 2×10^{-6} units bee venom PLA_2 (○—○), 2×10^{-6} units cobra venom PLA_2 (▲—▲), 2×10^{-6} units rattlesnake venom PLA_2 (■—■), and 5×10^{-6} units porcine pancreatic PLA_2 (●—●). Data points are the mean of at least three independent experiments, each performed in triplicate. The standard error of the mean was 5% or less of the mean for each data point (not shown).

RESULTS

Effects of manoalide on purified phospholipase A_2. The sensitivities of four purified PLA_2's to manoalide were compared using a radiometric assay (Fig. 1). The enzymes differed with respect to primary sequence, isoelectric point, and kinetic behavior [2, 3, 24]. The four purified extracellular enzymes also exhibited marked differences in their sensitivities to manoalide. PLA_2 from bee venom (*A. mellifera*) was the most sensitive to inhibition by manoalide, exhibiting an IC_{50} of 0.12 μM. This value was in good agreement with a previous report using different assay methods [19]. Phospholipase A_2 from rattlesnake and cobra venoms exhibited IC_{50} values of 0.7 and 1.9 μM respectively. Porcine pancreas phospholipase A_2 was found to be the most resistant to inhibition, with an IC_{50} of 30 μM. Manoalide at concentrations of 1 and 3 μM consistently produced a slight stimulation of pancreatic PLA_2. As previously reported [19, 20], inhibition of PLA_2 was dependent on the time of preincubation (data not shown).

Inhibition of intracellular phospholipases with manoalide. The effect of manoalide on mammalian intracellular PLA_2 was determined using cytosol fractions (100,000 *g* supernatant fractions) from guinea pig uterus and lung, rat basophilic leukemia cells (RBL-1) and a smooth muscle-like cell line (BC_3H_1). The specific activity of phospholipase A_2 in the cytosol fractions varied from 1.2 pmoles/mg/min to 470 pmoles/mg/min (Table 1). The effects of manoalide on PLA_2 were analyzed at equivalent enzyme concentrations. The intracellular enzymes were relatively resistant to inactivation by manoalide (Fig. 2), with IC_{50} values more than 200-fold greater than the value obtained for bee venom (Table 1). There was a consistent 25% stimulation of PLA_2 activity in BC_3H_1 cell cytosol by 30 μM manoalide ($P > 0.01$). It was not possible to obtain an accurate IC_{50} value for PLA_2 in the cytosol fraction of the two cell lines due to the limited solubility of manoalide in aqueous buffers and the relative insensitivity of the enzymes to manoalide under these conditions.

Protection against inhibition of phospholipase A_2 by manoalide. The lack of sensitivity of intracellular phospholipases to manoalide may be due to an inherent insensitivity of the enzymes, as was the case for purified porcine pancreas PLA_2. Alternatively, components in the cytosol fraction may inactivate manoalide. The second possibility was examined by the following experiment. Purified bee venom PLA_2 was co-incubated with guinea pig uterus cytosol and increasing concentrations of manoalide. The concentration–response curve for manoalide against the bee venom enzyme was shifted to the right in the presence of cytosol (Fig. 3). The manoalide concentration–response curve for bee venom PLA_2 in the presence of cytosol was equivalent to the concentration–response curve for inhibition of cytosolic PLA_2 (Fig. 3). This result suggests that a substance in the cytosol fraction protected the purified PLA_2 from inactivation by manoalide.

A previous report [20] suggested that manoalide inactivates *N. naja* PLA_2 by forming a covalent adduct with lysine residues. Because most proteins contain lysine, it was possible that manoalide would react with other proteins in the cytosol and prevent manoalide from reacting with cytosolic PLA_2. The ability of proteins to protect purified bee venom PLA_2 was tested by using BSA and poly-L-lysine (M_r = 57,000). Both BSA and poly-L-lysine shifted the concentration–response curve of manoalide to the right (Fig. 4). Poly-D-lysine also protected bee venom PLA_2 against inactivation by manoalide (data not shown), indicating that the reaction with lysine was not stereospecific. The protection of bee venom PLA_2 by both poly-L-lysine and BSA was concentration dependent (Fig. 5), and both agents were capable of 100% protection. Half-maximal protection against 500 nM manoalide was obtained with 3 nM poly-L-lysine and 100 nM BSA. Expressing the data as moles lysine per mole manoalide reveals that poly-L-lysine as four times more potent than BSA, suggesting that not all the lysine residues in BSA were equally reactive with manoalide.

The effects of L-lysine and L-alanine on inactivation of bee venom PLA_2 by manoalide were also determined. Co-incubation of bee venom PLA_2 with increasing concentrations of alanine in the presence of 0.5 μM manoalide did not result in protection of the enzyme (Fig. 6). High concentrations of lysine (>3 mM) resulted in partial protection of PLA_2. Lysine at a concentration of 10 mM, a 20,000 molar excess over manoalide, protected purified PLA_2 by only 25%. These data suggest that lysine residues in cytosolic macromolecules are probably the PLA_2 protective agents, not free amino acids.

During the course of these experiments, it was observed that incubation of manoalide with proteins or lysine resulted in the formation of a yellow product. As shown in Fig. 7, there was a change in the absorption spectrum of manoalide (100 μM) at

Table 1. Inhibition of intracellular PLA_2 by manoalide

Enzyme source	Specific activity (pmole/mg/min)	Manoalide IC_{50} (μM)
Guinea pig uterus	4.6	30
Guinea pig lung	4.7×10^2	100
Rat basophilic Leukemia cells (RBL-1)	1.2	>300
Mouse smooth muscle cells (BC_3H_1)	4.6	>300

Phospholipase A_2 activities were determined using phosphatidylcholine (1-palmitoyl-2-arachidonyl) as a substrate as described in Materials and Methods. The IC_{50} value for manoalide was determined by preincubating the enzyme with manoalide for 20 min at 37°, pH 8.0, as described in Materials and Methods.

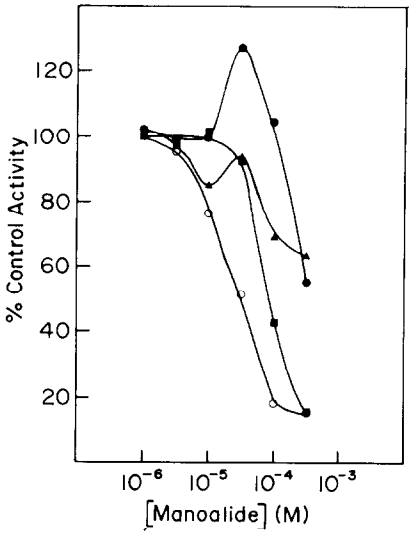

Fig. 2. Inhibition of cytosolic PLA_2 by manoalide. Manoalide was preincubated with cytosolic proteins for 20 min at 37° and then assayed for PLA_2 activity as described in Materials and Methods. Cytosol was obtained from guinea pig uterus, 45 μg protein, (○—○); guinea pig lung, 2 μg protein, (■—■); rat basophilic leukemia cells, 47 μg protein, (▲—▲); and a smooth muscle-like cell line, 27 μg protein (●—●). Data points represent the mean of at least three experiments, each performed in triplicate. The standard error of the mean was 5% or less of the mean for each data point.

462 nm when incubated with 40 μM BSA (Fig. 7A). As previously noted [25], the formation of the chromogenic product was also observed with lysine, λ_{max} = 436 nm (Fig. 7B), and to a limited extent with arginine (Fig. 7C). In each case the reaction was completed by 60 min.

Other amino acids also formed a chromophore when incubated with manoalide, though not as obviously prominent as lysine. Histidine reacted with manoalide at a rate and extent comparable to arginine (data not shown). Neutral amino acids such as alanine (Fig. 7D), glycine, phenylalanine, and glutamine formed a colored product with manoalide, but to a very limited extent. The acidic amino acids, aspartic and glutamic acid, did not form any detectable product with manoalide within 1 hr (data not shown). The sulfhydryl containing amino acid, cysteine, at concentrations greater than 3.0 mM failed to produce a chromophore with 100 μM manoalide; however, lower concentrations of cysteine readily reacted with manoalide (Fig. 7E). Thus, cysteine at concentrations greater than 30-fold molar excess to manoalide may undergo secondary reactions with manoalide preventing formation of the chromophore. Glutathione rapidly reacted with manoalide forming a chromophore with a strong absorption at 460 nm within 1 min after addition of manoalide (Fig. 7F, lower scan). The reaction between 25 mM glutathione and 0.1 mM manoalide was essentially complete within 10 min (Fig. 7F). 2-Mercaptoethanol also formed a chromophore with manoalide. Thus, free amino acids and sulfhydryl containing compounds are capable of reacting with manoalide, but do not appear to protect PLA_2 from inactivation.

Incubation of lysine and manoalide at a molar ratio of 250:1 for 20 min resulted in the formation of 40% of the maximal amount of chromogenic product. The lack of protection by lysine was apparently not due to insufficient preincubation time, as much greater molar ratios of lysine to manoalide failed to protect bee venom PLA_2 from inactivation by manoalide. Glutathione, which rapidly forms a chromogenic product with manoalide, did not protect purified PLA_2 from inactivation by manoalide at a 200-fold molar excess over manoalide (data not shown). Higher concentrations of glutathione (> 0.1 mM) inhibited the enzyme, possibly by reducing critical disulfide bonds.

Inhibition of intracellular PLA_2 by topical application of manoalide. Previous investigators have suggested that manoalide prevents phorbol myristate acetate, but not arachidonic acid, induced inflammation in the mouse ear by inhibiting intracellular PLA_2 [16, 18, 19]. However, the data presented above suggest that intracellular PLA_2 was relatively insensitive to inactivation by manoalide when compared to the venom enzymes. To determine if topical application of manoalide inhibited intracellular PLA_2, manoalide was applied to the mouse ear and the intracellular PLA_2 activities measured at various times after application. Treatment of the ears with 10 mM manoalide solution (170 μg/ear) did not produce a significant change in intracellular PLA_2 activity 60 min after application (data not shown). However, 15 mM manoalide solution (250 μg/ear) reduced the intracellular PLA_2 activity by 56%, 15 min after application (Table 2). The enzyme activity remained reduced for at least 60 min (Table

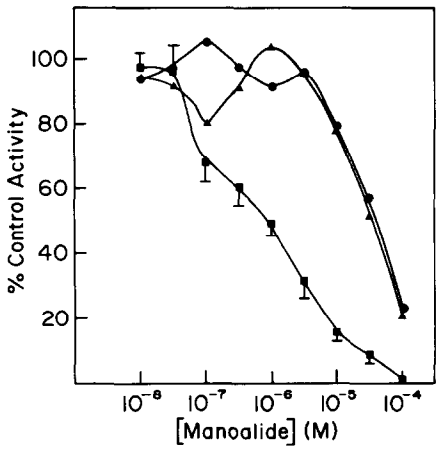

Fig. 3. Protection of bee venom PLA_2 against inactivation by manoalide. Purified bee venom PLA_2 (2×10^{-6} units) was incubated with increasing concentrations of manoalide in the absence (■—■) or presence (●—●) of 45 μg uterus cytosol protein for 20 min. The PLA_2 activity remaining was determined as described in Materials and Methods. The inactivation of PLA_2 in guinea pig uterus cytosol (45 μg protein) is shown (▲—▲) for the same set of experiments.

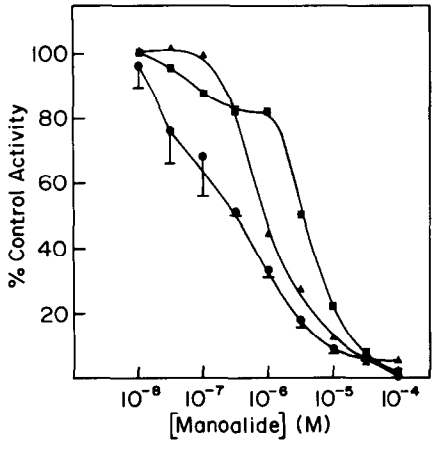

Fig. 4. Protection of bee venom PLA_2 with macromolecules. Purified bee venom PLA_2 (2×10^{-6} units) was preincubated alone (●—●), and in the presence of 5 nM poly-L-lysine (▲—▲) or 500 nM bovine serum ablumin (■—■) with increasing concentrations of manoalide. The PLA_2 activity was determined as described in Materials and Methods.

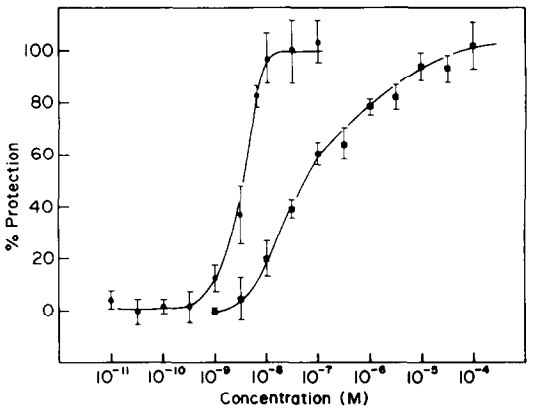

Fig. 5. Concentration-dependent protection of PLA_2 by macromolecules. Purified bee venom PLA_2 (2×10^{-6} units) was incubated with increasing concentrations of bovine serum albumin (■—■) or poly-L-lysine (●—●) and 0.5 μM manoalide for 20 min at 37°. The PLA_2 activity remaining was determined as described in Materials and Methods. Each data point represents the mean of two experiments, each performed in triplicate.

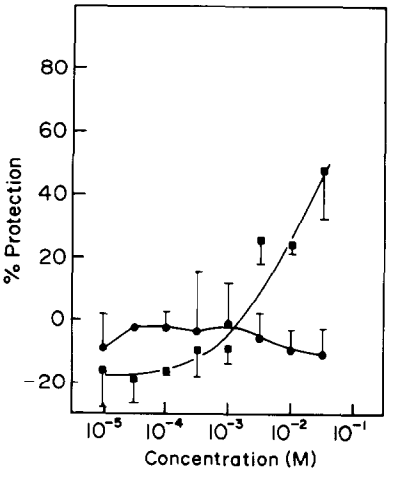

Fig. 6. Protection of PLA_2 with amino acids. Bee venom PLA_2 (2×10^{-6} units) was co-incubated with increasing concentrations of either L-lysine (■—■) or L-alanine (●—●) in the presence of 0.5 μM manoalide for 20 min at 37°. Preincubation of PLA_2 with 0.5 μM manoalide for 20 min inhibited the enzyme by 60%.

2). The cellular extract used for these studies contained both soluble and membrane bound PLA_2. Although the intracellular concentration of manoalide achieved after topical application is unknown, the IC_{50} value of manoalide inhibition of PLA_2 in the mouse ear homogenates was 60 μM (data not shown). The dose of manoalide required to inhibit intracellular PLA_2 significantly was 2.5-fold greatet than the ED_{50} required to prevent phorbol ester-induced inflammation [17].

DISCUSSION

Manoalide is a novel sesterterpenoid isolated from the sponge *L. variabilis* [15]. Previous studies

Table 2. Effect of topically administered manoalide on intracellular PLA_2

Time (min)	PLA_2 enzyme activity (pmoles/mg/min)	% Control
Control	8.8 ± 0.4	100
15	3.9 ± 0.4	44
30	4.4 ± 0.3	50
60	4.8 ± 0.2	55

The phospholipase A_2 activity in mouse ear homogenates was determined at the indicated times following application of a 15.0 mM solution of manoalide (20 μl) to both surfaces of the ears (two mice per group). Assays were performed in quadruplicate, two separate times as described in Materials and Methods.

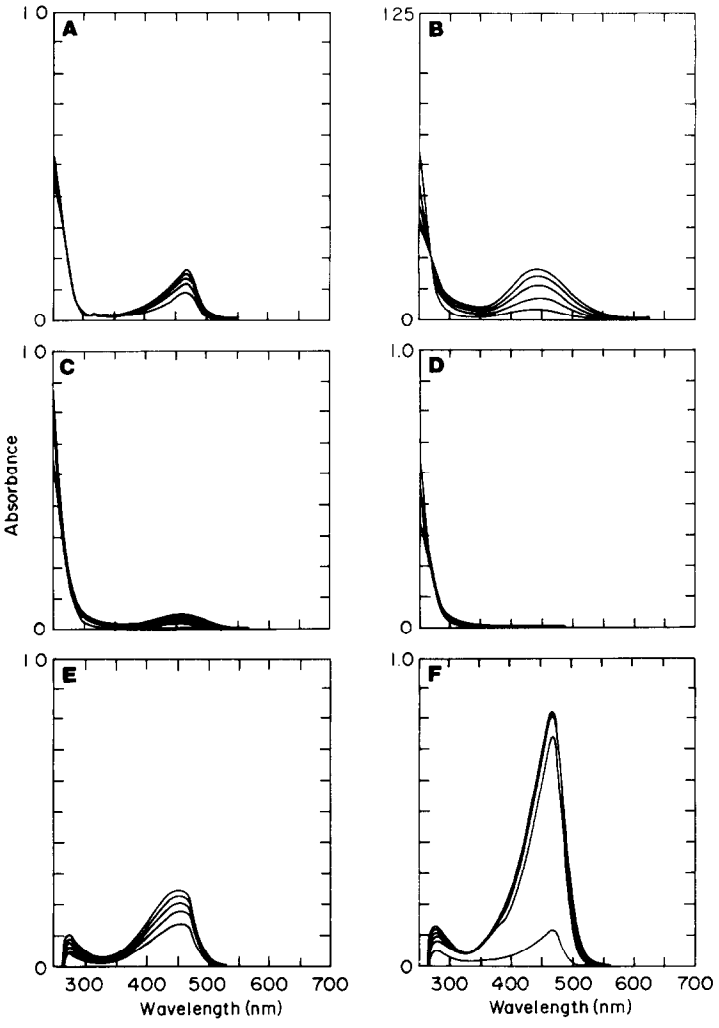

Fig. 7. Scanning spectrophotometry of manoalide conjugates. Manoalide (100 μM) was incubated with 40 μM bovine serum albumin (A), 25 mM L-lysine (B), 25 mM L-arginine (C), 25 mM L-alanine (D), 1.0 mM L-cysteine (E), or 25 mM glutathione (F) in 100 mM sodium borate buffer, pH 8.5. Scans were performed over 50 min at 10-min intervals with a scanning speed of 600 nm/min.

[19, 20] have demonstrated that it is a potent inhibitor of purified PLA_2 isolated from bee and cobra venoms. The inactivation of PLA_2 by manoalide is irreversible [19, 20], possibly due to the formation of a lysine adduct in the region required for binding of the enzyme to the phospholipid interface [20]. In the present study, inhibition of four purified PLA_2's by manoalide was demonstrated. The four enzymes exhibited a 250-fold difference in their sensitivities to manoalide.

The mammalian cytosolic PLA_2 were relatively resistant to inactivation by manoalide, with IC_{50} values greater than 30 μM. This lack of sensitivity of soluble intracellular PLA_2 was not due to an unusual characteristic of the cytosolic form of the enzyme; membrane-associated PLA_2 exhibited similar IC_{50} values (data not shown) and liver microsomal PLA_2 exhibits an IC_{50} of 94 μM [26]. From the data presented, manoalide appears to interact with proteins in the cytosol, thereby preventing manoalide from interacting with PLA_2. At the present time it is not possible to determine if purified intracellular phospholipases may prove to be more sensitive to inactivation by manoalide than indicated by the data in Table 1.

High doses of manoalide were required to produce significant inhibition of intracellular PLA_2 when applied topically to mouse ear. These doses were higher than those required to prevent phorbol-ester-induced inflammation in the mouse ear [17]. These data, combined with the finding that proteins were capable of protecting PLA_2 from inactivation, suggest that the observed anti-inflammatory activity of manoalide may be the result of interacting with other intracellular targets.

Attempts to correlate the chemical characteristics of the purified enzymes with their sensitivities to manoalide were unsuccessful. There are 11 lysine residues in the bee venom enzyme, 6 in *N. naja* PLA_2, and 9 in both porcine pancreas and *C. durissus* PLA_2. Structurally, the two snake venom enzymes are more similar to porcine pancreas than to *Apis*;

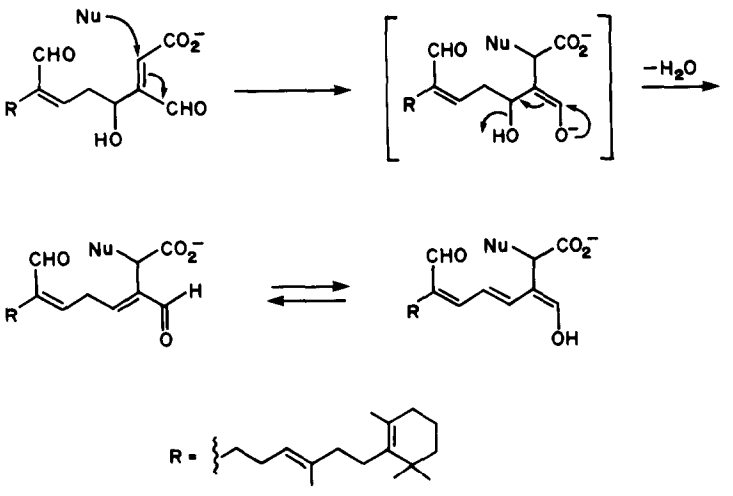

Fig. 8. Proposed model for manoalide reacting with nucleophiles to form a chromophore.

and their sensitivity to manoalide is intermediate between *A. mellifera* and porcine pancreas PLA_2. Two lysine-rich regions of bovine pancreatic PLA_2 are implicated in binding to the phospholipid interface [27, 28]. One region corresponds to residues 53, 56, 57 and 62; the other to the C-terminus. Both porcine pancreas PLA_2 and bee venom PLA_2 contain a cluster of lysine residues near the C-terminus [3]. However, this cluster is missing in the snake venom enzymes. Therefore, it is difficult from this type of comparative study to predict which lysine residues react with manoalide. There is also a lack of correlation between the specific activity of an enzyme and its sensitivity to manoalide. In summation, it appears that the marked differences in sensitivity to manoalide exhibited by the purified enzymes reflect unidentified structural differences, presumably near the phospholipid binding domain of the enzymes.

Spectrophotometric data allow an extension of the suggestion of Lombardo and Dennis [20] that nucleophilic groups such as the ε amine group of lysine may interact with manoalide via a Michael addition. Simple Michael addition of a nucleophilic group to a conjugated aldehyde is insufficient to produce the observed spectral changes, in particular a chromophore with λ_{max} at 465 nm which indicates a highly conjugated system. However, if Michael addition occurs to the ring-opened form of manoalide as shown in Fig. 8, the resulting intermediate, an enolized aldehyde, would be expected to undergo β-elimination of the C-4 hydroxyl group, thereby leading to a hepta-2,4-diena-1,7-dial system. This dial, in the enolized or enolate form, will be a highly conjugated chromophore. Moreover, this proposed mechanism for chromophore formation accounts for the observed independence (Fig. 7) of λ_{max} upon the nature of the nucleophile (nitrogen or sulfur) since the final product has an sp^3 carbon–carbon bond between chromophore and the attached nucleophile. Lastly, the spectral characteristics of the proposed adduct are reproduced (λ_{max} = 455 nm) by the simple hepta-2,4-diene-1,7-dial chromophore [29].

The fact that lysine is not very active in protecting PLA_2 from inactivation by manoalide can also be explained with the model presented above. The hydrophobic tail of manoalide may compete with fatty acids for the phospholipid binding domain on PLA_2. A lysine residue near the PLA_2 lipid binding domain undergoes a Michael addition with manoalide, thus irreversibly inactivating the enzyme. It is predicted from this model that lysine–manoalide conjugates can still interact at the phospholipid binding domain on PLA_2. In contrast, manoalide–protein adducts are no longer active because they are sterically prevented from interacting with PLA_2. Recently, Glaser and Jacobs [25] reported that lysine peptides with greater than 4 residues prevent manoalide from interacting with PLA_2, supporting the model that manoalide may be sterically prevented from interacting with PLA_2 when conjugated to macromolecules. Final proof of this model requires purification and characterization of manoalide–lysine adducts.

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REFERENCES

1. L. L. M. van Deenen and G. H. de Haas, *Adv. Lipid Res.* **2**, 167 (1964).
2. H. M. Verheij, A. J. Slotboom and G. H. de Haas, *Rev. Physiol. Biochem. Pharmac.* **91**, 91 (1981).
3. E. A. Dennis, in *The Enzymes* (Ed. P. D. Boyer), Vol. 16, p. 307. Academic Press, New York (1983).
4. D. H. Albert and F. Snyder, *J. biol. Chem.* **258**, 97 (1983).
5. B. Samuelsson, M. Goldyne, E. Granström, M. Hamberg, S. Hammarstrom and C. Malmsten, *A. Rev. Biochem.* **47**, 997 (1978).
6. S. Hammarström, *A. Rev. Biochem.* **52**, 355 (1983).
7. A. C. Rosam, J. L. Wallace and B. J. R. Whittle, *Nature, Lond.* **319**, 54 (1986).
8. B. B. Vargatfig, M. Chignard, J. Benveniste, J. Lefort and F. Wal, *Ann. N.Y. Acad. Sci.* **370**, 119 (1981).
9. P. J. Piper, in *The Leukotrienes: Chemistry and Biology* (Eds. L. W. Chakrin and D. M. Bailey), p. 215. Academic Press, New York (1984).

10. G. Nalbone and K. Y. Hostetler, *J. Lipid Res.* **26**, 104 (1985).
11. S. P. Sedlis, P. B. Corr, B. E. Sobel and G. G. Ahumada, *Am. J. Physiol.* **244**, H32 (1983).
12. R. J. Flower and G. J. Blackwell, *Nature, Lond.* **278**, 456 (1979).
13. F. Hirata, E. Schiffmann, K. Venkatasubramanian, D. Salomon and J. Axelrod, *Proc. natn. Acad. Sci. U.S.A.* **77**, 2533 (1980).
14. F. Russo-Marie, M. Paing and D. Duval, *J. biol. Chem.* **254**, 8498 (1979).
15. E. D. de Silva and P. J. Scheuer, *Tetrahedron Lett.* **21**, 1611 (1980).
16. R. S. Jacobs, P. Culver, R. Langdon, T. O'Brien and S. White, *Tetrahedron* **41**, 981 (1985).
17. E. S. Burley, B. Smith, G. Cutter, J. K. Ahlem and R. S. Jacobs, *Pharmacologist* **24**, 117 (1982).
18. J. C. DeFreitas, L. A. Blankemeier and R. S. Jacobs, *Experientia* **40**, 864 (1984).
19. K. B. Glaser and R. S. Jacobs, *Biochem. Pharmac.* **35**, 449 (1986).
20. D. Lombardo and E. A. Dennis, *J. biol. Chem.* **260**, 7234 (1985).
21. C. F. Bennett and S. T. Crooke, *Fedn. Proc.* **45**, 1559 (1986).
22. M. A. Clarke, D. Littlejohn, T. M. Conway, S. Mong, S. Steiner and S. T. Crooke, *J. biol. Chem.* **261**, 10713 (1986).
23. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
24. A. Plückthun and E. A. Dennis, *J. biol. Chem.* **260**, 11099 (1985).
25. K. B. Glaser and R. S. Jacobs, *Fedn. Proc.* **45**, 580 (1986).
26. M. M. Master and R. S. Jacobs, *Fedn. Proc.* **45**, 581 (1986).
27. B. W. Dijkstra, J. Drenth and K. H. Kalk, *Nature, Lond.* **289**, 604 (1981).
28. B. W. Dijkstra, K. H. Kalk, W. G. J. Hol and J. Drenth, *J. molec. Biol.* **147**, 97 (1981).
29. S. S. Malhotra and M. C. Whiting, *J. chem. Soc.* 3812 (1960).