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INACTIVATION OF HUMAN SYNOVIAL FLUID PHOSPHOLIPASE A_2 BY THE MARINE NATURAL PRODUCT, MANOALIDE*

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Abstract—The marine natural product, manoalide (MLD), was investigated to determine if this drug inhibited purified human synovial fluid phospholipase A_2 (HSF-PLA_2). Utilizing classical Michaelis–Menten kinetics, apparent K_m and V_{max} values for HSF-PLA_2 of 1.34 mM and 0.47 μmol [^{3}H]palmitic acid released/min/mg protein were obtained using dipalmitoylphosphatidylcholine (DPPC) as the substrate, and 38.0 μM and 18.8 μmol [^{3}H]arachidonic acid released/min/mg protein with *Escherichia coli* as a natural substrate. These kinetic parameters were utilized subsequently to evaluate the inhibitory effects of manoalide on HSF-PLA_2. Inhibition of HSF-PLA_2 by MLD was concentration and time dependent with IC_{50} values of 0.2 and 0.02 μM for DPPC and *E. coli* respectively. Dialysis studies and examination of DPPC or *E. coli* hydrolysis versus enzyme concentration indicate that MLD is an irreversible inhibitor of HSF-PLA_2. Substrate specificity was also examined in the absence and presence of MLD using dipalmitoylphosphatidylethanolamine (DPPE) as a substrate. MLD inhibited the hydrolysis of DPPE (>90% inhibition at 2 μM), and preliminary results indicate that DPPC was more readily hydrolyzed than DPPE under the substrate conditions of the assay. While the cellular source of secreted HSF-PLA_2 is unknown, these studies indicate that MLD can inactivate secreted phospholipase A_2 isolated from patients with inflammatory joint disease.

Phospholipase A_2 (PLA_2‖; EC 3.1.1.4) mediated hydrolysis of fatty acids, specifically arachidonate, from the sn-2 position of membrane phospholipids is significant because this rate-limiting step liberates the requisite substrate for the biosynthesis of eicosanoids [1], several of which have been implicated in the pathogenesis of many disease states [2–5]. Additionally, PLA_2 is an important enzyme in the generation of platelet-activating factor (PAF), another lipid mediator known to possess potent pro-inflammatory actions [6]. The activation, therefore, of PLA_2 by various physiological stimuli may prove to be a critical site for pharmacological intervention in the management of inflammatory diseases.

While the molecular biology for several venom PLA_2s has been studied extensively, the physiological relevance of these enzymes and their inhibitors to mammalian eicosanoid biosynthesis and inflammation remains to be addressed. In recent years, PLA_2s from a variety of human sources have been isolated and purified [7–9]. Of particular importance to our laboratories is the biochemical characterization of the PLA_2 isolated from the synovial fluid of human patients with inflammatory joint disease (HSF-PLA_2). Pruzanski and coworkers [10] recently reported that PLA_2 activity in synovial fluid aspirated from the inflamed joints of rheumatoid arthritics directly correlates with disease progression. The existing PLA_2 activity was found to exhibit the following general characteristics: Ca^{2+} dependence, neutral pH optimum, preference for natural over artificial substrates, molecular weight around 14K, and decreased activity in the presence of detergents [11, 12]. While the cellular source of HSF-PLA_2 is not known, there is evidence that human synoviocytes are capable of releasing PLA_2 into the extracellular space following stimulation by interleukin 1 (IL-1) [13]. Furthermore, during acute inflammation, polymorphonuclear leukocytes (PMNs) isolated from the synovial fluid of arthritic patients have been shown to exhibit elevated PLA_2 activity [14]. This increase in PMN-associated PLA_2 activity, however, may be due to nonspecific adsorbance of secreted PLA_2(s) to the PMN membrane [15]. Regardless of the source of HSF-PLA_2, strong evidence exists that PLA_2 activation exacerbates arthritis, thereby suggesting that inactivation of this enzyme may mitigate the progression of this and many other inflammatory diseases.

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‖ Abbreviations: PLA_2, phospholipase A_2; HSF-PLA_2, human synovial fluid phospholipase A_2; AA, arachidonic acid; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; PE, phosphatidylethanolamine; PMN, polymorphonuclear leukocyte; THF, tetrahydrofuran; MLD, manoalide; and BPB, *p*-bromophenacylbromide.

In recent years, manoalide (MLD), a marine natural product, has been studied extensively as a potent anti-inflammatory drug. MLD was shown to antagonize phorbol-induced inflammation of murine epidermis but not that induced by arachidonic acid [16], suggesting direct inhibition of PLA_2 as a potential mechanism for the antiinflammatory effects of MLD [16–19]. Subsequently, MLD was shown to irreversibly inactivate the extracellular bee [20] and cobra [21] venom PLA_2s in a time- and pH-dependent manner, possibly by modifying specific lysine residues on the PLA_2 molecule [21, 22].

The present investigation describes the hydrolytic activity of column purified HSF-PLA_2 using both natural and artificial substrates. In addition, HSF-PLA_2 activity is examined for the first time in the presence of manoalide in order to compare these effects with those previously described for manoalide inhibition of venom PLA_2s.

MATERIALS AND METHODS

Materials

Tris(hydroxymethyl)aminomethane, calcium chloride dihydrate, 1-α-phosphatidylcholine dipalmitoyl, 1-α-phosphatidylethanolamine dipalmitoyl, *p*-bromophenacylbromide, mepacrine, and tetrahydrofuran were purchased from the Sigma Chemical Co. (St Louis, MO). Aminopropyl (NH_2) solid phase columns (100 mg) were purchased from Burdick & Jackson (Muskegan, MI). Phosphatidylcholine L-α-dipalmitoyl[2-palmitoyl-9,10-^{3}H(N)] (sp. act. 50.0 mCi/μmol), palmitic acid, [1-^{14}C]palmitoyl (sp. act. 8.9 mCi/μmol) and Aquasol universal LSC mixture were purchased from New England Nuclear Research Products (Boston, MA). L-α-Phosphatidylethanolamine, 1,2-di[1-^{14}C]palmitoyl (sp. act. 110 mCi/μmol) was purchased from Amersham (Arlington Heights, IL). Manoalide was isolated and supplied by Allergan Pharmaceuticals (Irvine, CA). [^{3}H]Arachidonic acid labeled *Escherichia coli* was supplied by Dr Richard C. Franson, Virginia Commonwealth University (Richmond, VA). Human synovial fluid was collected from patients with rheumatoid arthritis by Dr Arthur S. Huppert, Office of Arthritis and Rheumatology (Philadelphia, PA), and was subsequently purified by Dr Richard Franson [23].

PLA_2 enzyme analysis

PLA_2-DPPC vesicle radioassay. DPPC vesicles were prepared by hydrating 1.11 mM DPPC with standard assay buffer (100 mM Tris, 5.0 mM $CaCl_2$, pH 7.4) at 4° for 20 min and then sonicating the substrate on ice using a Branson microprobe sonifier, model 185, for approximately 5 min at a setting of 5 for approximately 4–6 min until the emulsion was transluscent. Labeled DPPC (10 μCi) was added to the unlabeled DPPC and then sonicated with five short bursts (at the same setting) to ensure homogeneity. Final activity of the labeled substrate mixture was 0.32 μCi/100 μL substrate (426,000 cpm). Aliquots of the labeled substrate were equilibrated at 37° for 5 min prior to the addition of enzyme. Purified HSF-PLA_2 (7.3 μg protein/mL as determined by the method of Bradford [24]) was added to the substrate yielding a final concentration of 73 ng protein/100 μL total reaction volume; hydrolysis continued for 15 min (unless otherwise noted) at 37° in a shaking water bath. The reaction was quenched with 2 mL of ice-cold tetrahydrofuran (THF).

PLA_2-DPPE vesicle radioassay. Due to the difficulty of solubilizing DPPE, the following changes were made to the aforementioned protocol. For this comparative study, DPPC and DPPE were partially solubilized in methanol before the addition of 100 mM Tris buffer, pH 7.4 (1% methanol final volume). After hydrating for 30 min at 4°, the substrates were probe sonicated on ice until transluscent (approximately 15–20 min). Calcium was then added to the substrate solutions to a final concentration of 0.5 mM (higher concentrations precipitated DPPE); 0.1 μCi [^{3}H]DPPC and [^{14}C]DPPE were also added to the respective unlabeled substrates. The solutions were again sonicated with five short bursts, and subsequently maintained at 4° until needed. Hydrolysis was conducted as previously described, except that the reaction time was increased to 30 min due to the slow hydrolysis of DPPE. The reaction was quenched with 2 mL of ice-cold THF.

PLA_2-[^{3}H]AA-E. coli radioassay. PLA_2 activity was measured by the hydrolysis of [^{3}H]AA-labeled autoclaved *E. coli* phospholipid [25]. Only *E. coli* membrane fragments, and not extracted lipids, were used in this study. Lipid phosphorus was quantitated according to the method of Hurst [26]. The reaction mixture (100 μL total reaction volume) contained standard assay buffer, 5 μmol *E. coli* phospholipid (sp. act. 7.88 μCi/μmol phospholipid phosphorus) and approximately 35 ng HSF-PLA_2. The mixture was incubated at 37° in a shaking water bath for 10 min and then quenched by the addition of 2 mL of ice-cold THF.

Assessment of free fatty acid release

The quenched reaction mixtures were vortexed and applied to aminopropyl solid phase columns preconditioned with THF and THF:H_2O (20:1, v/v). Hydrolyzed [^{3}H]palmitic acid or [^{3}H]AA was eluted from the column with THF:glacial acetic acid (49:1, v/v).* Extraction efficiencies were determined in control assays (without enzyme) by the addition of ^{14}C-labeled palmitic acid before the extraction process; recoveries ranged from 95 to 99%. Radioactivity of the eluant was quantitated by liquid scintillation counting. Sample counts were corrected by subtracting counts in the blank containing no enzyme and then dividing by the extraction efficiency. Final enzyme activity is expressed in terms of velocity (micromoles of free fatty acid released per minute per milligram protein) as calculated from the specific activities of the radiolabeled phospholipid mixtures and the protein concentration of the enzyme.

* Steinder S and Tramposch K, Assay method for the detection of phospholipase A_2 inhibitors. Bristol-Meyers Pharmaceutical Research, Buffalo, NY. Abstract No. 96 presented at the Third International Conference poster session of the Inflammation Research Association, October 22, 1986.

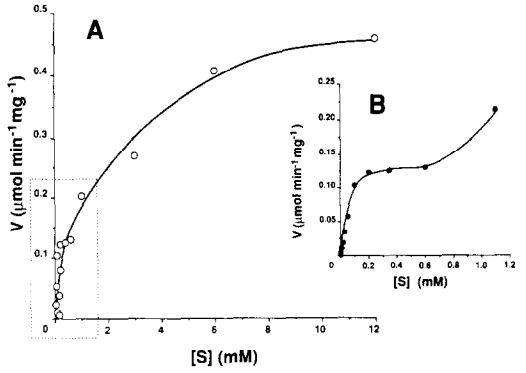

Fig. 1. (A) HSF-PLA_2 activity (V) as a function of DPPC concentration. (B) is an enlargement of the area within the dotted lines in (A) illustrating the biphasic response of the enzyme with DPPC vesicles as the substrate. Standard assay conditions were employed (N = 3).

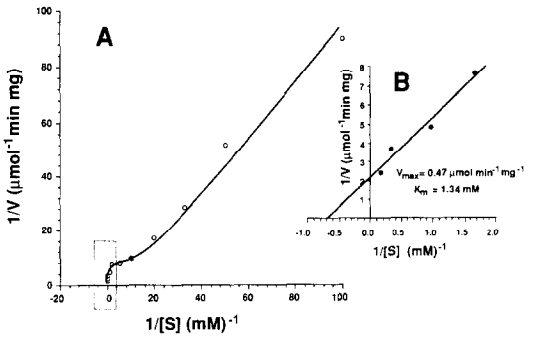

Fig. 2. (A) Double-reciprocal analysis of data in Fig. 1A illustrating a nonlinear $1/[S]$ vs $1/V$ relationship. Data selected for determination of K_m and V_{max} (Fig. 2B) are those which bracketed the apparent K_m estimated from the saturation curve seen in Fig. 1A. These points are enclosed within the dotted box in Fig. 2A for reference.

Treatment of PLA_2 with manoalide

When using the DPPC method, HSF-PLA_2 and MLD were preincubated at 4× the desired final concentration at 37° for 30 min. In control assay tubes, identical volumes of vehicle (100% methanol) were incubated with the enzyme. Because MLD inactivation of PLA_2 is pH dependent, standard assay buffer was included in the preincubation mixture to raise the pH from 4.5 to an optimal 7.4 [20]. Following preincubation, aliquots of the drug/enzyme mixture were added to equilibrated substrate and the reaction was allowed to proceed as previously described. Prior to analysis using the *E. coli* method, enzyme and MLD were preincubated with the complete standard assay buffer (at 1.25× final concentration), excluding substrate, for 30 min. Vehicle effects of dimethyl sulfoxide (DMSO) on the enzyme were examined similarly in control assay tubes. The reactions were started by the addition of substrate to the preincubation mixtures. In both systems, percent inhibition relative to control (vehicle) samples was calculated. Inhibition studies using *p*-bromophenacylbromide (BPB) were conducted similarly using the *E. coli* system.

Dialysis studies

HSF-PLA_2 was incubated with 1 μM MLD as mentioned above for 60 min. Equal aliquots were placed in two separate dialysis bags with a molecular weight cutoff of 6000–8000. One bag was dialyzed with a 1:500 ratio of enzyme mixture to buffer for 2 hr at 4° with two changes of buffer; the other bag was kept at 4° for 2 hr. At the end of 2 hr, [^{3}H]AA-labeled *E. coli* was added to both dialysis bags and hydrolysis was allowed to occur for 30 min at 37° with gentle shaking. Aliquots of the reaction mixtures were removed, liberated [^{3}H]AA was determined, and the hydrolytic activity of the inactivated enzyme was compared between dialyzed and non-dialyzed samples. Controls were analyzed in a similar manner.

Statistical analysis

All data points represent means of triplicate samples where the standard error was less than the size of the symbols used in the figures; therefore, standard error bars have not been included. Parallelism was determined by comparison of the slopes of two regression lines using Student's *t*-test [27].

RESULTS

Application of HSF-PLA_2 to classical Michaelis–Menten kinetics

To ensure that all kinetic measurements were conducted under initial rate conditions, time–course studies were carried out using both substrate systems. Results indicate that DPPC hydrolysis was linear through 180 min, and that the reaction velocity became constant after an initial lag period of 12 min, indicating negligible end-product inhibition (data not shown). Total hydrolysis after 180 min was 7.2% of the original phospholipid present. Using [^{3}H]AA-labeled *E. coli* as a natural substrate, per cent [^{3}H]AA hydrolysis was linear through 120 min; subsequent analyses were performed at 10 min. Analysis of HSF-PLA_2 activity versus DPPC vesicle concentration demonstrated apparent saturation kinetics, where the substrate concentration ratio at 90% V_{max} versus 10% V_{max} ($[S]_{0.9}/[S]_{0.1}$) calculates as 81, suggesting a valid hyperbolic relationship typical of Michaelis–Menten kinetics [28]. However, close inspection of Fig. 1A revealed a biphasic response in the activity of the enzyme using the DPPC vesicle assay. Figure 1B is an enlargement of Fig. 1A, illustrating the inflection seen at concentrations between 400 and 600 μM; at concentrations above 600 μM, enzyme activity again increased, eventually to plateau at an apparent V_{max} of 0.47 μmol/min/mg. Double-reciprocal analysis of DPPC vesicle data using velocity values at all substrate concentrations was biphasic (Fig. 2A), indicating complex rather than pure Michaelis–Menten kinetic behavior. Therefore, those values which bracketed the apparent K_m estimated from the saturation curve were used for estimating K_m and V_{max} (Fig. 2B). Apparent K_m and V_{max} values were estimated by least squares regression analysis to be

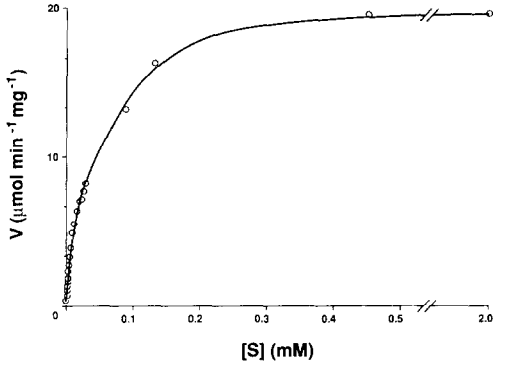

Fig. 3. HSF-PLA_2 activity (V) as a function of *E. coli* concentration demonstrating a monophasic saturation response. Standard assay conditions were employed (N = 3).

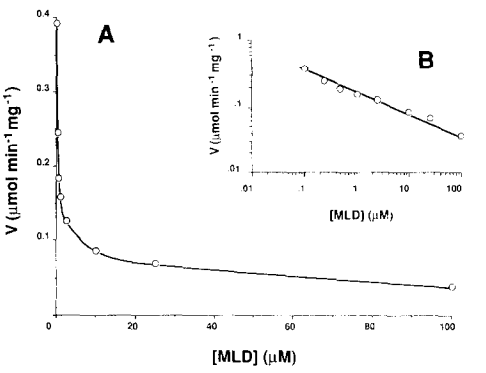

Fig. 5. (A) Concentration-dependent reduction of HSF-PLA_2 hydrolysis (V) of DPPC by MLD (N = 3). MLD was preincubated with HSF-PLA_2 at pH 7.4 for 30 min at 37° prior to the addition of the substrate. Therefore, standard assay conditions were employed. A bilogarithmic transformation (B) of the data in (A) illustrates a linear reduction in velocity typical of irreversible inactivation. All drug concentrations represent final concentrations in the enzyme assay.

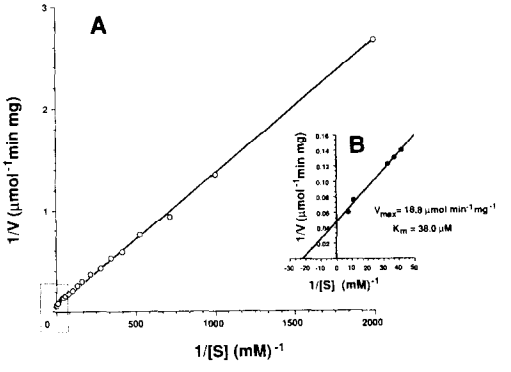

Fig. 4. (A) Double-reciprocal analysis of *E. coli* data shown in Fig. 3 illustrating a linear $1/[S]$ vs $1/V$ response. Data points chosen for the determination of K_m and V_{max} (Fig. 4B) are those which bracketed the apparent K_m estimated from the saturation curve in Fig. 3.

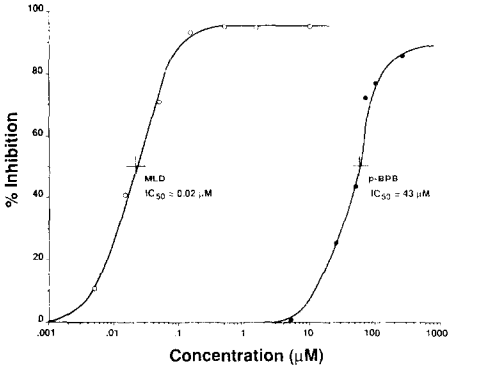

Fig. 6. Percent inhibition of HSF-PLA_2 activity as a function of increasing MLD (○) and BPB (●) concentrations using *E. coli* as the substrate (N = 3). Both drugs were preincubated at pH 7.4 for 30 min at 37° prior to the addition of substrate; standard assay conditions were employed thereafter. All drug concentrations represent final concentrations in the enzyme assay.

1.34 mM and 0.47 μmol/min/mg protein respectively. The HSF-PLA_2 saturation curve generated using [^{3}H]AA-labeled *E. coli* as the substrate is shown in Fig. 3. A biphasic response was not apparent using this system, and the double-reciprocal plot was linear over the entire range indicating more typical Michaelis–Menten kinetics (Fig. 4A). Values for K_m and V_{max} using the *E. coli* phospholipid system were 38.0 μM and 18.8 μmol/min/mg protein (Fig. 4B). Thus, HSF-PLA_2 has a demonstrably greater ($\times 10^4$) affinity for *E. coli* phospholipids than artificial DPPC vesicles.

Inactivation of HSF-PLA_2 by MLD

Because MLD has been shown to inactivate PLA_2 from bee [20] and cobra [21] venom PLA_2, it was of interest to determine if a human PLA_2 was equally susceptible to the inhibitory effects of MLD. Figure 5A shows the effects of increasing concentrations of MLD on reaction velocity of HSF-PLA_2 hydrolysis of DPPC vesicles. Reaction velocity decreased exponentially over the concentration range examined. A bilogarithmic transformation of the data (Fig. 5B) demonstrates a linear reduction in velocity typical of irreversible inactivation. The estimated IC_{50} for MLD in the DPPC model was 0.2 μM. Figure 6 illustrates MLD and BPB inhibition of *E. coli* hydrolysis by HSF-PLA_2. As can be seen, typical sigmoidal concentration–response curves were obtained for both drugs, with BPB being at least a full order of magnitude less potent than MLD. The IC_{50} values calculated for MLD and BPB in this substrate system were 0.02 and 43 μM respectively. The effect of mepacrine on HSF-PLA_2 was also examined using the *E. coli* system, but no inhibition was evident at 50 μM, and appreciable inhibition was only observed at mepacrine concentrations exceeding 800 μM (data not shown). These results indicate that MLD is a direct inactivator of HSF-PLA_2 at

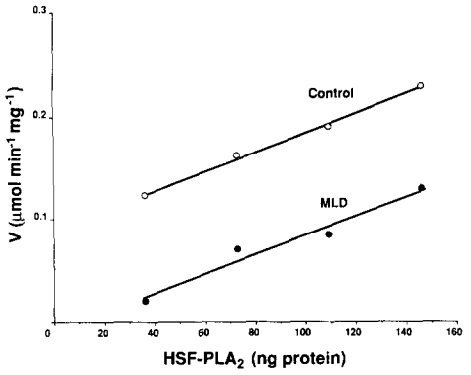

Fig. 7. HSF-PLA_2 activity (V) as a function of HSF-PLA_2 concentration in the absence (○) and presence (●) of 1.0 μM MLD using DPPC vesicles as the substrate (N = 3). Statistical analysis of the slopes of each linear regression line indicated no significant difference between the two, nor was there any visible evidence of convergence within the range of concentrations tested. Similar data were obtained using *E. coli* as the substrate. All drug concentrations represent final concentrations in the enzyme assay.

concentrations comparable to those which inhibit bee and cobra venom PLA_2 [20, 21]. Furthermore, HSF-PLA_2 was sensitive to BPB. This is in contrast to studies with the PLA_2 isolated from the $P388D_1$ macrophage-like cell line which was inhibited by MLD but not by BPB [29].

Binding characteristics of MLD

Irreversibility of MLD binding to HSF-PLA_2 was determined by dialysis methods and by kinetic analysis of enzyme activity versus enzyme concentration in the absence and presence of MLD. Using *E. coli* as the substrate, non-dialyzed (ND) and dialyzed (D) samples of HSF-PLA_2 (preincubated at pH 7.4 for 60 min at 37° with 1 μM MLD) showed no significant differences in their degrees of inactivation (Exp. I, ND = 91.6% vs D = 85.7%; Exp. II, ND = 60.6% vs D = 52.2%; Exp. III, ND = 98.9% vs D = 99%). Figure 7 illustrates the effects of HSF-PLA_2 activity as a function of enzyme concentration in the absence and presence of 1 μM MLD using DPPC as the substrate. There is an apparent parallel shift to the right for MLD-treated samples. Statistical analysis of the slopes of the concentration–response curves did not indicate significant differences [27] nor was there any visible evidence of convergence of the two concentration–response curves over the concentrations examined. Similar data were obtained using *E. coli* as the substrate. The collective results of these three experiments (linear bilog concentration–response relationship, inability to reverse MLD inactivation by dialysis, and a parallel shift in the concentration–response relationship as a function of enzyme concentration) indicate that MLD acts as an irreversible inactivator of HSF-PLA_2.

Comparison of DPPC and DPPE as substrates for HSF-PLA_2

HSF-PLA_2 hydrolysis of DPPC and DPPE was also examined in the absence and presence of 2 μM MLD in order to assess the relative substrate specificity of the enzyme and to determine if a different polar head group alters MLD inactivation of HSF-PLA_2. It should be noted that the dipalmitoyl form of PE is extremely difficult to solubilize due to its high transition temperature. As a result, low substrate concentrations (<100 μM) were used to avoid precipitation of the phospholipid. Calcium concentrations less than 1 mM were also used to avoid precipitation of DPPE. Results indicate that under identical conditions, as outlined in Materials and Methods, DPPC was hydrolyzed to a greater degree than DPPE (57 μmol/min/mg protein versus 20 μmol/min/mg protein respectively). Preincubation of HSF-PLA_2 with 2 μM MLD at pH 7.4 for 30 min at 37° resulted in similar degrees of inhibition using both DPPC and DPPE vesicles (98.6 and 90.7% respectively).

DISCUSSION

The relationship of PLA_2 activation to the inflammatory response has assumed an increasing role in the development of pharmacological agents which mitigate inflammatory diseases. While the benefits of such research are forthcoming, most structural and mechanistic studies have, by necessity, concentrated on the characterization of venom sources of PLA_2 [17, 21, 30]. Such work has demonstrated, however, that differences exist between the various forms of PLA_2, not only in regard to their interaction with phospholipids [1, 17, 21, 30], but also with respect to the compounds which inhibit their activity [17, 27]. As a result, increasing attention has been aimed at the isolation, purification, and characterization of human and other mammalian sources of PLA_2 in an effort to identify specific inhibitors as potential therapeutic agents. Manoalide is therefore representative of a novel class of compounds which directly inhibits eicosanoid biosynthesis through the irreversible inactivation of phospholipase A_2.

Kinetics of HSF-PLA_2

We have demonstrated that HSF-PLA_2 hydrolysis of DPPC vesicles and *E. coli* phospholipids is a saturable process, and that K_m and V_{max} values representative of each substrate system can be derived. While these kinetic values were quantitatively and qualitatively different between systems, they provided meaningful estimates for the substrate concentrations and reaction intervals used to conduct the drug studies described herein. One kinetic feature apparently unique to the DPPC vesicle assay was the biphasic nature of the saturation curve. The portion of the curve above 0.6 mM could be adequately described by Michaelis–Menten kinetics, but we were unable to resolve the kinetic behavior of the enzyme below this concentration. Similar observations were reported by Lister *et al.* [31], using the PLA_2 isolated from the $P388D_1$ macrophage-like cell line. Several explanations for the biphasic response of HSF-PLA_2 toward phospholipid vesicles are possible. One is that more than one isozyme is present in the purified enzyme preparation used, each with different substrate affinities and specific activities.

Another possibility is that the enzyme may be allosteric in nature, and under these conditions exhibits activity indicative of negative cooporativity [28]. This behavior would not be inconceivable as a control mechanism for phospholipid metabolism in a biological system. Vesicular non-uniformity may also have produced the biphasic saturation response seen in the vesicle assay, but evidence from our laboratory that bee venom PLA_2 exhibits a monophasic saturation curve over the same phospholipid vesicle concentration range would indicate this is not the case (unpublished observations). Other possibilities described in detail elsewhere include the presence of two or more substrate binding sites on the enzyme [21], or conformational changes in the enzyme following its reincorporation into the phospholipid vesicle [31]. The fact that HSF-PLA_2 did not display a biphasic response with *E. coli* as the substrate is difficult to resolve due to the natural mixture of phospholipids present in this system which may interact in a complex manner, thereby obscuring single substrate phenomena. While the observation of a biphasic saturation response is intriguing, whether or not it is critical for the structural, functional and pharmacological characterization of this enzyme must await further studies.

A final noteworthy feature of these initial kinetic studies of HSF-PLA_2 is the significant differences obtained for K_m and V_{max} values in each substrate system (1.34 mM and 0.47 μmol/min/mg for DPPC, respectively, and 38.0 μM and 18.8 μmol/min/mg with *E. coli*). Vadas and co-workers [12] reported findings where HSF-PLA_2 hydrolysis of *E. coli* phospholipids was greater than the hydrolysis of DPPC mixed micelles. The values obtained by Vadas and co-workers, qualitatively similar, differed quantitatively (4-fold versus 40-fold in the present study). This discrepancy may be attributable to the use of DPPC sodium deoxycholate mixed micelles as opposed to DPPC vesicles employed in the present study. The comparable qualitative differences invariably involve the form in which the phospholipid is presented to the enzyme [1]. While both substrate systems are composed of phospholipid bilayers, factors such as thermotropic phase transitions, varying degrees of fatty acid saturation, and the absence or presence of proteins and other macromolecules in the lipid bilayer could have profound effects on enzyme hydrolysis [32–34]. Since *E. coli* is a natural source of phospholipids, it may provide a more interactive and dynamic surface for the enzyme than the rigid and uniform interface characteristic of reconstituted phospholipid vesicles or mixed micelles. An additional reason for the greater enzymatic activity seen using *E. coli* may include the head group specificity of the enzyme. While some studies indicate that little difference exists between PLA_2 activity using PE or PC as substrates [12, 31], head group specificity may be an important factor in the activity of other enzymes such as the cobra venom PLA_2 [21]. Because *E. coli* is primarily composed of PE-containing phospholipids [35], it was therefore surprising in the present study that DPPC vesicles were the preferred substrate for HSF-PLA_2 over those containing DPPE. This can be explained by the possible presence of activator phospholipids in the *E. coli* membrane which could potentially increase the binding affinity and/or hydrolysis rate of PE-containing phospholipids. This idea has been demonstrated with cobra venom PLA_2, which like HSF-PLA_2 hydrolyzes DPPE poorly; however, when a phosphorylcholine-containing phospholipid "activator" is added to PE, the affinity of cobra venom PLA_2 for this mixed substrate is enhanced greatly [36]. These observations therefore demonstrate the importance of using both natural and artificial substrates in studying enzyme behavior. Natural phospholipid sources such as *E. coli* may provide a more efficient and physiologically relevant substrate for PLA_2 studies. On the other hand, substrates composed of single phospholipid species can provide detailed kinetic information and serve as controls for substrate specificity studies.

Inhibitors of phospholipase A_2

Compounds which have been identified as inhibitors of PLA_2 include BPB, mepacrine, and manoalide. BPB covalently modifies essential histidine residues associated with the catalytic site on the enzyme [37]. While labeled as a selective inhibitor of PLA_2, BPB inactivates a wide variety of other enzymes through similar mechanisms [38]. Mepacrine has been shown to inhibit PLA_2 activity *in situ* but not *in vitro* by altering calcium availability to the enzyme [39] and possibly by modifying the structure of membrane phospholipids [40]. Manoalide, a nonsteroidal sesterterpenoid isolated from the sponge *Luffariella variabilis* [41] has been demonstrated to be a potent inactivator of bee [20] and cobra [21] venom PLA_2 with IC_{50} values of 0.05 and 2.0 μM respectively. BPB and mepacrine, on the other hand, require greater concentrations to inactivate PLA_2. Other work has shown MLD to inhibit the release of labeled arachidonic acid from both phosphatidylcholine and phosphatidylinositol from prelabeled PMNs stimulated with the calcium ionophore A23187 [42]. In cultured mouse peritoneal macrophages stimulated with phorbol myristate acetate and A23187, MLD likewise produced a dose-dependent inhibition of arachidonic acid and prostglandin E_2 release [43]. In addition, MLD has been shown to inactivate PLA_2 isolated from the rabbit PMN [42], the PLA_2 isolated from the $P388D_1$ macrophage-like cell line [29] and now human synovial fluid PLA_2. These collective results provide evidence that manoalide is a potent inactivator of intracellular and extracellular sources of PLA_2, and that the mechanism of inactivation, based upon venom PLA_2 data, is probably due to structural modification of specific lysine residues near the active site of the enzyme [21, 22].

These results contrast to those of Bennett *et al.* [44] who suggested that MLD was a nonspecific and relatively poor inhibitor of mammalian cytosolic PLA_2s (MLD IC_{50} values: guinea pig uterus, 30 μM; guinea pig lung, 100 μM; rat basophilic leukemia cells (RBL-1), >300 μM; mouse smooth muscle cells (BC_3H_1), >300 μM). In these studies, crude cytosolic enzyme preparations were used which had low specific activities (pmol/min/mg range) towards PC mixed micelles. Manoalide-sensitive forms of PLA_2,

however, have been derived from mammalian membrane-associated fractions [29, 42] and secreted venom sources [20, 21]. Thus, the results of the present study are relevant in that they demonstrate manoalide sensitivity of a secreted PLA_2 of human origin whose cytosolic or membrane source is as yet unknown.

In summary, the present study has demonstrated several important points: (1) purified HSF-PLA_2 hydrolyzes natural or artificial phospholipid sources with a relatively high specific activity (μmol/min/mg range) and that this is a saturable process, (2) *E. coli* may be a more efficient substrate for this enzyme, but the examination of specific phospholipids can reveal subtle features in the kinetics of the enzyme, (3) MLD is a potent and irreversible inactivator of HSF-PLA_2 with IC_{50} values similar to those reported for the venom PLA_2s, and (4) MLD inactivates HSF-PLA_2 whether PC- or PE-containing phospholipids are used as substrates. Future studies will examine MLD–HSF-PLA_2 complexes in order to study MLD-specific binding regions and to better understand the mechanism(s) involved in the apparent irreversibility of the reaction. Additionally, investigations studying the cellular source(s) of HSF-PLA_2 and other secreted PLA_2s will be critical for defining the role of phospholipase A_2 in the pathogenesis of inflammatory diseases.

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