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MOLECULAR PHARMACOLOGY OF MANOALIDE

INACTIVATION OF BEE VENOM PHOSPHOLIPASE A_2*

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Abstract—The marine natural product manoalide (MLD) was shown to directly inactivate bee venom phospholipase A_2 (PLA_2). Inactivation was pH dependent (maximum inactivation occurred at pH 8.0), time dependent and concentration dependent. The IC_{50} was estimated at 0.05 μM and virtually complete inactivation of the enzyme occurred at 4.0 μM. The time-dependent loss of PLA_2 activity suggested that inactivation does not follow typical Michaelis–Menten kinetics. Reversibility was studied directly by dilution and dialysis; both methods were ineffective in dissociating the MLD–PLA_2 complex. A kinetic plot of initial velocity (v) versus [PLA_2] supported our hypothesis that MLD apparently inactivates bee venom PLA_2 by an irreversible mechanism.

In recent years, the role of PLA_2‡ (EC 3.1.1.4) as the rate-limiting enzyme in the biosynthesis of pro-inflammatory eicosanoids (prostaglandins, leukotrienes and thromboxanes) has been well demonstrated [1]. Pharmacological investigation of the marine natural product MLD [2], a sesterterpenoid isolated from the marine sponge *Luffariella variabilis*, has led to the identification of a potent anti-inflammatory property that appears to be selective in that it blocks phorbol myristate acetate (PMA) but not arachidonic acid induced local inflammation in the mouse ear. MLD has a greater potency than indomethacin, a qualitatively different mechanism than either indomethacin or hydrocortisone, and demonstrates a time-dependent antagonism of PMA-induced inflammation, indicating that its action is at an early stage, before arachidonic acid release, in the arachidonic acid cascade.§ Subsequently, MLD was shown to directly inactivate bee venom PLA_2 and prevent β-bungarotoxin neurotoxicity at submicromolar concentrations [3–5].

The present study investigates the mechanism of inactivation of purified bee venom PLA_2 by MLD and provides evidence that the formation of the drug–enzyme complex is pH-dependent, time-dependent, concentration dependent and Ca^{2+} independent. Dissociation of MLD from PLA_2 was not evident following dilution or dialysis, suggesting that inactivation may be irreversible.‖

MATERIALS AND METHODS

Materials. DL-α-Phosphatidylcholine dipalmitoyl and purified bee venom phospholipase A_2 (one unit will hydrolyze 1 μmole of phosphatidylcholine to lysophosphatidylchloline and a fatty acid per min at pH 8.5 and 37°) were obtained from the Sigma Chemical Co. (St. Louis, MO). L-3-Phosphatidylcholine, 1-palmitoyl-2-[9,10(n)-^{3}H]palmitoyl (sp. act. 35 Ci/mmole) and [^{14}C]palmitic acid (sp. act. 56 mCi/mmole) were obtained from Amersham (Arlington Heights, IL). Manoalide was isolated and supplied by D. J. Faulkner, Scripps Institute of Oceanography (La Jolla, CA).

pH-stat assay. PLA_2 activity was measured with a Copenhagen Radiometer pH-stat apparatus using a modified method of Dennis [6].

Standard assay conditions were 1.36 mM phosphatidylcholine dipalmitoyl, 2.76 mM Triton X-100, 1 mM $CaCl_2$ and 0.495 units/ml bee venom PLA_2 in a final volume of 5.0 ml at 41° and pH 7.4; pH was maintained throughout the reaction by continuous addition of 0.0025 M NaOH. PC was dispersed in 1 mM $CaCl_2$ with a Polytron sonicator at setting 7.5 for 2 min. After sonication the substrate was degassed with nitrogen (g) for 2 hr while stirring. The reactants were brought to assay temperature and pH under nitrogen before addition of the enzyme. The reaction was initiated by addition of enzyme, diluted to 0.495 units/ml, and allowed to proceed for 1 min for determinations of v.

Radioassay of PLA_2 activity. Unlabeled phosphatidylcholine dipalmitoyl (1.36 mM), 2.76 mM Titron X-100, 10 mM Tris, 1 mM $CaCl_2$, pH 7.4, at 41° were dispersed with a Wheaton glass homogenizer and Teflon pestle and subsequently sonicated

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‡ Abbreviations: PLA_2, phospholipase A_2; MLD, manoalide; v, initial velocity (μmoles FFA released/min); FFA, free fatty acid; PC, phosphatidylcholine; and BPB, *p*-bromophenacyl bromide.

§ R. S. Jacobs, E. L. Clason, E. S. Burley, J. E. Hochlowski and D. J. Faulkner, manuscript submitted for publication.

‖ A preliminary report of this work was presented in *Fedn. Proc.* **44(3)**, 501 (1985).

for 30 sec to allow formation of a homogeneous monomolecular substrate. L-3-Phosphatidylcholine, 1-palmitoyl-2-[9,10(n)-^{3}H]palmitoyl was added to the unlabeled phosphatidylcholine after sonication to give a final activity of 0.014 μCi/0.5 ml substrate (12,000 cpm). Aliquots of 0.5 ml of the labeled substrate were equilibrated at 41° for 10 min before addition of the enzyme. Enzyme was added to yield a final concentration of 0.495 units/ml, and hydrolysis was allowed to continue for 30 sec at 41°. The reaction was quenched, and [^{3}H]palmitic acid was extracted according to the method of Dole and Meinertz [7] as modified by Gatt and Barenholz [8]. Briefly, the reaction was terminated by addition of 5 vol. of extraction mixture (isopropyl alcohol–heptane–0.5 M H_2SO_4, 40:10:1, by vol.) and vortexed; to achieve phase separation 4 vol. (2.0 ml) of *n*-heptane and 2 vol. (1.0 ml) of H_2O were added and vortexed; to clarify the layers, the mixture was centrifuged in an IEC clinical centrifuge at low speed for 2 min; 1 ml of the upper heptane layer was removed and diluted in 1.0 ml of *n*-heptane; 100–150 mg of silica gel 60 HR (Merck) was added, vortexed and centrifuged at 800 *g* for 5 min to pellet the silica gel. Then 1.0 ml of the heptane supernatant fraction was removed, added to 10 ml of Hydrofluor scintillation fluid, and counted in a Packard Tri-Carb liquid scintillation counter. Final counts were corrected for normal background and for [^{3}H]PC co-extracted with [^{3}H]palmitic acid. Extraction efficiency was determined in control assays (without enzyme) by addition of [^{14}C]palmitic acid before extraction.

RESULTS

Effect of MLD on PLA_2 activity. Inactivation of PLA_2 by MLD at pH 7.4 occurred in a concentration-dependent manner (Fig. 1). A 100% reduction of enzyme activity occurred at concentrations $\geq$ 4.0 μM MLD. The reduction in v by MLD was linear on a bilogarithmic plot over the concentration range studied. The estimated IC_{50} was approximately 0.05 μM. A double-reciprocal plot of the data was non-linear (not shown), suggesting a type of inhibition which does not follow typical Michaelis–Menten kinetics.

Mepacrine, a widely studied inhibitor of PLA_2 *in situ*, did not inactivate bee venom PLA_2 *in vitro* at concentrations up to 100 μM.* These results and those of others [9] support the evidence that mepacrine does not directly inactivate PLA_2, whereas the time-dependence of the inactivation of PLA_2 by MLD indicates a direct reaction between MLD and PLA_2.

Time-dependence of inactivation of PLA_2 by MLD. We observed that there was no inactivation of PLA_2 when MLD was preincubated with the substrate and that it was necessary to preincubate the enzyme–MLD mixture to achieve inactivation. The

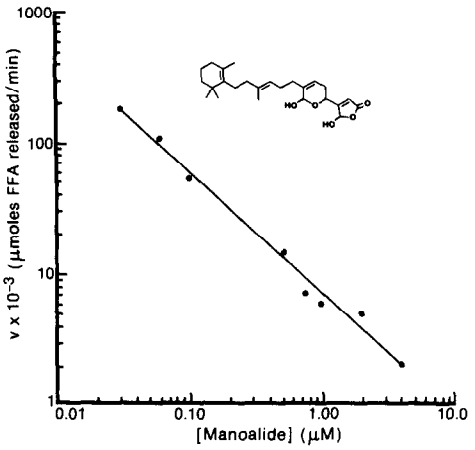

Fig. 1. Concentration-dependent reduction of PLA_2 activity (v) by MLD. Standard assay conditions for the radioassay method were employed. MLD was preincubated with PLA_2 for 60 min at 41° and pH 7.4 prior to assay (N = 3). (Structure, courtesy of D. J. Faulkner).

activity–decay curves (Fig. 2) demonstrate this phenomenon. The time-dependent and the concentration-dependent inactivation of PLA_2 suggests that the reaction is irreversible. At micromolar concentrations of MLD, 85% of the PLA_2 activity was lost during the first 8 min of preincubation. The loss of PLA_2 activity was non-linear with respect to time on a logarithmic plot, indicating that the reaction does not follow pseudo-first-order kinetics under these conditions. At concentrations above 1.0 μM the degree of inactivation appeared to reach a maximum over the preincubation time course.

Effect of pH and Ca^{2+} on the inactivation of PLA_2. The v of PLA_2 hydrolysis was found to vary with pH as shown in Fig. 3A. A characteristic bell shaped

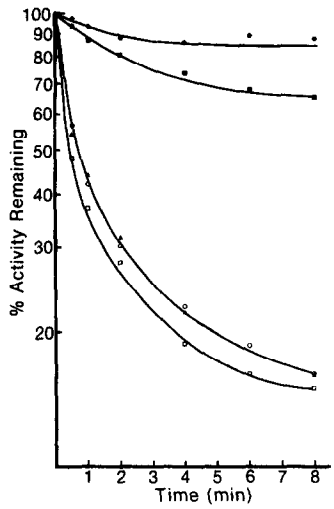

Fig. 2. Degree of inactivation of PLA_2, expressed as percent activity remaining, with respect to preincubation time (min). Preincubation was at 41° and pH 7.4. Standard assay conditions for the radioassay method were employed. For each curve, N = 1 at MLD concentrations of 0.03 μM (●—●), 0.10 μM (■—■), 1.0 μM (▲—▲), 2.0 μM (○—○), and 4.0 μM (□—□).

* Inhibition of PLA_2 activity by mepacrine at 4, 8, 10 and 100 μM was 0, 0, 8.92 and 9.00%, respectively, for 0.495 units/ml bee venom PLA_2. Mepacrine was preincubated with PLA_2 for 60 min at 41° (pH 7.4) prior to assay.

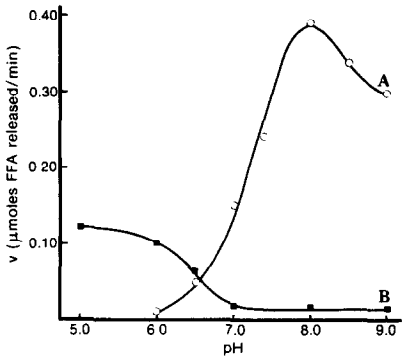

Fig. 3. Effect of pH on enzyme activity (○—○) and inactivation of PLA_2 by 0.5 μM MLD (■—■). Standard assay conditions for the pH-stat method were employed, except for (A) variation of end-point pH for the determination of the optimum pH of bee venom PLA_2 and (B) variation of preincubation pH of the MLD–PLA_2 mixture with final assay of PLA_2 activity under standard conditions (pH 7.4). (N = 3).

curve for v versus pH is evident. The optimum pH for bee venom PLA_2 under these conditions was approximately 8.0, which agrees with the optimum reported by Shipolini *et al.* [10]. Figure 3B shows the effect of varying preincubation pH of the MLD–PLA_2 mixture. There was a 3-fold reduction in v between pH 6.0 and 7.0. Maximal inactivation was achieved between pH 7.0 and 8.0.

Ca^{2+} has a specific binding site on PLA_2 and is an absolute requirement for hydrolytic activity [11]. At calcium concentrations from 0 to 12 mM there was no significant effect on the degree of inactivation obtained with 0.5 μM MLD (Fig. 4).

Binding characteristics and reversibility. Reversibility of the MLD–PLA_2 complex was investigated directly by dilution and dialysis methods (Table 1, A and B, respectively) and graphic analysis of a plot of v versus PLA_2 concentration in the presence and absence of MLD (Fig. 5). Table 1A, column 1 (0-min preincubation), shows the effect of simultaneous addition of PLA_2 and MLD to the substrate mixture. The observed v was identical to that obtained by addition of PLA_2 alone which indicates that, without preincubation, inactivation was not obtained.

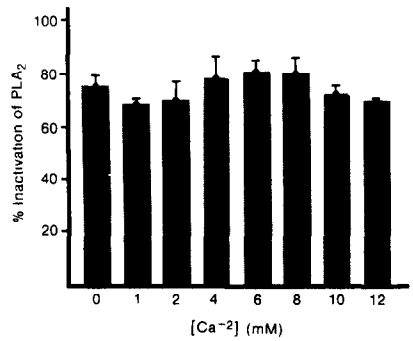

Fig. 4. Effect of $[Ca^{2+}]$ (mM) on the inactivation of PLA_2 by 0.50 μM MLD. Standard assay conditions for the pH-stat method were employed. PLA_2 was preincubated with MLD and the indicated $[Ca^{2+}]$ (mM) for 60 min at 41° and pH 7.4 prior to assay (N = 3).

Table 1. Irreversibility of the MLD–PLA_2 complex

(A) Dilution following 60-min preincubation

MLD (μM)	(μmoles FFA released/min) 0-min Preincubation 0.495 units/ml PLA_2	60-min Preincubation 49.5 units/ml PLA_2
6	0.121	0.035*
50	0.102	0.0023*

* Following preincubation, samples were diluted 100× to final assay concentrations of 0.495 units/ml PLA_2, 0.06 and 0.50 μM MLD.

(B) Dialysis following 60-min preincubation

MLD (μM)	% Reduction of enzyme activity Before dialysis	After dialysis
0.25	86.4	84.9
0.50	91.6	93.0
1.00	92.3	95.0

There was no significant difference between pre- and post-dialysis values at $P < 0.05$, Student's t-test, N = 3.

Standard assay conditions for the radioassay method were employed. (A) Dilution: 0-min preincubation—simultaneous addition of PLA_2 and MLD to the substrate mixture; 60-min preincubation—concentrated MLD–PLA_2 mixture preincubated at 41° and pH 7.4 for 60 min prior to assay. A 100× dilution of the MLD–PLA_2 mixture reduced PLA_2 to the equivalent control concentration (0.495 units/ml) and reduced the MLD concentration to 1/100 of control levels (0.06 and 0.50 μM respectively). (B) Dialysis: MLD–PLA_2 mixtures were preincubated at 41° and pH 7.4, for 60 min prior to pre-dialysis sampling (Before dialysis); the remainder of the MLD–PLA_2 mixture was dialyzed in Spectr/Por MWCO 12000-14000 cellulose tubing at 4° for 24 hr with two buffer changes during the 24-hr period, after which post-dialysis samples were assayed (After dialysis). Enzyme activity is reported as percent reduction in enzyme activity as compared to control (without MLD) samples which were treated identically to MLD–PLA_2 mixtures.

Column 2 shows the effect of preincubation of MLD with PLA_2 60 min prior to assay at pH 7.4. A 100× dilution of the concentrated MLD–PLA_2 complex reduced PLA_2 to the equivalent control concentration and reduced the concentration of MLD to 1/100 of control levels (0.06 and 0.5 μM respectively). If the MLD–PLA_2 complex were readily reversible, the observed v in column 2 would be expected to be equal to or greater than the v observed in column 1. In contrast, v was markedly lower than control values, indicating that the MLD–PLA_2 complex was not significantly reversed by dilution.

Dialysis for 24 hr at 4° of concentrated MLD–PLA_2 mixtures, preincubated for 60 min at 41°, showed equivalent degrees of inactivation as compared to pre-dialysis samples (Table 1B).

Figure 5 is a plot of v versus PLA_2 concentration; comparisons were made between identical samples in the presence and absence of 0.50 μM MLD (MLD-treated samples were preincubated for 60 min at 41° prior to assay). As can be seen, there was a parallel shift of the plot to the right which we interpret to mean that a uniform number of PLA_2 molecules have been inactivated [12]. This form of kinetic

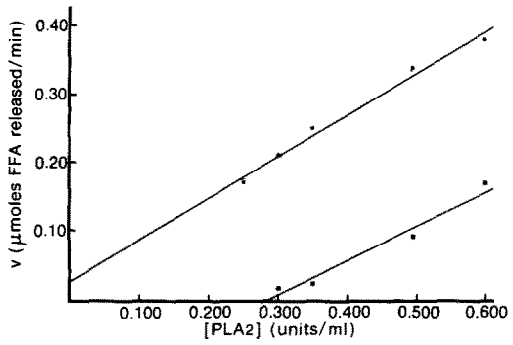

Fig. 5. Variation of [PLA_2] (units/ml) in the absence (●—●) and in the presence of 0.5 μM MLD (■—■). Preincubation of the MLD-treated samples was at pH 6.0 and 41° for 60 min prior to assay. Standard assay conditions for the pH-stat method were employed, except for variation of enzyme concentration (best graphical representation of four experiments, all demonstrating the identical relationship between v in the presence and the absence of MLD).

analysis supports our hypothesis that MLD inactivates bee venom PLA_2 by an irreversible mechanism.

DISCUSSION

Two classes of agents have been reported in the literature to inhibit PLA_2, *p*-bromophenacyl bromide (BPB) and its analogs and mepacrine (quinacrine, N^4-(6-chloro-2-methoxy-9-acridinyl)-N^1,N^1-diethyl-1,4-pentanediamine). BPB covalently modifies histidine and methionine residues of proteins and has the potential to modify other nucleophilic residues [13]. BPB inactivates pancreatic PLA_2 by modification of His-53, which is an essential active site residue [14]. Mepacrine, on the other hand, has been reported to interact directly with membrane phospholipids and form mepacrine-phospholipid derivatives, thus altering the substrate rather than competing for or binding to sites on the enzyme [9]. Both of these compounds require high concentrations (mM) to inactivate or reduce PLA_2 activity and, as such, are limited in their use as pharmacological probes.

MLD, on the other hand, has proven to be a potent inhibitor of PLA_2 *in vitro*. The IC_{50} reported here is approximately 0.05 μM at pH 7.4 for bee venom PLA_2. Recently, MLD has also been shown to inactivate the allosteric enzyme *Naja naja* PLA_2 [15].

In our study, inactivation of bee venom PLA_2 by MLD was pH dependent. There was a significant increase in the degree of inactivation corresponding to a change in pH from 6.0 to 8.0, indicating that ionization of specific residues is essential for maximum inactivation and that these residues are also essential for hydrolytic activity.

Hydrolytic activity of PLA_2 was also dependent on Ca^{2+} which has a specific binding site on the enzyme [11]. Ca^{2+} concentrations up to 12 mM afforded no protection against inactivation of PLA_2 by MLD. This result demonstrates that MLD did not alter PLA_2 activity by antagonism of the Ca^{2+} binding site. These results, along with previous studies,* suggest that the pharmacological mechanism of action of MLD may be the result of a direct and possibly specific inactivation of phospholipases of the A type. Recently, Lombardo and Dennis [16] have shown that MLD inhibits cobra venom PLA_2 by specific, covalent modification of lysine residues.

MLD inactivation of PLA_2 is a time-dependent process that does not follow true Michaelis–Menten kinetics and is not reversed by dilution or dialysis. Our data collectively indicate that the inactivation is apparently irreversible. The non-linearity of the activity–decay curves, resulting from non-first-order kinetics, did not allow kinetic analysis of the irreversible interaction between MLD and PLA_2. The non-linearity might be explained by the occurrence of multiple reactions between MLD and PLA_2 [17].

In animal models, MLD is effective in the control of inflammatory responses, possibly due to its ability to directly inactivate PLA_2 and prevent the biosynthesis of the proinflammatory eicosanoids.

It should also be noted that recent literature implicates an alteration of phospholipase A activity in disease processes that affect general membrane function. This has been proposed in several degenerative diseases of muscle and nerve [18–21]. If an altered PLA_2 activity is associated with the pathogenesis of these degenerative diseases, MLD might be useful in altering the time course of certain clinical features of these diseases.

* R. S. Jacobs, E. L. Clason, E. S. Burley, J. E. Hochlowski and D. J. Faulkner, manuscript submitted for publication.

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