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TWO-STEP INACTIVATION OF BEE VENOM PHOSPHOLIPASE A_2 BY SCALARADIAL*†

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Abstract—Scalaradial (SLD), a marine natural product isolated from the sponge (*Cacospongia sp.*, possesses anti-inflammatory properties *in vivo* and *in vitro* (*Pharmacologist* **32**: 168, 1990). In this study we characterize its effects against bee venom phospholipase A_2 (PLA_2; EC 3.1.1.4). SLD is a potent inactivator of bee venom PLA_2 with an IC_{50} value of 0.07 μM. Inactivation of bee venom PLA_2 occurred in a time-dependent, irreversible manner. The rate of inactivation followed first-order reaction kinetics and was dependent on the concentration of SLD. Kinetic analysis suggested a two-step mechanism of inactivation: an initial apparent noncovalent binding ($K_i = 4.5 \times 10^{-5}$ M) followed by covalent modification. The rate of inactivation was reduced markedly in the presence of excess phosphatidylcholine, suggesting that modification of the enzyme occurs at or near the substrate binding site.

During our investigations of novel anti-inflammatory agents from marine invertebrates [1, 2], we observed that scalaradial (SLD§) (5α,12a,17$a\beta$)-12-(acetyloxy) - 4,4,8 - trimethyl - D - homoandrost - 16 - ene - 17,17a-dicarboxaldehyde), originally isolated by Cimino *et al.* [3], had a basic ring structure which resembled an adrenalcorticoid except that the "D" ring was a cyclohexene ring instead of a cyclopentane ring. Due to the resemblance of SLD to the steroid molecule and the presence of dialdehyde functional groups, which are believed necessary for the potent activity of the anti-inflammatory marine natural product manoalide [4, 5], we hypothesized that SLD could be an important pharmacophore useful for the investigation of inflammation.

Preliminary experiments showed that SLD has potent anti-inflammatory properties *in vivo* when administered topically or systemically against phorbol 12-myristate 13-acetate (PMA)-induced skin inflammation, whereas it does not inhibit arachidonic acid-induced inflammation [6]. In *in vitro* cellular studies, SLD proved to be a potent inhibitor of PMA-stimulated release of arachidonic acid in the mouse peritoneal macrophage [6]. Further experiments showed that desacetylscalaradial, a SLD structural analog with similar anti-inflammatory properties, inhibits macrophage phospholipase A_2 (PLA_2; EC 3.1.1.4) activity in cell-free homogenates [6]. SLD, in other experiments not reported here, also inhibited macrophage PLA_2 in cell-free homogenates.‖

In the present study, we investigated the molecular mechanism of action of SLD using bee venom PLA_2. As reported here, SLD is a potent, apparent irreversible inactivator of bee venom PLA_2, acting at a site near the substrate binding site. Kinetic studies indicate that SLD inactivation of PLA_2 occurs with the formation of an initial noncovalent, reversible Michaelis-type complex prior to covalent modification of the enzyme.

MATERIALS AND METHODS

Materials

L-α-Dipalmitoyl phosphatidylcholine, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid (HEPES), and Triton X-100 were obtained from the Sigma Chemical Co. (St. Louis, MO). Bee venom phospholipase A_2 (2400 units/mg protein) was purchased from Boehringer Mannheim (Indianapolis, IN). L-α-Dipalmitoyl-[2-palmitoyl-9,10-^{3}H (N)] phosphatidylcholine (sp. act. 50 Ci/mmol) and [1-^{14}C]palmitic acid (sp. act. 8.9 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Silica gel 60 was purchased from Merck (Darmstadt, Germany). BioSafe scintillation fluid was obtained from Research Products (Mount Prospect, IL).

PLA_2 radioassay

Phosphatidylcholine substrate was prepared at 1.36 mM as mixed micelles. Briefly, unlabeled L-α-dipalmitoyl phosphatidylcholine was homogenized in 10 mM HEPES buffer, 1 mM $CaCl_2$, pH 7.4, at

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§ Abbreviations: SLD, scalaradial; PMA, phorbol 12-myristate 13-acetate; PLA_2, phospholipase A_2; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; and p-BPB, *p*-bromophenacyl bromide.

‖ Manuscript in preparation.

41° containing 0.15% (w/v) Triton X-100. ^{3}H-Labeled L-α-dipalmitoyl phosphatidylcholine was added to a separate test tube and organic solvent was removed. Unlabeled substrate solution was added and the solution was sonicated until clear in an ice bath for 30 min (final activity of 0.25 μCi/0.5 mL substrate). Bee venom PLA_2, 5 μM determined by the Bradford protein assay [7] using bovine gamma globulin as the protein standard, was prepared in 10 mM HEPES buffer, 1 mM $CaCl_2$, pH 7.4, at 41°. SLD was dissolved in methanol. The final methanol concentration in SLD and control tubes was 3% before dilution into substrate. SLD/PLA_2 mixtures were incubated at 41° for variable time periods. Enzyme assays were run under conditions previously described [8]. Five microliters of SLD/PLA_2 mixture was added to 0.5 mL of substrate (final enzyme concentration = 50 nM with substrate). After 15 sec at 41° the reaction was terminated with 2 mL isopropyl alcohol/heptane/0.5 M H_2SO_4 (40:10:1, by vol.) and free palmitic acid was extracted as previously reported [8], with modifications, as follows. An additional 2 mL of heptane and 1 mL of H_2O were added and tubes were vortexed and centrifuged. Then 2 mL of the heptane layer was removed and added to tubes containing 150 mg silica. After vortexing and centrifugation at 800 *g* for 10 min, 1 mL of heptane was removed and added to 3 mL of BioSafe scintillation fluid and counted in an LKB scintillation counter. Extraction efficiency was determined by co-extraction of ^{14}C-labeled palmitic acid in tubes containing no enzyme.

Dialysis studies

Prior to dialysis, 1 mL of enzyme was preincubated at 41° for 1 hr with methanol or SLD. Then 5 μL was removed and assayed for enzyme activity. The remaining solution was dialyzed against 1 L of HEPES buffer at 4° in SpectraPor mol. wt 10,000 cutoff dialysis tubing for 24 hr with two buffer changes before post-dialysis assay.

Progressive inactivation studies

The rate of inactivation of PLA_2 by SLD was determined as follows. SLD was preincubated with 5 μM enzyme and at various times 5 μL was removed and diluted 100-fold into substrate for assay of enzyme activity. Percent enzyme activity remaining at different times was calculated by dividing enzyme activity in the presence of SLD by control enzyme activity from samples that were incubated with methanol and assayed at identical time points.

Kinetic analysis

In initial experiments it was determined that inactivation of PLA_2 followed first-order kinetics; thus, the analysis used by Kitz and Wilson [9] was applied to analyze the SLD–PLA_2 interaction as follows.

Inactivation rates. The rates of inactivation of PLA_2 by SLD were determined by plotting the percent residual enzyme activity versus time of preincubation on a semilogarithmic plot. At least four data points, each the average of duplicate measurements, were used to determine the pseudo first-order rates of inactivation (k'). The slopes and intercepts were calculated using linear regression by least squares.

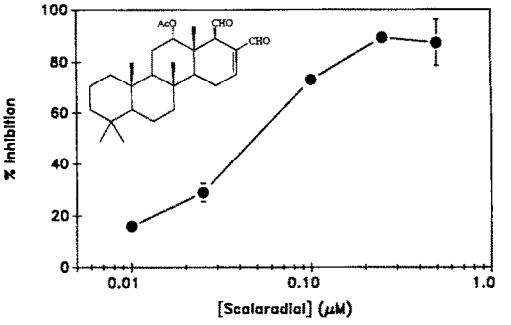

Fig. 1. Inhibition of PLA_2 by SLD. SLD was preincubated with PLA_2 for 1 hr, pH 7.4, 41°. Enzyme activity was assayed as detailed in Materials and Methods. SLD concentrations shown are the final concentration in assay with 50 nM enzyme. Data are means ± SE (N = 3) of percent inhibition as compared to controls. Control enzyme activity = 0.205 μmol/min.

The relationship between the pseudo first-order rate constant (k') and the concentration of SLD was used to estimate the binding constants, K_i and k_3, for SLD (I) with the bee venom PLA_2 (E). The following reaction scheme (1) represents the hypothetical reaction model where $K_i = k_2/k_1$ is the inhibitor dissociation constant for formation of an initial reversible complex and k_3 is the rate formation of the irreversibly inactivated enzyme (EI*) from the reversible complex (E − I).

$$E + I \underset{k_2}{\overset{k_1}{\rightleftarrows}} E - I \xrightarrow{k_3} EI^*. \qquad (1)$$

Statistical analysis

Data are presented as means ± SE. Where standard error bars are not visible, the standard error bar was less than or equal to the size of the symbol.

RESULTS

Inhibition of PLA_2 by scalaradial

SLD is a potent inactivator of bee venom PLA_2 (Fig. 1). When SLD was incubated with PLA_2 at 41° for 1 hr prior to assay with substrate, a concentration-dependent inhibition occurred over 3 log units with an apparent IC_{50} of 0.07 μM with 50 nM enzyme. At this enzyme concentration complete inactivation could be achieved at SLD concentrations of 0.25 μM and above. During preincubation there was no visible precipitation of the protein giving no evidence of nonspecific denaturation, cross-linking of protein or formation of insoluble aggregates. Additionally, during preincubation of SLD with PLA_2, lysine or cysteine, there was no visible evidence of chromophore formation typical of observations made during the

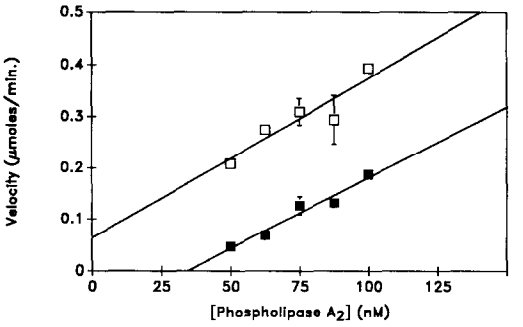

Fig. 2. Inhibition by SLD as a function of PLA_2 concentration. SLD was preincubated for 1 hr at pH 7.4, 41° with increasing molar concentrations of PLA_2 before addition of an aliquot to the substrate. Concentrations shown are final concentrations in assay. Key: (□) control enzyme activity, and (■) enzyme activity with 0.1 μM SLD. Data are means ± SE (N = 3). The difference between the slopes was not significant, $P \geq 0.05$ (Student's *t*-test).

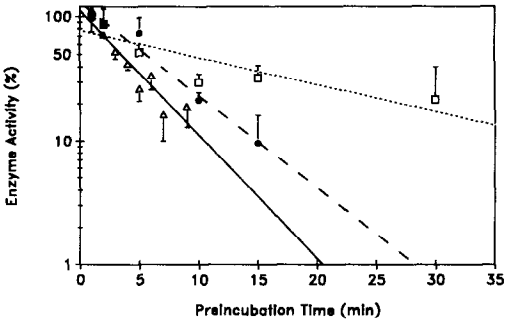

Fig. 3. Progressive inactivation of PLA_2 by SLD. SLD was preincubated with 5 μM PLA_2 at pH 7.4, 41°. Enzyme activity is expressed as percent of control samples. Data are means ± range (N = 2–3) from three experiments. Key: (– – – –) 10 μM SLD, control enzyme activity = 0.112 to 0.148 μmol/min over preincubation time; (— — —) 25 μM SLD, control enzyme activity = 0.116 to 0.170 μmol/min; and (———) 100 μM SLD, control enzyme activity = 0.08 to 0.121 μmol/min.

investigation of the chemical reactivity of manoalide [10].

Irreversible properties

When 10 μM SLD was preincubated with 5 μM PLA_2 for 1 hr at 41°, the enzyme was inhibited by 67% compared to control samples with no SLD. Following dialysis for 24 hr against a 1000-fold volume of HEPES buffer at 4°, no reversal of inhibition was detected when compared against paired control samples dialyzed simultaneously. Post-dialysis control samples retained 84% of activity as compared to pre-dialysis controls (data not shown).

Further evidence that SLD binding was irreversible is based on analysis of plots of the relationship between the initial velocity versus PLA_2 concentration in the presence and absence of SLD (Fig. 2). As can be seen in Fig. 2, there was a parallel shift in the lines indicating a constant reduction in the amount of available enzyme. This relationship is typically obtained with irreversible inhibitors [8, 11]. On the other hand, with reversible inhibitors the two enzyme activity lines converge at their origin [11].

Kinetics of inactivation

Progressive inactivation of PLA_2. When SLD was preincubated with PLA_2 in the absence of substrate, the loss of PLA_2 activity was progressive with time. The time dependence of PLA_2 inactivation for three concentrations of SLD (10, 25, and 100 μM) with 5 μM PLA_2 is shown in Fig. 3. As can be seen, the rate of inactivation increased as the concentration of SLD was increased. At concentrations of less than 25 μM, as the SLD concentration approached the enzyme concentration the data were not linear through 100% inactivation. To accurately determine the degree of irreversibly modified enzyme at the various time points, the PLA_2/SLD mixtures were diluted extensively (100-fold) to dissociate any reversible complexes. At the concentrations used, there was no Michaelis–Menten type of kinetics apparent at the time of addition as the lines routinely intercepted the y axis at 100% control activity.

Calculation of the pseudo first-order rate constants (k') for inactivation of PLA_2. The inactivation of bee venom PLA_2 by SLD exhibited pseudo first-order kinetics. Inactivation was first order through 20% residual enzyme activity at 10 μM SLD and first order through 100% at concentrations of 25 μM and higher with 5 μM enzyme. From these data the pseudo first-order rate constants for inactivation were calculated from the slopes of the inactivation lines from SLD concentrations of 10–100 μM, or from SLD/PLA_2 ratios of 2 to 20 (Fig. 4A). The correlation coefficients for the inactivation lines were greater than 0.86 for all sets of data. The estimated values of k' were dependent on the concentration of SLD and increased from 0.05 min^{-1} to 0.19 min^{-1} with an apparent saturation at an SLD/enzyme ratio of 20 (Fig. 4A).

Estimation of the kinetic constants (k_3 and K_i) for SLD inactivation of PLA_2. The binding properties of SLD can be described from a double-reciprocal plot of the relationship between the pseudo first-order rate constant, k', and the concentration of SLD; and is analogous to the estimation of the K_m and V_{max} for enzyme–substrate interactions (Fig. 4B). The regression line through the points intercepted the positive y axis suggesting that there were reversible complexes formed between the enzyme (illustrated in the kinetic scheme No. 1 in Materials and Methods) and allowed an estimation of the dissociation constant for this complex. The dissociation constant for reversible binding of SLD to bee venom PLA_2 was calculated from the intercept on the x axis, as $K_i = 1/x$ intercept and was equal to 4.5×10^{-5} M. Estimation of the first-order rate constant for formation of irreversibly inactivated enzyme from the reversible complex (k_3) was made from the 1/y intercept. The estimated k_3 was 0.27 min^{-1} at pH 7.4, 41°.

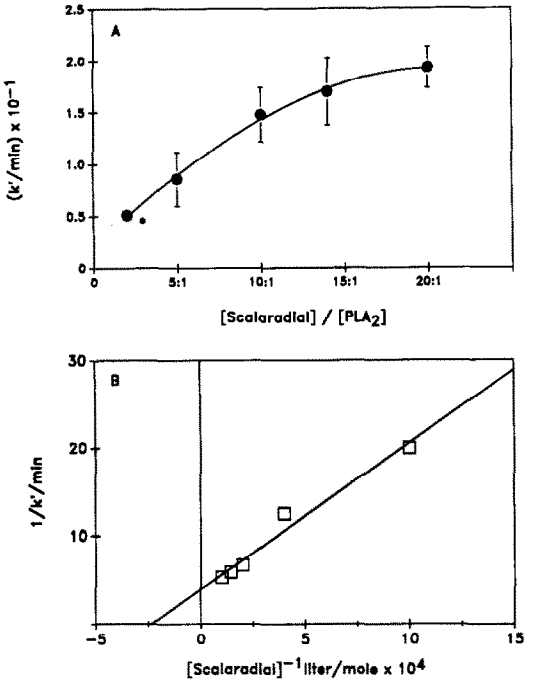

Fig. 4. (A) Changes in pseudo first-order rate constant as a function of SLD concentration. The pseudo first-order rate constant for each SLD/PLA_2 ratio was derived from the slope of the inactivation lines (e.g. Fig. 3). Data are means ± SE from two experiments (four replicates). * One experiment (two replicates). (B) Double-reciprocal plot $1/k'$ vs 1/(SLD) for determination of the kinetic parameters of SLD inhibition of bee venom PLA_2. Data from panel A are plotted in reciprocal form. Correlation coefficient: $r = 0.99$.

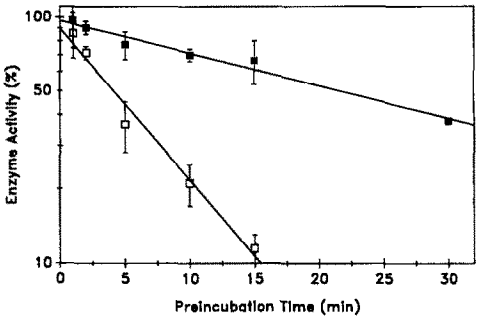

Fig. 5. Effect of substrate on the rate of inactivation of PLA_2 by SLD. SLD was preincubated with PLA_2 at pH 7.4, 41°, in the presence and absence of a 10-fold molar excess of substrate. Enzyme activity is expressed as percent of control samples. Key: (□) SLD (50 μM), and (■) SLD (50 μM) + phosphatidylcholine (500 μM). Data are means ± range (N = 2). Control enzyme activity = 0.111 to 0.167 μmol/min.

Protection of inactivation by substrate

We tested the ability of phosphatidylcholine to alter the rate of inactivation of PLA_2 by SLD. If SLD is binding at the active site, the presence of substrate during the preincubation period should slow the inactivation rate.

The substrate, dipalmitoyl phosphatidylcholine, was prepared as a sonicated dispersion, and was added to the enzyme–inhibitor preincubation mixture in a 10-fold molar excess over the SLD concentration. As can be seen in Fig. 5, in the presence of substrate the inhibition was still time dependent, but the rate of inactivation by SLD was decreased markedly (4.6 fold), suggesting that SLD was competing with the substrate for a binding site on the enzyme. Preincubation of enzyme alone with this concentration of substrate prior to assay did not alter enzyme specific activity.

DISCUSSION

In this study we investigated the mechanism of inactivation of bee venom PLA_2 by the marine natural product SLD. Our data indicate that SLD irreversibly inactivated PLA_2 through a two-step mechanism. As the concentration of SLD was increased, there was an apparent saturation of the inactivation rate suggesting that a reversible step, prior to covalent modification, limits the rate of irreversible inactivation. The noncovalent complex was characterized by an apparent dissociation constant of 4.5×10^{-5} M. The irreversible step occurred relatively quickly with a rate constant of 0.27 min^{-1}. These two binding characteristics result in a potent inhibition of bee venom PLA_2.

Noncovalent interactions

The data indicating that SLD formed a noncovalent complex with the enzyme raised the question as to whether SLD had an affinity for the active site. When SLD was incubated with the enzyme along with a 10-fold molar excess of substrate the inactivation rate was reduced markedly, suggesting that the substrate may be competing with SLD for a site on the enzyme though the possibility of a hydrophobic interaction between SLD and the phosphatidylcholine cannot be excluded as an alternative explanation for these results. Further experiments to elucidate the binding site on PLA_2 will determine whether SLD shares a binding site with substrate.

Noncovalent, hydrophobic bonding interactions have been proposed to be involved in the binding of a number of PLA_2 inhibitors. A kinetically distinct noncovalent complex, with an estimated dissociation constant of 1×10^{-3} M, was observed between the inhibitor, *p*-bromoacetophenone and cobra venom PLA_2 (*Naja naja naja*). This compound contains an aromatic group which was suggested to be involved in hydrophobic interactions with the enzyme [12]. Studies done in our laboratory with the PLA_2 inhibitor, manoalide, have suggested that the degree of hydrophobicity of the manoalide structure is an important property, conferring the potency of this inhibitor [4]. The binding constants for manoalide could not be determined due to nonlinear kinetics over a wide range of inhibitor concentrations [8]. In contrast, with SLD the binding constants could be estimated. This will allow further studies to determine the role of hydrophobic interactions between

effective inhibitors and the active site of PLA_2. It is conceivable that this property is important in allowing an inhibitor access to the active site.

Irreversible modification

The irreversible inhibitor *p*-bromophenacyl bromide (p-BPB) has been used to define and study the residues present at the active site of extracellular PLA_2s. p-BPB covalently modifies a histidine residue at the active site of PLA_2 [12, 13]. The rate of inactivation of bee venom PLA_2 occurred at a faster rate with SLD at pH 7.4 (at 41°, 100 μM) compared to p-BPB inactivation [14]. This suggests that these two inhibitors differ in their specificity and kinetic properties.

The marine natural product, manoalide, also covalently modifies PLA_2. When compared to manoalide (IC_{50} = 0.02 μM, bee venom PLA_2), SLD had a similar potency under identical incubation conditions. Manoalide had been shown to inactivate PLA_2 through a complex mechanism. The proposed mechanism of action involves the opening of manoalide's two rings and the subsequent unmasking of two $\alpha\beta$-unsaturated aldehyde groups. The aldehyde groups are proposed to react with the bee venom enzyme at lysine residues near the active site [15]. These aldehydes do not appear to react synchronously or may not react in the same plane due to the flexible properties of the manoalide carbon backbone. Asymmetric binding to more than one lysine residue may also contribute to the complex inactivation kinetics, since manoalide reacts with three of eleven lysine residues on bee venom PLA_2 [15] and four of the six on cobra venom PLA_2 [5].

On the other hand, the linear inactivation kinetics obtained with SLD suggest that the interaction between SLD and bee venom PLA_2 is less complex than the manoalide reaction. Initial experiments using an analog of SLD have shown that the acetyloxy group is not necessary for inactivation of bee venom PLA_2, suggesting that the reactive groups are the aldehydes [6]. Though SLD has two aldehyde functional groups similar to those which are proposed to occur upon opening of the manoalide rings, SLD has only one $\alpha\beta$-unsaturated aldehyde. The aldehydes are present in an unmasked form fixed in a rigid position relative to the hydrophobic portion of the molecule, thus SLD represents a simple molecule for use in studying the structural requirements for inhibition of PLA_2. Cimino *et al.* [16] proposed the hypothesis that SLD reacts with primary amines and that the reactivity is dependent on the three-dimensional relationship of the two aldehyde groups in space. Their studies with molecules which exhibited a hot taste and contained 1,4-dialdehydes suggested that SLD reacted with primary amines under biomimetic conditions to form a pyrrole derivative, as other hot-tasting molecules did. Even though SLD was reactive with primary amines, it did not possess a hot taste. These investigators suggested that SLD was too bulky of a molecule to reach the site in the taste receptor.

The possibility that the aldehyde groups on SLD react independently and form other types of adducts with PLA_2 should be considered. Future investigations are directed toward determining the number of binding sites for SLD on bee venom PLA_2 and their location.

Currently, there are a limited number of PLA_2 inhibitors, even though there has been a vigorous attempt to design inhibitors of PLA_2 as this enzyme has been proposed to have an important rate-limiting role in inflammation [17]. The design of affinity reagents that have an affinity for the active site of PLA_2 and are able to subsequently inactivate the enzyme is an important mechanism for the design of PLA_2 inhibitors with a degree of selectivity. Our data suggest that defining the structural requirements necessary for inhibition of PLA_2 by SLD could provide information for the development of a new class of PLA_2 inhibitors.

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