CINATRINS, A NOVEL FAMILY OF PHOSPHOLIPASE A_2 INHIBITORS

II. BIOLOGICAL ACTIVITIES

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Cinatrins A, B and C_3 inhibited phospholipase A_2 purified from rat platelets in a dose-dependent manner. Cinatrin C_3, the most potent component (IC_{50} 70 μM), was noncompetitive with a *Ki* value of 36 μM. Cinatrins B and C_3 also inhibited both porcine pancreas and *Naja naja* venom phospholipase A_2. Inhibition of rat platelet phospholipase A_2 by cinatrin C_3 was independent of Ca^{2+} and substrate concentration. Comparison with duramycin, another phospholipase A_2 inhibitor, displayed inhibition dependent on substrate concentration when phosphatidylethanolamine was the substrate. These results indicate that the inhibition of phospholipase A_2 by cinatrin C_3 may result from direct interaction with the enzyme.

Phospholipase A_2 [EC 3.1.1.4] (PLA_2) is a lipolytic enzyme that specifically hydrolyzes the 2-acyl position of a glycerophospholipid. PLA_2 plays a crucial role in the rate-limiting step in the biosynthesis of pro-inflammatory eicosanoids (prostaglandins, leukotrienes, thromboxanes)[1]. It is becoming increasingly obvious that lysosomal and granular PLA_2s secreted into interstitial, intraarticular, or intravascular compartments are involved in the pathogenesis of inflammatory processes[2]. Recently, secretory PLA_2 was purified from a medium of rat platelets stimulated with thrombin[3]. By using this enzyme, we searched for a new PLA_2 inhibitor from culture filtrates of microorganisms and found cinatrins A, B, C_1, C_2 and C_3. Their structures are reported in our previous paper[4]. The present study was done to evaluate the effects of cinatrins on the lipolytic activity of PLA_2 and to clarify their other biological properties.

Materials and Methods

Cinatrins (A, B, C_1, C_2 and C_3) and their derivatives (methyl esters and seco acids) were prepared as described in a previous paper[4]. Duramycin was prepared as previously described[5]. L-α-Phosphatidylethanolamine (from egg yolk), PLA_2 from *Naja naja* venom and porcine pancreas were purchased from Sigma. 1-Palmitoyl-2-[1-^{14}C]linoleoylphosphatidylethanolamine (59 mCi/mmol) was purchased from Amersham Corp. PLA_2 released from thrombin-stimulated rat platelets[3] was prepared by immunoaffinity chromatography[6,7]. All PLA_2s used for these studies were homogeneous as confirmed by SDS-polyacrylamide gel electrophoresis. All other reagents were analytical grade or better.

Phospholipase A_2 Assay

The substrate, [^{14}C]phosphatidylethanolamine aqueous suspension, was prepared by diluting 1-palmitoyl-2-[1-^{14}C]linoleoylphosphatidylethanolamine with L-α-phosphatidylethanolamine for a specific activity of 2,000 dpm/nmol. The lipids were then dried under N_2 and suspended in deionized water with a probe sonicater[8]. The standard reaction mixtures in a total volume of 250 μl contained Tris-HCl buffer (100 mM, pH 7.4), $CaCl_2$ (3 mM), 40 μM [^{14}C]phosphatidylethanolamine and enzyme. The reaction was started by addition of the enzyme solution. The amount of PLA_2 was adjusted to optimize the linear kinetics for quantitation; *i.e.*, hydrolysis of the substrate was less than 20% in all experiments. Following incubation at 37°C for 20 minutes, the reactions were terminated by addition of 1.25 ml of DOLE's reagent[9].

To determine the release of ^{14}C-label from the phospholipid substrate, free fatty acid was extracted by the method of NATORI *et al.*[8], and counted in 7 ml of Liquifluor (Du Pont, New England Nuclear). Before the reaction was started, inhibitor was added to the assay tube. Inhibition is expressed as a %; with enzyme control as 100% reaction *i.e.* 0% inhibition. All data are the average of at least duplicate determinations corrected for none enzymatic hydrolysis (0.5% or less in all experiments). Both cinatrins and their seco acid derivatives were dissolved in Tris-HCl buffer (100 mM, pH 7.4). Cinatrin methyl ester derivatives and duramycin were added to assay tubes as DMSO solutions (2% of final volume), using a DMSO-enzyme control. Control experiments showed that DMSO at concentrations up to 2% had no effect on enzymatic activities. IC_{50} values were determined graphically from plots of percent inhibition versus log concentration of inhibitors.

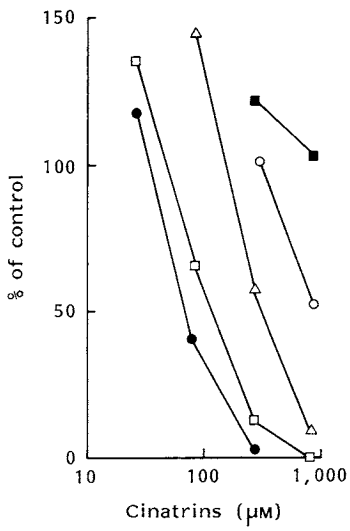
Results

Inhibition of Various PLA_2 by Cinatrins

The effects of cinatrins A, B, C_1, C_2 and C_3 at the concentrations from 30 to 800 μM on rat platelet PLA_2 are shown in Fig. 1. Cinatrins A, B, C_2 and C_3 inhibited PLA_2 activity in a dose-dependent manner, while cinatrin C_1 showed no inhibition in this concentration range. The dose required for 50% inhibition IC_{50} were as follows; 320 μM for A, 120 μM for B, 800 μM for C_2 and 70 μM for C_3 (Table 1). Thus, cinatrin C_3 had the maximal inhibitory activity among the members against rat platelet PLA_2.

Fig. 1. Inhibition of rat platelet phospholipase A_2 by cinatrins.

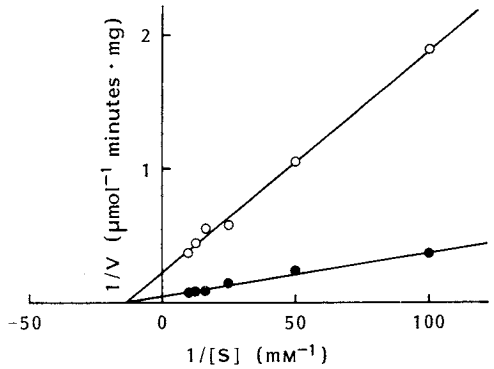
Standard reaction mixtures contained the indicated amounts of cinatrins A (△), B (□), C_1 (■), C_2 (○) and C_3 (●). Inhibition is expressed as the % of the enzyme control.



All data are the average of at least duplicate determinations corrected for none enzymatic hydrolysis.

Fig. 2. Noncompetitive inhibition of rat platelet phospholipase A_2 by cinatrin C_3.

Double reciprocal plot of rat platelet PLA_2 activity toward phosphatidylethanolamine in the presence of (140 μM, ○) or absence (●) of cinatrin C_3.



Standard assay conditions were employed and the lines were drawn on the basis of regression analysis.

Table 1. Inhibition of various phospholipase A_2s by cinatrins.

Cinatrin	IC_{50} (μM) on PLA_2 from		
	Rat platelet	*Naja naja* venom	Porcine pancreas
A	320	430	>800
B	120	110	460
C_1	>800	>800[a]	>800
C_2	800	120	>800
C_3	70	140	390

[a] Cinatrin C_1 exhibited 45% inhibition at 800 μM.

A double reciprocal Lineweaver-Burk plot of cinatrin C_3 is shown in Fig. 2. Cinatrin C_3 inhibited rat platelet PLA_2 activity noncompetitively with a *Ki* of 36 μM.

Because cinatrins A, B, C_1, C_2 and C_3 exhibited differential potency in inhibiting on rat platelet PLA_2, we also investigated their effects on *Naja naja* venom and porcine pancreas PLA_2 to determine whether these characteristics are common to various PLA_2s. As shown in Table 1, all the PLA_2 tested were inhibited by cinatrins B and C_3, but the IC_{50} values varied with the enzyme tested: rat platelet $\geqq$ *Naja naja* venom > porcine pancreas PLA_2. Similar results were obtained with cinatrin A, but it was less potent than cinatrins B and C_3. Cinatrin C_2 preferentially inhibited *Naja naja* venom PLA_2. Cinatrin C_1 showed little ability to inhibit these PLA_2s over this range of concentration.

Effect of Substrate and Ca^{2+} Concentration on the Inhibition of Rat Platelet PLA_2 by Cinatrin C_3 and Duramycin

To survey the mechanism of inhibition by cinatrins on rat platelet PLA_2, we examined the extent of inhibition by cinatrin C_3 as a function of substrate concentration. PLA_2 activity in the presence or absence of cinatrin C_3 increased linearly with substrate concentration (data not shown). Fig. 3(A) shows that the % inhibition by cinatrin C_3 remained constant over the entire range of substrate concentrations. Thus, the inhibition of rat platelet PLA_2 by cinatrin C_3 is independent of the substrate concentration. Recently, the revised structure of duramycin, a polypeptide antibiotic[10], was reported[5] and it was identified as a PLA_2 inhibitor[11]. In addition, duramycin has the ability to interact specifically with two lipids: phosphatidylethanolamine and monogalactosyl-diacylglycerol[12]. We examined the extent of inhibition by duramycin as a function of substrate concentration. It is apparent from Fig. 3(B) that the extent of the inhibition by duramycin is dependent on the substrate concentration, since the inhibition was substantially relieved or even abolished at higher substrate concentrations. The inhibitory potency of cinatrin C_3 on rat platelet PLA_2 was not affected by the Ca^{2+} concentration (3~30 mM) in the reaction mixture (data not shown).

Fig. 3. Effects of substrate concentrations on the inhibition of rat platelet phospholipase A_2 by cinatrin C_3 (A) and duramycin (B).

Standard reaction mixtures contained 10 ng of rat platelet PLA_2, the indicated concentrations of [^{14}C]phosphatidylethanolamine and (A) 34 μM (□), 67 μM (△) of cinatrin C_3; (B) 12.5 μM (□), 25 μM (△), 50 μM (○) of duramycin. Inhibition is expressed as % of the enzyme control.

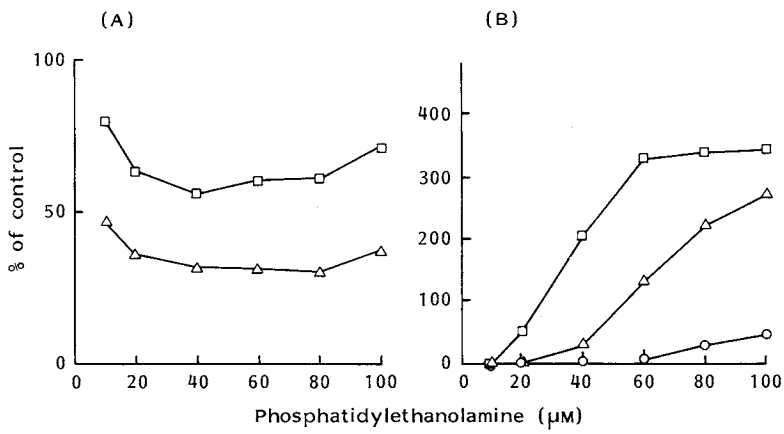
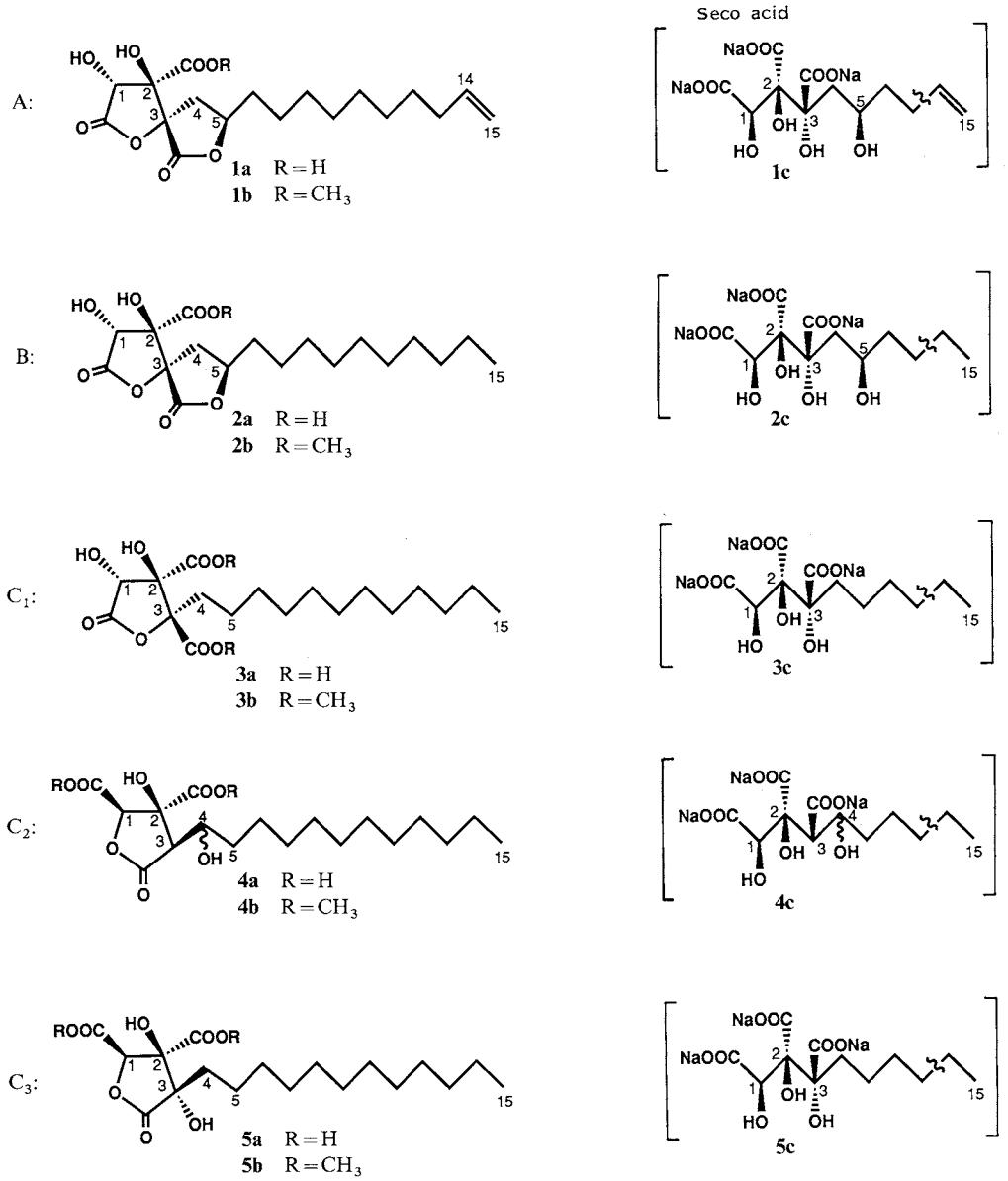


Fig. 4. Structures of cinatrins.



Inhibition of Rat Platelet PLA_2 by Cinatrin Derivatives

To explore in more detail the inhibitory mechanism of the cinatrins on PLA_2, we examined the effect of cinatrin derivatives (Fig. 4) on rat platelet PLA_2 (Table 2). Cinatrin C_1 (**3a**) increased its inhibitory activity on methylation (**3b**), while the activities of cinatrins A, B, C_2 and C_3 were not markedly altered.

Table 2. Inhibition of rat platelet phospholipase A_2 by cinatrin derivatives.

Cinatrin	IC_{50} (μM)		
	Intact	Methyl ester	Seco acid
A	320	220	120
B	120	220	65
C_1	>800	140	n.d.
C_2	800	750	38
C_3	70	130	60

n.d.: Not done (see text).

We also examined the cinatrin seco acids, in which the spiro-γ-dilactones of A (**1a**) and B (**2a**), and the γ-lactones of C_1 (**3a**), C_2 (**4a**) and C_3 (**5a**) were opened (the structures of cinatrin C_1 seco acid (**3c**) and cinatrin C_3 seco acid (**5c**) are identical). All cinatrin seco acid derivatives exhibited more potent inhibitory activity than the original compounds. About 20 times stronger inhibition was noted for cinatrin C_2 seco acid (**4c**) than cinatrin C_2 (**4a**).

Discussion

Our data demonstrated that, although the chemical structures of cinatrins A, B, C_1, C_2 and C_3 are similar, their inhibition activities against rat platelet PLA_2 were different. The most potent activity came from cinatrin C_3 (IC_{50} 70 μM), which was found to be a noncompetitive inhibitor with a *Ki* of 36 μM. Some alkaloids and non-steroidal anti-inflammatory agents displace Ca^{2+} and thus the inhibition by some of these agents appear to be dependent on Ca^{2+} concentration[13]. However, we found the inhibition by cinatrin C_3 to be independent of the Ca^{2+} ion content. Many non-specific PLA_2 inhibitors have been thought to affect the "quality of the interface" by modifying phospholipid bilayer properties that render phospholipid inaccessible to the enzyme[14]. For example, DAVIDSON *et al.*[15] found that lipocortin, which is thought to be an important steroid-inducible inhibitor, inhibits PLA_2 by sequestering the phospholipid substrate; the inhibition can be overcome by high phospholipid substrate concentrations. In the experiments presented herein, the extent of inhibition by duramycin, recently reported as a PLA_2 inhibitor[11], was dependent on the substrate concentration. Since NAVARRO *et al.* reported that duramycin specifically interacts with phosphatidylethanolamine[12], its activity is probably due to direct interaction with the substrate of phosphatidylethanolamine. On the other hand, the inhibition of rat platelet PLA_2 by cinatrin C_3 was independent of the substrate concentration. This evidence strongly suggests that the inhibition of rat platelet PLA_2 by cinatrin C_3 is due to direct interaction with the enzyme.

Since PLA_2 is believed to be the key enzyme responsible for arachidonic acid release[1], it should be investigated whether cinatrin C_3 can inhibit this release from the cell membrane. However, cinatrin C_3 has been shown to cause hemolysis of human erythrocytes (ED_{50} 290 μM) as well as loss of cell viability (cytotoxicity, ED_{50} 160 μM) (data not shown).

BALLOU *et al.*[16] found that unsaturated fatty acids inhibit human platelet PLA_2, and the methylation of unsaturated fatty acids caused complete loss of inhibitory activity. Our finding that methylation of cinatrins did not affect the inhibitory activity suggests the mechanism of PLA_2 inhibition by cinatrins to be different from that of unsaturated fatty acids. Manoalide, a sesterterpenoid PLA_2 inhibitor isolated from marine sponge, contains two cyclic moieties; a six-membered hemiacetal ring and a γ-lactone ring[17,18]. DEEMS *et al.*[19] reported that the γ-lactone ring may play an important role in manoalide inhibition, although this does not offer a complete explanation of its activity. Cinatrins also contain a γ-lactone ring, and we examined their derived seco acid, which are derived by opening this ring. All cinatrin seco acids showed similar IC_{50} values. Although we do not know whether the mechanisms of PLA_2 inhibition by cinatrins and cinatrin seco acids are the same, our finding suggests that the γ-lactone ring may play a significant role in the inhibitory activity of the cinatrin family.

In conclusion, although cinatrins are toxic to cells, they can serve as valuable tools for revealing the structure-function relationships of extracellular PLA_2.

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