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Suicide-inhibitory Bifunctionally Linked Substrates (SIBLINKS) as Phospholipase A₂ Inhibitors

MECHANISTIC IMPLICATIONS*

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Novel phospholipids that function as mechanism-based inhibitors for phospholipase A₂ (PLA₂) are described. PLA₂-catalyzed hydrolysis of the *sn*-2 ester of these suicide-inhibitory bifunctionally linked substrates (SIBLINKS) followed by a cyclization reaction generates a cyclic anhydride at the active site of the enzyme which leads to inhibition. Structure/activity relationships for the SIBLINKS substituents in the *sn*-1 and *sn*-2 position are delineated. Time courses and efficiency of SIBLINKS inhibition are reported and compared for extracellular PLA₂s obtained from *Naja naja naja*, porcine pancreas, bee venom, *Crotalus atrox* and *Crotalus adamanteus*. SIBLINKS-inhibited PLA₂s cannot process either monomeric or micellar substrates consistent with inhibition at the catalytic site. Some SIBLINKS efficiently inactivate 1 mol of *N. naja naja* and *C. adamanteus* PLA₂/6–10 mol of SIBLINKS hydrolyzed. Inhibition of *N. naja naja* PLA₂ can be reversed by hydroxylamine, suggesting that a tyrosine residue is acylated.

Phospholipase A₂ (PLA₂)¹ comprises a ubiquitous set of intracellular and extracellular hydrolytic enzymes that hydrolyze the *sn*-2 ester of phospholipids; the extracellular PLA₂s are relatively well characterized, particularly those secreted by the pancreas or contained in various venoms (1, 2). The extracellular enzymes are low molecular mass (about 13,000 daltons) thermally stable proteins with an absolute dependence on Ca²⁺ for activity. These enzymes have been grouped into at least three major classes based on their different disulfide bond patterns (3, 4). Certain phospholipases liberate arachidonic acid, which is the precursor of prostaglandins, leukotrienes, thromboxane, prostacyclin, and HETEs (5). Interest in modulators or inhibitors of PLA₂ activity has been stimulated by the finding that a plethora of biological responses are induced by these metabolic products (5). Since phospholipase A₂ has been postulated to play a central role in septic shock, rheumatoid arthritis, and the production of inflammatory eicosanoids in general, developing inhibitors of this enzyme is important (5, 6).

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¹ The abbreviations used are: PLA₂, phospholipase A₂; SIBLINKS, suicide-inhibitory bifunctionally linked substrates; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine; DCPC, 1,2-dicaproyl-*sn*-glycero-3-phosphorylcholine; P, partition ratio corresponding to the number of mol of SIBLINKS hydrolyzed per mol of enzyme inactivated; HPLC, high pressure liquid chromatography.

We have reported recently the first example of mechanism-based inhibitors for PLA₂, termed *suicide-inhibitory bifunctionally linked substrates* (SIBLINKS) (7, 8). The SIBLINKS reaction scheme is illustrated in Fig. 1 for PLA₂. SIBLINKS employ a dibasic acid as a spacer to link a leaving group to a molecular ensemble conveying enzyme recognition. Upon enzymatic hydrolysis of the ester bond, the nascent carboxylate acts as an internal nucleophile to expel the leaving group (in this case *p*-nitrophenoxide) thereby forming a five- or six-membered anhydride. If cyclization is fast relative to diffusion, the anhydride will be formed at the active site or in the solvation sphere of the enzyme. Under these conditions, enzyme acylation resulting in modulation of catalytic activity could occur. Nonspecific enzyme acylation would be precluded for those anhydrides that diffuse away from the active site because of facile trapping of the anhydride by water.

This study focuses on the consequences of hydrolysis of phospholipids 1–5 (Fig. 1) containing the following five dibasic acids: glutaric, 2,2-dimethylglutaric, 3,3-dimethylglutaric, succinic, and 2,2-dimethylsuccinic, respectively. In each instance, the dibasic acid serves as a link to join the *sn*-2 position of a 1-acyl-*sn*-glycerol-3-phosphorylcholine to *p*-nitrophenoxide. Enzymatic cleavage of the *sn*-2 ester produces a 1-acyl-*sn*-glycerol-3-phosphorylcholine and a *p*-nitrophenyl ester 6–10. The rate of cyclization of the esters to anhydrides 11–15, respectively, is a function of both the number of intervening methylenes between carbonyls and the specific substituents on the methylenes (9). For the series of half-esters 6–10, the half-life for cyclization in buffer varies from 150 s for the unsubstituted ester 6 to 1 s for 10 at 20 °C (8).

Herein, we describe mechanistic and substrate dependence studies that delineate salient features of the SIBLINKS in inhibiting the cobra venom (*Naja naja naja*) PLA₂. This includes *sn*-1 acyl chain length dependence and identification of the acylation site. In addition, we compare the effectiveness and efficiency of SIBLINKS-induced inhibition of five PLA₂s, which include type I (*N. naja naja* and porcine pancreatic), type II (*Crotalus atrox*, and *Crotalus adamanteus*), and type III (bee venom) enzymes (4).

EXPERIMENTAL PROCEDURES

Materials

All lysophospholipids, 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC), and 1,2-dicaproyl-*sn*-glycero-3-phosphorylcholine (DCPC) were purchased from Avanti Polar Lipids. All cyclic anhydrides except 2,2-dimethylsuccinic anhydride were obtained from Aldrich Chemical Co. The latter was prepared by treating dimethylsuccinic acid (Aldrich) with a 3-fold excess of trifluoroacetic anhydride in CH₂Cl₂ for 2 h, removing the volatiles, and using the residual solid without further purification. All solvents and reagents were of reagent quality.

PLA₂ obtained from cobra venom (*N. naja naja*) was purified as

FIG. 1. PLA₂ catalyzed hydrolysis of phospholipid SIBLINKS 1–5 to produce lysophospholipids and *p*-nitrophenyl esters 6–10 as products. These esters spontaneously cyclize to release *p*-nitrophenoxide and anhydrides 11–15, which react with PLA₂. The acyl chain length (*R*) in the *sn*-1 position is designated by a–f in the text and in Table I.

described previously (10). Bee venom phospholipase A₂ and porcine pancreatic phospholipase A₂ were from Boehringer Mannheim, and *C. adamanteus* PLA₂ was from Sigma. *C. atrox* PLA₂ was a gift from Professor Paul Sigler, Yale University.

Preparation of Phospholipids 1–5

Phospholipids 1–5 with various acyl chains in the *sn*-1 position were synthesized (see Table I). The general procedure employed for the synthesis of all phospholipids is described for compound 2b. To 42 mg (0.1 mmol) of 1-decanoyl-*sn*-glycerol-3-phosphorylcholine in 3 ml of CH₂Cl₂ was added 60 mg (0.43 mmol) of 2,2-dimethylglutaric anhydride followed by 50 μ l (0.35 mmol) of Et₃N. The reaction was heated for 24 h at 45 °C. If the reaction was not complete by TLC analysis (1:10:22 H₂O/MeOH/CHCl₃ using Analtech Silica Gel G-250 glass plates with UV indicator visualized with molybdate spray), an additional 30 mg (0.21 mmol) of anhydride and 40 μ l (0.28 mmol) of Et₃N were added and the reaction heated for an additional 10 h until all starting lipid was consumed. After removal of all volatile components, the residue was leached three to four times with 10-ml portions of dry Et₂O to remove unreacted anhydride and triethylamine glutaric acid salts. The remaining crude half-acid phospholipid after evacuation at 0.1 torr was converted to the acid chloride upon treatment with 2 ml of CH₂Cl₂ containing 0.3 ml of oxalyl chloride for 5 h at 20 °C. Alternatively the half-acid phospholipid was isolated by washing with 0.1 N HCl the crude acylation product dissolved in 2:1 CHCl₃/MeOH. Pure half-acid phospholipid was obtained after chromatography on silica gel using 1:4:30:65 HOAc/H₂O/MeOH/CHCl₃ as the eluant.

The acid chloride was separated from oxalyl chloride by removal of the volatiles *in vacuo* followed by two cycles of dissolution in 2 ml of dry benzene and evaporation. A solution of 60 mg (0.43 mmol) of *p*-nitrophenyl and 60 μ l (0.42 mmol) of Et₃N in 4 ml of CH₂Cl₂ was added to a 1-ml CH₂Cl₂ solution of the acid chloride. If necessary, additional phenol and amine were added to ensure that an excess of *p*-nitrophenoxide was present. After standing overnight at 20 °C, the volatiles were removed and the residue taken up in 5 ml of 2:1 CHCl₃/MeOH and washed with 4 ml of 0.1 N HCl. The solvent was removed, and the residue containing phospholipids 2, 3, and 5 was chromatographed on silica gel using 10–33% MeOH/CHCl₃ as the eluant to give the desired phospholipid in 80–90% yield from starting material. Phospholipids 1 and particularly 4 underwent hydrolysis on silica gel, florisil, and alumina. A more satisfactory purification procedure for these two lipids entailed repeated leaching with Et₂O until all *p*-nitrophenyl was removed.

Pure samples of 1–5 were obtained after HPLC chromatography using anhydrous MeOH to elute the lipid from a Brownlee C₁₈ column. The *p*-nitrophenyl esters of SIBLINKS 1 and 4 are especially prone to hydrolysis in polar solvents such as alcohols. Storage of these compounds at –10 °C in CHCl₃ or CH₂Cl₂ greatly enhances the

stability. Therefore HPLC-purified SIBLINKS were immediately transferred to CHCl₃ for storage. In an aqueous environment, the stability is enhanced when incorporated into vesicles or Triton micelles. The following are representative spectral data for lipids 2, 3, 4, and 5 containing a decanoyl (C₁₀) chain moiety at the *sn*-1 position; that for 1 was reported previously (7).

2b—¹H NMR (CDCl₃) δ 0.9 (t, 3H), 1.3 (s, 12H), 1.4 (s, 6H), 1.6 (m, 2H), 2.05 (t, 2H), 2.25 (t, 2H), 2.45 (t, 2H), 3.3 (s, 9H), 3.7 (m, 2H), 4.0 (m, 2H), 4.15 (m, 1H), 4.3 (m, 2H), 4.4 (m, 1H), 5.2 (m, 2H), 7.3 (ABq, 2H), 8.25 (ABq, 2H) MS(FAB) *m/e* 675.

Exact mass calculated: 675.3258

Found: 675.3267

3—¹H NMR (CDCl₃) δ 0.9 (t, 3H), 1.25 (s, 6H), 1.3 (s, 12H), 1.6 (m, 2H), 2.25 (t, 2H), 2.55 (s, 2H), 2.75 (s, 2H), 3.4 (s, 9H), 3.75 (m, 2H), 4.0 (m, 2H), 4.15 (m, 1H), 4.3 (m, 2H), 4.4 (m, 1H), 5.25 (m, 1H), 7.3 (ABq, 2H), 8.3 (ABq, 2H) MS(FAB) *m/e* 675.

Exact mass calculated: 675.3258

Found: 675.3245

4a—¹H NMR (CDCl₃) δ 0.9 (t, 3H), 1.3 (s, 12H), 1.6 (m, 2H), 2.25 (t, 2H), 2.75 (t, 2H), 2.9 (t, 2H), 3.3 (s, 9H), 3.75 (m, 2H), 4.0 (m, 2H), 4.15 (m, 1H), 4.3 (m, 2H), 4.4 (m, 1H), 5.25 (m, 1H), 7.3 (ABq, 2H), 8.3 (ABq, 2H) MS(FAB) *m/e* 633.

Exact mass calculated: 633.2788

Found: 633.2800

5—¹H NMR (CDCl₃) δ 0.9 (t, 3H), 1.3 (s, 12H), 1.42 (s, 3H), 1.45 (s, 3H), 1.6 (m, 2H), 2.25 (t, 2H), 2.8 (s, 2H), 3.3 (s, 9H), 3.75 (m, 2H), 4.0 (m, 2H), 4.15 (m, 1H), 4.35 (m, 2H), 4.4 (m, 1H), 5.25 (m, 2H), 7.3 (ABq, 2H), 8.3 (ABq, 2H) MS(FAB) *m/e* 661.

Exact mass calculated: 661.3101

Found: 661.3079

Preincubation Conditions

Unless otherwise stated, lipids 1–5 were purified by HPLC. SIBLINKS vesicles were prepared by sonicating 2–4 mg of phospholipid in 1 ml of 100 mM KCl using four 30-s pulses of a MSE 100-watt sonicator. The resulting solution was centrifuged (25 min at 9,500 $\times$ *g*, 4 °C). The vesicles after separation from the pellet were analyzed spectrophotometrically for free *p*-nitrophenol and *p*-nitrophenyl ester

by measuring the OD at 400 nm at first pH 8 and then pH 12 using 1.5 and 1.8×10^4 , respectively, as the extinction coefficient for *p*-nitrophenoxide. To minimize slow hydrolysis of the aryl ester, the vesicles were stored at 4 °C. Further purification of the above vesicle preparation by ultracentrifugation (4 h at $50,000 \times g$, 4 °C) did not significantly alter inhibition time courses.

Standard preincubation conditions utilized 100 μ M SIBLINKS as sonicated vesicles with PLA₂ in 20 mM Tris-HCl (pH 8.0), 10 mM CaCl₂, and 100 mM KCl at 20 °C. The same conditions were used for preincubation with mixed micelles except for the presence of Triton X-100. The concentration of *N. naja naja* PLA₂ was 0.37 μ M (5 μ g/ml). The preincubation concentrations of the PLA₂s from other sources ranged from 5 to 20 μ g/ml.

Residual Activity Determination

To determine the amount of residual enzyme activity remaining after preincubation, a titrametric assay was employed in which 5–20 μ l of the preincubation solution was added to 1.7 ml of the substrate solution containing 10 mM CaCl₂ at 40 °C. Thus, the preincubation mixture was diluted so activity toward residual SIBLINKS was negligible, and activity toward added substrate was maximized. PLA₂s obtained from *N. naja naja* and bee venoms were routinely assayed with 5 mM DPPC in mixed micelles with 20 mM Triton X-100. The assay utilized 50 ng of protein (11). Porcine pancreatic PLA₂ was assayed titrimetrically with 100 ng of protein/assay and an egg lecithin/sodium deoxycholate mixture as substrate (12). *C. adamanteus* and *C. atrox* PLA₂s were assayed titrimetrically using 70 ng of protein/assay with the same DPPC/Triton X-100 assay described above for *N. naja naja* except for the addition of 1 mg/ml of bovine serum albumin (13). Residual activities (percent) were calculated from the mol of base consumed titrimetrically relative to a PLA₂ solution preincubated under the same conditions in the absence of the SIBLINKS.

SIBLINKS Inhibition

Typically, the reaction was simultaneously monitored spectrophotometrically and titrimetrically during preincubation of PLA₂ with SIBLINKS vesicles. Continual spectral monitoring of *p*-nitrophenoxide release (400 nm; $\epsilon_{\text{max}} = 1.5 \times 10^4$ at pH 8) revealed the extent of hydrolysis (number of mol) of the inhibitor SIBLINKS as a function of time. The initial rates of PLA₂-catalyzed hydrolysis of SIBLINKS were based on the *p*-nitrophenoxide produced during the first 1% or less of the reaction, assuming instantaneous cyclization, as assumption that is not valid for substrates 1a and 1b. Titrametric assays, as described above, revealed the amount of residual enzymatic activity from which the number of mol of PLA₂ inactivated could be calculated. To ascertain maximum inhibition, preincubations were continued for 24 h or less if no further loss of activity with time occurred within experimental error. The partition ratio (*P*) of the number of mol of SIBLINKS hydrolyzed per mol of enzyme inactivated was calculated using the above independent determinations of mol of *p*-nitrophenol released and mol of PLA₂ inactivated. *P* values were calculated several times during the time course of inactivation studies. *P* was essentially constant between 20 and 70% inactivation (see Table III under "Results"). The value of *P* reported in Tables I and II for a specific SIBLINKS is an average of the three or four values measured during the determination of each inactivation time course.

Cyclic Anhydride Inhibition

The following procedure was utilized to measure PLA₂ inhibition by cyclic anhydrides. A CH₂Cl₂ solution of the anhydride was evaporated under an N₂ stream. Immediately, 400 μ l of the appropriate PLA₂ in 20 mM Tris-HCl (pH 8.0), 10 mM CaCl₂, and 100 mM KCl at 20 °C was added followed by vortexing to ensure rapid mixing. Aliquots were assayed titrimetrically after 5 min; no further change in activity was observed after an additional 1–2 h.

Preparative Scale Inhibition of PLA₂

When large amounts of PLA₂ inhibited by cyclic anhydride were needed, the following procedure was employed. To 0.5–0.9 ml of buffer (0.7 M Tris-HCl, pH 8.0) containing 0.15–0.2 mg of PLA₂ was added 6.5–9 mg of cyclic anhydride 12. After vortexing and standing for 1 h, a second portion of anhydride was added to ensure that maximum inhibition was obtained. The suspension was applied to a Pharmacia LKB Biotechnology Inc. G-25 PD-10 column that was preequilibrated

with buffer (10 mM K₂HPO₄, pH 8.0), and the protein was eluted with the same buffer. Similar conditions were employed to obtain PLA₂ inhibited by SIBLINKS except for a 20-h preincubation with 500 μ M SIBLINKS vesicles and the inclusion of 10 mM CaCl₂ and 100 mM NaCl. The 0.5-ml chromatographic fractions were analyzed for protein and *p*-nitrophenyl ester; only protein fractions free of the SIBLINKS were utilized.

Hydroxylamine Studies

The following procedure was employed for hydroxylamine treatment. The appropriate amount of a freshly prepared stock solution of 50 mM NH₂OH HCl in 1 M Tris-HCl (pH 8.0) was added to the PLA₂ solution to a final concentration of 5 mM. After vortexing and before assaying titrimetrically for PLA₂ activity, the solution was allowed to stand 1–2 h at 20 °C. This reaction was performed in a closed vial to minimize NH₂OH oxidation.

RESULTS

Dependence of Inhibition on Acyl Chain Length—One of the parameters that could affect SIBLINKS inhibition is the acyl chain length at the *sn*-1 position. Since initial studies were all carried out on decanoyl *sn*-1 chains (8), the effect of increasing the *sn*-1 acyl chain length from 8 to 18 carbons was determined for the 2,2-dimethylglutarate series 2a–2f. As shown in Table I the initial rate of PLA₂ activity toward these SIBLINKS is rather insensitive to the *sn*-1 acyl chain length whereas *P* appears more sensitive to chain length. A longer acyl group enhanced the hydrolysis rate of the succinate ester 4b over 4a with a small increase in *P*. Thus, initial rates and *P* values do vary somewhat with chain length, but since subtle packing differences in the phospholipid vesicles could contribute, it is difficult to attribute specific effects on inhibition efficiency to chain length.

Inhibition of PLA₂ from Various Sources—The susceptibility of different PLA₂s to inhibition by 2b is shown in Fig. 2; in Table II the inhibition is analyzed for two representative SIBLINKS 2b and 4b. In all cases, *p*-nitrophenoxide was released upon preincubation of the PLA₂ with these SIBLINKS. As measured by the rate of *p*-nitrophenoxide release from the SIBLINKS, the *N. naja naja* PLA₂ hydrolyzed the SIBLINKS faster than did the other four PLA₂s. The inhibition efficiency that is measured by *P* and the residual activity tabulated in Table II varied with the particular PLA₂. Under the conditions employed, *N. naja naja* PLA₂ was 90%

TABLE I
Rate of *N. naja naja* phospholipase A₂-catalyzed hydrolysis of various SIBLINKS and the residual activity and partition ratio (*P*)

SIBLINKS	<i>sn</i> -1 chain length	Rate $\mu\text{mol min}^{-1} \text{mg}^{-1}$	Residual activity %	<i>P</i> mol/mol
Glutarate				
1a	8	40 ^a	100	
1b	10	155 ^a	100	
Dimethylglutarate				
2a	8	0.11	15	52
2b	10	0.11	10	19
2c	12	0.14	18	32
2d	14	0.13		46
2e	16	0.15	15	45
2f	18	0.11		58
Dimethylglutarate 3	10	0.002	25	9
Succinate				
4a	10	0.6	10	35
4b	16	1.9	8	60
Dimethylsuccinate 5	10	0.005	25	11

^a 7.4 nM PLA₂ and 100 μ M SIBLINKS as sonicated vesicles at 40 °C. All other experiments were carried out on 370 nM PLA₂ and 100 μ M SIBLINKS as sonicated vesicles at 20 °C (standard conditions). Data on decanoyl *sn*-1 chains are from (8).

FIG. 2. Inhibition of activity of *N. naja naja* (○), *C. atrox* (●), *C. adamanteus* (□), porcine pancreatic (■), and bee venom (△) PLA₂ as a function of the preincubation time with 100 μM SIBLINKS 2b in vesicles. Preincubation of a ~270:1 (mol/mol) mixture of 2b to PLA₂ was at 20 °C in buffer (20 mM Tris-HCl, pH 8.0, 10 mM CaCl₂, and 100 mM KCl). The residual activity of the PLA₂ was measured titrimetrically using the appropriate assay for the specific PLA₂ as described under "Experimental Procedures."

TABLE II

Rate of hydrolysis of SIBLINKS 2b and 4b by various phospholipase A₂s and the residual activity and partition ratio (P)

PLA ₂	SIBLINKS	Rate μmol min ⁻¹ mg ⁻¹	Residual activity μmol min ⁻¹ mg ⁻¹	%	P mol/mol
<i>N. naja naja</i>	2b	0.11	122	9	14
Porcine pancreatic	2b	0.027	80	15	>200
<i>C. atrox</i>	2b	0.025	105	4	30
<i>C. adamanteus</i>	2b	0.005	35	7	6
Bee venom	2b	0.028	120	86	200
<i>N. naja naja</i>	4b	1.9	135	10	60
Porcine pancreatic	4b	0.46	220	40	200
<i>C. atrox</i>	4b	1.8	158	6	120
Bee venom	4b		165	120	

TABLE III

Partition ratio (P) as a function of remaining enzymatic activity after preincubation with SIBLINKS 2b

PLA ₂	Residual activity %	P mol/mol
<i>N. naja naja</i>	49	18
	31	19
	23	19
	20	22
	12	60
<i>C. adamanteus</i>	71	6
	30	6
	28	7
	7	12

inhibited upon hydrolyzing 14 SIBLINKS 2b/PLA₂. Preincubation of 2b with PLA₂ from bee venom resulted in a 14% decrease in catalytic activity after 3.5 h. Porcine pancreatic PLA₂ hydrolysis of 2b induced an 85% decrease in catalytic activity over 10 h. Porcine pancreatic and bee venom PLA₂ required processing of hundreds of SIBLINKS before significant inhibition occurred. *C. atrox* and *C. adamanteus*, after a 3-h preincubation, were almost totally inhibited upon processing 30 and 6 molecules of SIBLINKS 2b, respectively.

Table III shows the dependence on enzymatic activity of P, measured for *N. naja naja* and *C. adamanteus* PLA₂ during preincubation with 2b. For a given SIBLINKS, P was found to be essentially constant until about 75% inhibition. Although enzymatic activity continued to decrease slowly to ~10%, the failure to achieve complete inhibition was reflected in increasing values for P.

Control Studies for Inhibition—The inhibition of PLA₂ by the bulk addition of cyclic anhydride was determined to

exclude the possibility that the inhibition observed is due to free cyclic anhydride generated in the course of enzymatic processing of the SIBLINKS. Addition of 0.37 μM *N. naja naja* PLA₂ to a 3 mM solution of glutaric anhydrides 11, 12, or 13 or succinic anhydride 14 produced a maximum inhibition of ~50%; repeated treatments did not alter this value significantly. No inhibition was observed at 0.3 mM anhydride. When treated with a similar 8,000-fold excess of the 2,2-dimethylglutaric anhydride 12, porcine pancreatic, *C. atrox*, and *C. adamanteus* PLA₂ were 86, 92, and 95% inhibited, respectively; however, PLA₂ from bee venom was not affected. Thus, all sources of PLA₂ were not equally sensitive to cyclic anhydrides, but for those that were all required far higher concentrations to achieve inhibition than are generated during SIBLINKS preincubation conditions.

Substrate Specificity of the Residual Activity of PLA₂ Inhibited by SIBLINKS—*N. naja naja* PLA₂ and *C. adamanteus* PLA₂ were assayed (Table IV) with both monomeric (DCPC) and micellar (DPPC) substrates after preincubation with SIBLINKS 2b or with 2,2-dimethylglutaric anhydride 12. Prior treatment of *N. naja naja* with free cyclic anhydride 12 induced a 55% reduction in the ability to hydrolyze DPPC Triton micelles compared with a 20% inhibition for monomeric DCPC. In contrast, preincubation of the same enzyme with SIBLINKS 2b or 4b equally inhibited (~90%) the hydrolysis of both monomeric and micellar phospholipids. Similar results were obtained with *C. adamanteus*.

Modified Residue—In an attempt to determine which residue was modified by SIBLINKS, reversibility was investigated. Inhibition arising from preincubation of *N. naja naja* PLA₂ with 2b, 2d, 4a, or 4b for 24 h was not reversed upon separation of the inhibited PLA₂ from the inhibitor and reaction products by chromatography on a Pharmacia G-25 PD-10 column. Enzymatic activity of the chromatographed PLA₂ was also not restored upon standing 24 h at 20 °C, indicating that inhibition is not due to an unstable acylation product. Identical treatment of *C. adamanteus* PLA₂ after inhibition by 2b also failed to regenerate activity. Tight competitive binding with substrate was not the cause of inhibition since preincubation of *N. naja naja* PLA₂ with 20 μM 2b gave inhibition whereas none was observed upon titrametric assay of control PLA₂ with 5 mM DPPC as substrate in the presence of 20 μM 2b.

Some PLA₂s inhibited by SIBLINKS could be rescued partially by treatment with 5 mM hydroxylamine (Table V). For example, *N. naja naja* PLA₂, possessing ~10% initial activity after preincubation with 2b for 30 min to 24 h, was restored to 80% initial activity. Under identical conditions, *N. naja naja* PLA₂, rendered 90% inactive by preincubation with succinate 4b, recovered essentially full activity. Hydroxylamine treatment increased the activity of inhibited bee

TABLE IV

Comparison of residual activities toward various substrates of phospholipase A₂ inactivated by SIBLINKS or anhydrides

PLA ₂	Inhibitor	Residual activity			
		DPPC		DCPC ^a	
		μmol min ⁻¹ mg ⁻¹	%	μmol min ⁻¹ mg ⁻¹	%
<i>N. naja naja</i>	12 ^b	540	45	215	80
	4b	120	10	29	12
<i>C. adamanteus</i>	2b	132	11	37	15
	12 ^b	22	4	3.3	34
	2b ^b	6	1	0	0

^a 5 mM DCPC with 250 ng of PLA₂ (*N. naja naja*) and 10,000 ng of PLA₂ (*C. adamanteus*).

^b Preparative scale procedures were employed to inhibit PLA₂.

TABLE V
 Hydroxylamine restoration of activity of inactivated phospholipase A₂ after SIBLINKS inhibition

PLA ₂	Inhibitor ^a	Preincubation time min	Residual activity			
			Before NH ₂ OH		After NH ₂ OH	
			$\mu\text{mol min}^{-1} \text{mg}^{-1}$	%	$\mu\text{mol min}^{-1} \text{mg}^{-1}$	%
<i>N. naja naja</i>	12	600	540	45	530	44
	2b	40	250	18	1190	85
	2b	1440	140	10	1105	80
	4b	30	110	8	1340	97
	4b	1440	110	8	1410	102
Bee venom	2b	210	120	86	165	120
<i>C. atrox</i>	2b	1440	105	4	250	10
<i>C. adamanteus</i>	12	400	20	5	24	6
	2b	1440	0	0	26	5
Porcine pancreatic	2b	900	60	11	66	12

^a Concentration was 3 mM for 12 and 100 μM for SIBLINKS 2b and 4b.

venom PLA₂ from 86 to 120%. Hydroxylamine treatment induced little if any change in the activity of porcine pancreatic, *C. atrox*, and *C. adamanteus* PLA₂s inhibited by 2b. No increase in catalytic activity was observed upon hydroxylamine treatment of any of the PLA₂s inhibited by 2,2-dimethylglutaric anhydride 12 in bulk solution.

DISCUSSION

Inhibition by SIBLINKS—All SIBLINKS listed in Table I except for glutarate esters 1 were inhibitors of *N. naja naja* PLA₂ under standard assay conditions. Maximum inhibition of ~90% was achieved after a 0.5–16 h preincubation at 20 °C. Both enzyme inhibition and dye release were Ca²⁺ dependent, and the partition ratio ranged from 10 to 60 for SIBLINKS vesicles. Our preliminary work showed that the SIBLINKS concentration required for maximum inhibition was a function of the PLA₂ concentration and the *P* value of the inhibitor (8). At 40 °C, *P* increased somewhat, possibly reflecting enhanced diffusion of the hydrolysis products 7–10 out of the active site. The physical state of the SIBLINKS also influences the efficiency of inhibition. For example, the *P* values for inhibition in Triton X-100 mixed micelles, are ~7-fold higher than is observed for sonicated vesicles.

We have demonstrated that in bulk solution the hydrolysis products, half-esters 6–10, and the cyclic anhydrides, 11–15, induced detectable inhibition of PLA₂ only at concentrations > 100 times that required for comparable inhibition by the suicide substrate. Consequently, the inhibition of PLA₂ must be due to a reaction with the SIBLINKS hydrolysis products before they have diffused from the active site. This is consistent with mechanism-based inhibition. As outlined in Fig. 1, enzymatic processing should generate the reactive cyclic anhydride that can acylate and inhibit that particular enzyme molecule before being released to bulk solution, except for substrates 1a and 1b, in which the enzymatic product 6 leaves the active site before cyclization (7, 8). This hypothesis is supported by the control studies that show that anhydride at low concentration in bulk solution is not inhibitory, presumably because it reacts with H₂O before nonspecific acylation of the enzyme can occur.

One other possible mechanism for PLA₂ inactivation is for the SIBLINKS to act as direct acylating agents. This process would entail binding to the active site followed by nucleophilic attack of the enzyme on the *p*-nitrophenyl ester. This mechanism can be excluded for the following reasons. (i) Numerous physical organic studies have established that the steric encumbrance of geminal methyl groups α or β to an ester carbonyl retard external nucleophilic attack by a factor of ~40 (14). If the SIBLINKS were direct acylators, the unsub-

stituted glutarate SIBLINKS 1 should have been a better inhibitor than either dimethylglutarates 2 or 3, which is not the case. However, the parent succinate 4 was a stronger inhibitor than the dimethylsuccinate 5, but this may be due to interference of the dimethyl groups with enzyme catalysis. The failure to observe any inhibition with SIBLINKS 1 excludes direct acylation as the cause for PLA₂ inactivation. (ii) No inhibition was observed in the absence of Ca²⁺, indicating that catalytic activity is required for inactivation. In order to exclude the possibility that Ca²⁺ serves only to bind the SIBLINKS to the active site and not to catalyze the reaction, we have substituted 10 mM Ba²⁺ (which competes with Ca²⁺ (15) in order to enhance phospholipid binding (16)) in preincubations of PLA₂ with substrate 1b or inhibitors 2b and 4b (data not shown); in neither case did PLA₂ cause *p*-nitrophenoxide release during preincubation, nor was there any inhibition of the enzyme relative to controls when assayed for residual activity.

Dependence of SIBLINKS Hydrolysis Rate on Linker Structure—The rates, shown in Table I for *N. naja naja* PLA₂-catalyzed hydrolysis of SIBLINKS 1–5, are a function of the structural differences in the dibasic acid linkage incorporated into the *sn*-2 acyl chain. The unsubstituted glutarate 1b was hydrolyzed rapidly, suggesting that the unsubstituted glutaryl chain and phenolic group are a good substrate. Indeed, for *N. naja naja* PLA₂ acting on SIBLINKS 1b as substrate in mixed micelles with Triton X-100, k_{cat}/K_M can be estimated from kinetic data to be about 70 min⁻¹ μM^{-1} (7). This is similar to the k_{cat}/K_M which can be calculated to be about 100 min⁻¹ μM^{-1} for DPPC as substrate under similar experimental conditions (17). Furthermore, because of the relatively slow cyclization rate of 6, the *p*-nitrophenoxide liberated at short measurement times of initial rate conditions does not reflect the full amount of substrate hydrolyzed so that 1b is an even better substrate than this comparison implies. This confirms the similarity of this SIBLINKS substrate to the natural phospholipid substrate. Introduction of geminal methyl groups on either the γ carbon, 2b, or the β carbon, 3, of the glutaryl chain reduced the velocity by 1,400- and 80,000-fold, respectively. The velocity for the succinate ester 4a was reduced 250-fold relative to the glutaryl ester 1b, suggesting that upon removal of one methylene group, the phenyl ester and/or the *sn*-2 acyl chain could not be as optimally accommodated. Geminal β methylation of the succinate chain decreased the velocity of 5 a further 120-fold, analogous to the glutaric series. The time courses for inhibition of *N. naja naja* upon preincubation with vesicles of SIBLINKS 2b, 3, 4a, and 5 reflect these differences in initial velocity (8). Maximum inhibition was obtained with 4a and 2b in ~30 min whereas

16 h was required for 3 and 5, which have similar slow rates in Table I, reflecting the effect of geminal methyl substitution at the β carbon.

The observed sensitivity of velocity to geminal substituents at the β and γ positions was consistent with prior structure/activity studies of Roberts and co-workers (18). They found that *N. naja naja* PLA₂ hydrolyzed phospholipids containing a single methyl branch on either acyl chain at the α , β , or γ position with no reduction in velocity. However, a methyl branch on both acyl chains reduced the velocity 10–70-fold depending on the substitution position; likewise γ geminal methyls induced an 800-fold rate decrease. The decreased hydrolysis of these branched phospholipids was attributed to the inability of the PLA₂ to undergo a conformational change necessary for catalytic activity because of the spatial requirements of the multiple methyl groups.

Effect of Linker Structure on Partition Ratio—Comparison of the *P* values for inhibitors 2b, 3, 4a, and 5, which differ only with respect to the dibasic acid moiety, suggests that inhibition efficiency increases with methyl substitution on the linker. All three lipids with a geminal dimethyl group are more efficient inhibitors (lower *P* value) than the unsubstituted compounds. However, it is clear that the cyclization rates of the hydrolysis products do not quantitatively account for differences in the partition ratio. It is possible that the methyl groups on either the half ester and/or the cyclic anhydride could increase the residence time in the active site because of tighter binding. The net result would be a greater probability of protein acylation which would be reflected in smaller *P* values.

Acylation by Anhydrides—Most PLA₂s were inhibited upon treatment with excess bulk cyclic anhydrides. In the case of *N. naja naja* PLA₂, a prior study had established that excess phthalic anhydride modified lysine residues (19). From trinitrobenzenesulfonic acid analysis, at least four of the seven free amino groups (6 lysine residues and the amino terminus) (20) were acylated at 8 °C, resulting in 70% inhibition. It appears that dimethylglutaric anhydride 12 reacted similarly to acylate surface lysines of all the PLA₂s, accounting for their decreased activity when subjected to high concentrations of cyclic anhydride.

The profiles of residual activity after inactivation defined by utilizing DPPC and DCPC (which forms monomers) as substrate are quite different for *N. naja naja* and *C. adamanteus* PLA₂s treated with dimethylglutaric anhydride versus these enzymes inhibited with SIBLINKS. Preliminary results using 1,2-dilauryl-*sn*-glycero-3-phosphorylethanolamine in mixed micelles with Triton as a substrate indicated a similar difference in residual activity for *N. naja naja* PLA₂ inhibited by SIBLINKS versus cyclic anhydrides. As shown in Table IV, the bulk cyclic anhydride acylation of either *N. naja naja* or *C. adamanteus* PLA₂ resulted in the inhibition of the hydrolysis of interfacial substrates more than monomeric ones. In contrast, the SIBLINKS-inhibited enzymes appeared to have similar reductions in activity toward both interfacial and monomeric substrates.

These results emphasize that the dimethylglutaric anhydride 12 generated upon enzymatic processing of the SIBLINKS acylates a different residue(s) than the residues that are available to the same anhydride free in bulk solution. In addition, the low *P* values observed for these PLA₂s argue for a different very efficient pathway of inactivation. The simplest explanation is that the SIBLINKS are acting as a source of an active site-specific reagent that inhibits the enzyme. In contrast, the bulk anhydride acylation of the accessible surface lysines affects primarily the ability of *N. naja naja* and

C. adamanteus PLA₂s to process DPPC in organized surfaces.

The mechanism by which the catalytic activity for hydrolysis of organized substrates is diminished upon acylation of PLA₂ with bulk anhydrides is unclear. Two possibilities are perturbation of either (i) interfacial binding or (ii) the interfacial activation process. Interfacial binding could be disrupted due to modification of the lysines present on the surface of PLA₂. Presumably, acylation of these sites with a dibasic acid could disrupt binding due to the introduction of a negative charge where a positive charge existed on the native enzyme. An alternative explanation is that dibasic acid acylation of critical lysines disfavors dimer formation required for interfacial activation (16, 21–25) although its necessity has been questioned (26).

Hydroxylamine Reversal—The finding that hydroxylamine can restore the catalytic activity lost as a result of preincubation of *N. naja naja* PLA₂ with SIBLINKS is very intriguing. This result is particularly striking since hydroxylamine had no effect on the catalytic activity of PLA₂ inhibited by bulk 2,2-dimethylglutaric anhydride 12. These results require that the nascent anhydride formed upon enzymatic processing of the SIBLINKS substrate acylate some residue other than lysine (27). Acylated histidines are believed to be hydrolytically unstable (28). The failure to observe gradual restoration of catalytic activity with time after PD-10 column chromatography appears to exclude histidine acylation. Remaining possibilities are tyrosine and serine since hydroxylamine can cleave both serine and tyrosine esters. Tyrosine is the most reasonable possibility since x-ray crystallography shows that the catalytic site of the *N. naja naja* PLA₂ contains tyrosines, but no serines as is the case with other PLA₂s examined.² Tyrosine 52, 69, and 73 are attractive candidates.

The failure of hydroxylamine to restore *C. atrox* and *C. adamanteus* activity despite low *P* values suggests that a different critical amino group in or near the active site may be specifically acylated, perhaps a lysine. For some PLA₂s, SIBLINKS may inhibit in a nonspecific manner by merely providing a source of anhydride for surface lysine acylation. Under these circumstances, *P* should be large, i.e. > 100 and the acylation pattern similar to that obtained with bulk anhydride. The fact that *P* is > 200 for porcine pancreatic PLA₂ and that hydroxylamine induced no change in activity suggests that this may be the case for this enzyme.

Summary—This work demonstrates that (i) SIBLINKS 2, 3, 4, and 5 are mechanism-based inhibitors of PLA₂ from *N. naja naja* since inhibition occurs in a time-dependent irreversible fashion only in the presence of Ca²⁺, and natural substrates protect the enzyme. (ii) SIBLINKS-inactivated PLA₂ possesses reduced capacity to process both micellar and monomeric substrates. (iii) Hydroxylamine reverses the inhibition of *N. naja naja* PLA₂. (iv) Inhibition is very efficient; as few as one in nine SIBLINKS substrates are required for inactivation of *N. naja naja* PLA₂. (v) Inhibition of *N. naja naja* PLA₂ occurs via generation of a cyclic anhydride at the active site and is consistent with acylation of a tyrosine residue. (vi) SIBLINKS-derived cyclic anhydrides inactivate *N. naja naja* PLA₂ by a different mechanism than with preformed bulk cyclic anhydrides since the latter only partially inhibits hydrolysis of micellar substrates, does not inhibit processing of monomeric substrates as efficiently, and no catalytic activity is recovered upon hydroxylamine treatment. (vii) SIBLINKS are also efficient suicide inhibitors for some class II PLA₂s since *C. adamanteus* PLA₂ was inhibited after processing as few as six SIBLINKS. (viii) Hydroxyl-

² D. Fremont, D. Anderson, I. Wilson, N. Xuong, and E. A. Dennis, manuscript in preparation.

amine treatment does not restore activity to all SIBLINKS-inhibited PLA₂s, suggesting that residues other than tyrosine, such as lysine, may also be acylated. Clearly, additional work is required to demonstrate the precise amino acid residues acylated by SIBLINKS in each enzyme.

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REFERENCES

- Dennis, E. A. (1983) in *The Enzymes* (Boyer, P., ed) 3rd Ed., Vol. 16, pp. 307–353, Academic Press, New York
- Waite, M. (1987) *The Phospholipases*, pp. 155–240, Plenum Publishing Corp., New York
- Heinrikson, R. L., Krueger, E. T., and Keim, P. S. (1977) *J. Biol. Chem.* **252**, 4913–4921
- Dennis, E. A., and Davidson, F. F. (1990) *J. Mol. Evol.* **31**, 228–238
- Dennis, E. A. (1987) *Bio/Technology* **5**, 1294–1300
- Mobilio, D., and Marshall, L. A. (1989) *Annu. Rep. Med. Chem.* **24**, 157–166
- Washburn, W. N., and Dennis, E. A. (1990) *J. Am. Chem. Soc.* **112**, 2040–2041
- Washburn, W. N., and Dennis, E. A. (1990) *J. Am. Chem. Soc.* **112**, 2042–2043
- Kirby, A. J. (1980) *Adv. Phys. Org. Chem.* **17**, 183–278
- Hazlett, T. L., and Dennis, E. A. (1985) *Toxicon* **23**, 457–466
- Deems, R. A., and Dennis, E. A. (1981) *Methods Enzymol.* **71**, 703–710
- Nieuwenhuizen, W., Kunze, H., and de Haas, G. H. (1974) *Methods Enzymol.* **32b**, 147–154
- Plückthun, A., and Dennis, E. A. (1985) *J. Biol. Chem.* **260**, 11099–11106
- Taft, R. W. (1956) *Steric Effects in Organic Chemistry* (Newman, M. S., ed) pp. 556–675, John Wiley & Sons, New York
- Roberts, M. F., Deems, R. A., and Dennis, E. A. (1977) *J. Biol. Chem.* **252**, 6011–6017
- Roberts, M. F., Deems, R. A., and Dennis, E. A. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 1950–1954
- Deems, R. A., Eaton, B. R., and Dennis, E. A. (1975) *J. Biol. Chem.* **250**, 9013–9020
- DeBose, C. D., Burns, R. A., Donovan, J. M., and Roberts, M. F. (1985) *Biochemistry* **24**, 1298–1306
- Darke, P. (1982) *Phospholipase A₂ from Cobra Venom: Chemical Modification, Ligand Binding and Self-association Equilibria*. Ph.D thesis, pp 79–81, University of California, San Diego
- Davidson, F. F., and Dennis, E. A. (1990) *Biochim. Biophys. Acta* **1037**, 7–15
- Cho, W., Tomasselli, A. G., Heinrikson, R. L., and Kézdy, F. J. (1988) *J. Biol. Chem.* **263**, 11237–11241
- Tomasselli, A. G., Hui, J., Fisher, J., Zürcher-Neely, H., Reardon, I. M., Oriaku, E., Kézdy, F. J., and Heinrikson, R. L. (1989) *J. Biol. Chem.* **264**, 10041–10047
- Wells, M. A. (1973) *Biochemistry* **12**, 1086–1093
- Heinrikson, R. L. (1982) in *Proteins in Biology and Medicine* (Bradshaw, R. A., Hill, R. L., Tang, J., Liang, C., Tsao, T. C., and Tsou, C. L., eds) pp. 131–152, Academic Press, New York
- Romero, G., Thompson, K., and Biltonen, R. L. (1987) *J. Biol. Chem.* **262**, 13476–13482
- Jain, M. K., and Berg, O. G. (1989) *Biochim. Biophys. Acta* **1002**, 127–156
- Wilson, I. B., Bergmann, F., and Nachmansohn, D. (1950) *J. Biol. Chem.* **186**, 781–790
- Fife, T. H. (1965) *J. Am. Chem. Soc.* **87**, 4597–4600