Accelerated Publications

Inhibition of Mitochondrial Phospholipase A_2 by Mono- and Dilysocardiolipin†

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ABSTRACT: Phospholipase A_2 extracted from the acetone powder of previously frozen rat liver mitochondria is strongly inhibited compared to the activity manifest before acetone powder preparation. Activity is substantially recovered upon partial purification of the enzyme by gel filtration chromatography. Inhibitor activity elutes in the void volume from the column and is obtained in the chloroform layer when void volume fractions are subjected to a Folch extraction. Structural studies support the inhibitor being monolysocardiolipin. Under the assay conditions employed, 1 molecule of the inhibitor per 5000 substrate molecules or 40 nM on a nominal concentration basis is I_{50} for the mitochondrial enzyme. The agent is similarly effective against pancreatic and snake venom phospholipases A_2. Monolysocardiolipin and dilysocardiolipin prepared enzymatically from bovine heart cardiolipin are less potent than the material arising from rat liver cardiolipin by factors of 10- and 30-fold, respectively, yet are still highly potent compared to the other known inhibitors of this enzyme. Differences in acyl group composition, in the degree of acyl group oxidation, or in structural isomerism between the *sn*-1 and *sn*-2 positions of the lyso compounds may account for the difference in potency between the materials derived from rat liver and bovine heart.

Rat liver mitochondria contain phospholipase A_2 activity associated with their inner and outer membranes (Waite, 1969; Nachbaur et al., 1972; Zurini et al., 1981). Recently, highly purified preparations of this enzyme have been obtained (De Winter et al., 1982; Natori et al., 1983; Aarsman et al., 1984), and some properties of purified protein have been described (De Winter et al., 1984, 1987; Lenting et al., 1987).

The physiological roles of this enzyme are not well established. We have emphasized that the inner membrane component of phospholipase A_2 activity, together with inner membrane acyl coenzyme A–lysophospholipid acyltransferase activity (Nachbaur et al., 1969) and matrix space acyl coenzyme A synthetases (Galzigna et al., 1967; Groot et al., 1974), potentially allows cyclic deacylation and reacylation of inner membrane phospholipids (Pfeiffer et al., 1979). This cycle is proposed to establish and regulate inner membrane steady-state levels of lysophospholipids and free fatty acids and thereby to influence the permeability properties of the membrane [see Broekemeier et al. (1985) and references cited therein]. Thus, mitochondrial phospholipase A_2 may alter the efficiency of energy-linked processes, allow Ca^{2+} release from mitochondria, and otherwise affect the functional properties of these organelles (Broekemeier et al., 1985).

Endogenous phospholipase A_2 has been known for some time to be responsible for the aging of isolated mitochondrial preparations (Scarpa & Lindsay, 1972). It seems probable that a similar process, occurring in vivo, produces the damage to mitochondrial structure and function that occurs in cells injured by ischemia [e.g., Okayasu et al. (1985) and Starke et al. (1986)] or other means. Additional pathological conditions, including Reye's syndrome [e.g., Aprille (1977) and Martens et al. (1986)], muscle wasting diseases [e.g., Wrogemann et al. (1973)], and malignant hyperthermia [e.g., Cheah and Cheah (1981)], also result in abnormal appearing and functioning mitochondria. Since the abnormalities observed are similar to those seen in mitochondria subjected to attack by endogenous phospholipase A_2 (Broekemeier et al., 1985), it is possible that this enzyme is involved in the etiology of those disease states.

Further insights into the physiological functions and pathological roles of mitochondrial phospholipase A_2 can be expected from a further understanding of how the enzyme is regulated. In that context, we report here that mono- and dilysocardiolipin are potent inhibitors of the enzyme.

Experimental Procedures

Liver mitochondria were prepared from male Sprague-Dawley rats as described previously (Broekemeier et al., 1985). The preparations were stored at −80 °C prior to their use as a source of phospholipase A_2 or phospholipase A_2 inhibitor.

The assay of phospholipase A_2 was conducted at 37 °C, essentially as described by De Winter et al. (1982). The 0.5-mL incubations contained 10 mM $CaCl_2$, 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 8.5), and 100 nmol of 1-acyl-2-[1-^{14}C]linoleoylphosphatidylethanolamine (sp act. 300 dpm/nmol), which was dispersed in the aqueous medium by sonication. In most cases, when an inhibitor of phospholipase A_2 was present, the media also contained Triton X-100 at a one-to-one mole ratio with respect to phosphatidylethanolamine. The presence of this detergent improved assay precision, presumably by facilitating the mixing of substrate and inhibitor. After preincubation of the substrate, inhibitor, and Triton X-100 for approximately 30 min, reactions were initiated by the addition of an appropriate amount of protein and were terminated 30 min later by performing a Dole extraction (Van den Bosch et al., 1974). Free fatty acid was then isolated from the heptane phase by chromatography on silica gel minicolumns and was quantitated by scintillation counting (De Winter et al., 1982).

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Table I: Phospholipase A_2 Purification Summary[a]

fraction	protein recovery (mg)	activity recovery (milliunits/min)	SA (milliunits/mg)	x-fold purification
mitochondria	1670	1607	1.0	1
acetone powder extract	1310	78	0.1	0.1
AcA-54 gel filtration column	1.2	880	746	808
ultrafiltrate	1.7	234	136	144
dialysate	1.6	165	109	114
affinity column fractions 22–28	0.03	131	4503	4740
fraction 23 (max)	0.005	39	7923	8340

[a] The enzyme was assayed, solubilized, and purified by employing literature procedures described under Experimental Procedures.

To solubilize mitochondrial phospholipase A_2, acetone powder, prepared from isolated mitochondria, was extracted with 1.0 M KCl, 50 μM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4), 10 mM β-mercaptoethanol, and 10% glycerol (De Winter et al., 1982). The enzyme was then purified approximately 8000-fold by gel filtration chromatography on Ultrogel AcA-54 (De Winter et al., 1982), followed by affinity chromatography on the resin as described by Aarsman et al. (1984). Highly purified porcine pancreatic phospholipase A_2 was a generous gift from Dr. Howard L. Brockman. The snake venom enzyme (*Naja naja*) was purchased from Sigma.

To determine the fatty acid content and composition of phospholipase A_2 inhibitors, the purified (see Results) material was transesterified in basic methanol. Fatty acid methyl esters were then separated and quantitated by capillary gas–liquid chromatography (Holman et al., 1983). Heptadecanoic acid (17:0) was present throughout the procedure as an internal standard to allow correction of the data for incomplete recovery. To determine the inhibitor glycerol content, the purified material was subjected to acid hydrolysis (Kates, 1972). Glycerol was then quantitated in aliquots of the hydrolysate by a sensitive enzymatic procedure that is based on the action of glycerol-3-phosphate oxidase and detection of its product, H_2O_2 (Esders & Goodhue, 1976). Phosphate was determined by the method of Bartlett (1959).

Results

The summary of a typical purification run on the mitochondrial phospholipase A_2 is shown in Table I. The results are similar to those described by Van den Bosch and coworkers (De Winter et al., 1982; Aarsman et al., 1984) except that we find a poor recovery (5%) of total activity in the acetone powder extracts. Subjecting the extract to gel filtration chromatography, however, increases the recovered activity by approximately 10-fold. These observations suggest the presence of a phospholipase A_2 inhibitor in the acetone powder extract and its separation from the enzyme during gel filtration.

Examination of the gel filtration column fractions for inhibitor activity showed that the agent responsible was located in the void volume (data not shown). Most of the inhibitor activity is recovered in the chloroform layer of the Folch extract from the void volume fractions (Figure 1), indicating that it is lipid in nature. Several components can be separated from the Folch extract by thin-layer chromatography (Figure 2, lane 2). When the individual fractions are collected, eluted, and tested as inhibitors of the purified enzyme, activity is associated with the spots labeled B, C, and E, all of which contain phosphorus. Component C is the most potent on a phosphorus basis and was, therefore, selected for the structural studies.

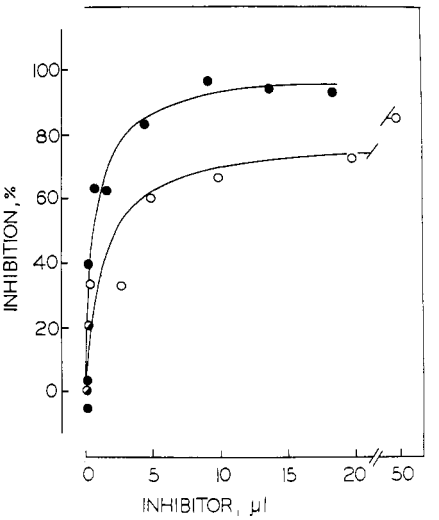

FIGURE 1: Recovery of phospholipase A_2 inhibitor activity from the Ultrogel AcA-54 column void volume fraction. Phospholipase A_2 assays were conducted in duplicate as described under Experimental Procedures and in De Winter et al. (1984) by utilizing the affinity column purified enzyme (see Table I). (●) Aliquots (1–20 μL) of the gel filtration column void volume fraction were added to the assay medium prior to the addition of enzyme. (○) Experiments were conducted as described for the closed circles, except that aliquots of a Folch extract preparation obtained from the column void volume fraction were substituted for the fraction per se. To obtain the extract preparation, the Folch lower phase (lipid-containing phase) was taken to dryness in a N_2 stream and then redissolved in chloroform/methanol (2:1). Aliquots of this solution, representing various volumes of the original Folch lower phase, were again taken to dryness and were resuspended in 0.5 mL of 10 mM Triton X-100. The resultant preparations were sonicated briefly and then 10 μL was taken for addition to the phospholipase A_2 assay mixtures (0.5-mL total volume per assay). This procedure yielded a final Triton X-100 concentration of 200 μM, regardless of the inhibitor concentration, and allowed the recovery of inhibitor activity from the void volume fraction to be assessed on a volume equivalent basis. At the Triton X-100 concentration employed, stimulation of control phospholipase A_2 activity by the detergent was less than 20%. The absolute amount of substrate hydrolysis was always less than 15% of the total.

Table II: Structural Components of the Purified Phospholipase A_2 Inhibitor[a]

component	mol/mol of phosphate
phosphate	1.00 ± 0.06 (n = 4)
glycerol	1.30 ± 0.02 (n = 5)
total acyl groups[b]	1.28 (n = 1)

[a] The determinations of phosphate, glycerol, and acyl group content of the purified inhibitor were conducted as described under Experimental Procedures. Values for phosphate and glycerol are given as means ± the standard deviation for the number of determinations specified. [b] Sixty-five percent of the total acyl groups were linoleic acid (18:2).

By use of preparative thin-layer chromatography, 300 μg of the material represented by spot C was accumulated. Figure 2, lane 3, shows that the accumulated material gives a single spot on analytical thin-layer chromatography, which migrates to the same position as spot C from the gel filtration column void volume extract. This material also gives a single spot when chromatographed on silica gel H containing 7.5% magnesium acetate and developed with chloroform/methanol/water/ammonia (65:35:5:1) or with chloroform/methanol/acetone/acetic acid/water (30:10:40:7:5) (data not shown).

The compositional properties of the isolated inhibitor are listed in Table II. The fact that the agent is a phospholipid that contains a large percentage content of linoleic acid (18:2)

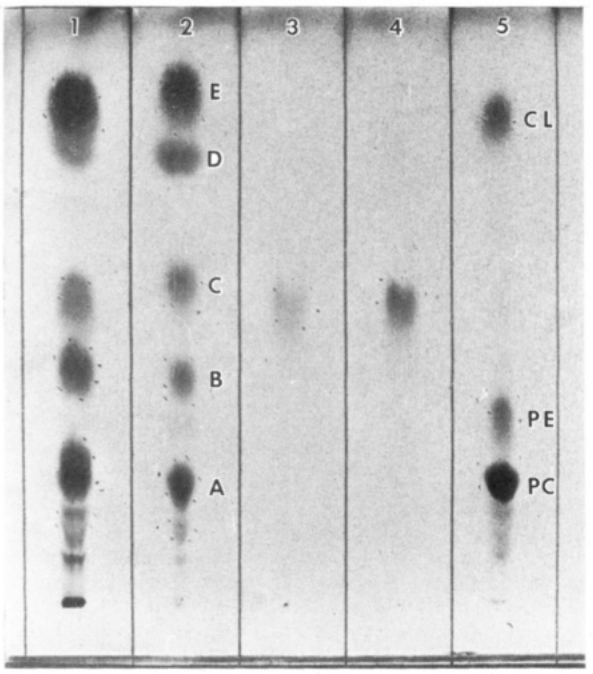

FIGURE 2: Thin-layer chromatography of lipid fractions. The silica gel H plate (0.5 mm) was developed with chloroform/methanol/water (65:25:4) and was subsequently charred. Lanes 1 and 2 are samples from Folch extracts of the acetone powder pellet (material not solubilized during extraction of phospholipase A_2 from mitochondrial acetone powder) and the gel filtration column void volume, respectively. Lanes 3 and 4 are the purified inhibitor and authentic bovine heart monolysocardiolipin, respectively. In lane 5, spots PC, PE, and CL are authentic phosphatidylcholine, phosphatidylethanolamine, and cardiolipin, respectively.

among the acyl groups suggests that it is a precursor of or a product derived from cardiolipin since, in rat liver mitochondria, only this phospholipid class contains that high an amount of linoleic acid (Pfeiffer et al., 1979). Phosphorus, glycerol, and total acyl groups were found at mole ratios of approximating 1:1.3:1.3 (Table II). These data are most consistent with the inhibitor being monolysocardiolipin, as were infrared spectra (not shown) of the isolated material. When authentic monolysocardiolipin was prepared from bovine heart cardiolipin by the method of Eichberg (1974), it was found to comigrate with the isolated inhibitor in all three solvent systems described in the preceding paragraph (e.g., Figure 2, compare lanes 3 and 4). Nevertheless, as shown in Figure 3, the authentic material is less potent than the purified inhibitor by a factor of approximately 10.

Dilysocardiolipin, also obtained by the Eichberg procedure (1974), was less potent than the authentic monolyso species by a factor of 3 (data not shown). Under the chromatography conditions employed in Figure 2, dilysocardiolipin migrates in the area of spot B whereas cardiolipin migrates in the area of spot E (lane 2). It is probable, therefore, that these lipid classes are responsible for the lesser inhibitory activity of components B and E, noted above. In the case of cardiolipin, inhibition may be a property of the lipid per se or it may be converted to mono- and dilyso derivatives during assay by action of the phospholipase A_2 itself (see Discussion).

A final point of interest regarding the isolated monolyso inhibitor is illustrated in Table III. These data show that the purified agent is essentially as effective against snake venom and pancreatic phospholipases A_2 as it is against the mitochondrial enzyme.

Discussion

According to the data in Figure 3, only 1 molecule of the

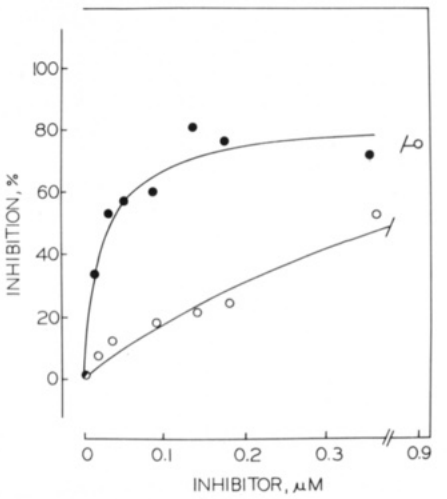

FIGURE 3: Comparison of apparent and authentic monolysocardiolipin as inhibitors of mitochondrial phospholipase A_2. Assays were conducted as described in the legend to Figure 1, open circles, except that purified component C (see Figure 2, lane 2) (●) or authentic bovine heart monolysocardiolipin (○) was substituted for the Folch extract lower phase as sources of enzyme inhibitor.

Table III: Effect of Purified Inhibitor on Activity of Phospholipase A_2 from Selected Sources[a]

enzyme source	% inhibition		
	0.02 μM	0.10 μM	0.23 μM
rat liver mitochondria			
(1) 800-fold purified	23	52	63
(2) 8000-fold purified	33	58	72
porcine pancreas	39	64	80
snake venom (*Naja naja*)		68	45

[a] Assays were conducted as described in the legends to Figures 1 and 3. The 800- and 8000-fold purified mitochondrial phospholipase refers to material obtained from the gel filtration and affinity chromatography columns, respectively (see Table I). Values are the means of duplicate determinations.

purified inhibitor per 5000 phosphatidylethanolamine substrate molecules (40 nM on a nominal concentration basis) is required to reduce the activity of mitochondrial phospholipase A_2 by 50%. This agent is therefore 2–3 orders of magnitude more potent than are the known inhibitors of this enzyme [see Broekemerier et al. (1985)] and, as shown in Table III, is similarly potent against snake venom and pancreatic phospholipases A_2. The mode of inhibition cannot be established from the present data; however, in view of the high potency, it seems probable that inhibition results from a direct interaction with the enzyme as opposed to an effect of the inhibitor on the physical state of the substrate.

Mono- and dilysocardiolipin prepared enzymatically from bovine heart cardiolipin, while being 10- and 30-fold less potent, respectively, than the material isolated from rat liver, are still very effective inhibitors when compared to the previously known compounds [see Broekemeier et al. (1985) and references cited therein]. The evidence that the isolated material is monolysocardiolipin includes the fact that it contains a high percentage content of linoleic acid and arises in significant quantities from mitochondrial phospholipids, its behavior in several thin-layer chromatography systems, infrared spectra, and the structural data presented in Table II. From Table II, the observed ratios of glycerol and acyl chains to phosphate are slightly lower than the values of 1.5:1 expected for monolysocardiolipin. Regarding the glycerol value, Courtade et al. (1967) reported that acid hydrolysis of cardiolipin normally results in a 15–20% degradation of the product glycerol and can, therefore, result in an underestimation of glycerols per

phosphate to that degree. In the present data, the glycerol content is lower than expected by 15%, which can be explained by those earlier findings. The 15% low value for the acyl chain per phosphate ratio may also be a result of degradation during the analysis or from an incomplete reaction of the starting material.

The potency difference between the two samples in Figure 3 could arise from variation in acyl group composition, from varying extents of acyl group oxidation, from a structural isomerism in the position of the missing acyl chain, or from a combination of these variations. Work is now in progress to determine what effects such compositional differences have on the potency of monolysocardiolipin as an inhibitor of phospholipase A_2.

The present findings are of considerable potential interest physiologically and pathologically. Assuming that cardiolipin degradation products are effective inhibitors when the enzyme is acting against bilayer phase phospholipids, these agents could severely limit degradation of the inner mitochondrial membrane by the associated enzyme. Indeed, De Winter et al. (1987) have noted that the enzyme does not readily degrade the total mitochondrial content of phosphatidylethanolamine or phosphatidylcholine and that it has some activity against cardiolipin. Inhibition of the enzyme by accumulating monolysocardiolipid may explain their finding. Limiting phospholipid degradation would favor reversal of the permeability increase produced by phospholipase A_2 activity and could be a factor in resisting the loss of mitochondrial function in injured cells.

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