The Effect of Resorcinolic Lipids on Phospholipid Hydrolysis by Phospholipase A_2

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Phenolic Lipids, Resorcinolic Lipids, Alk(en)ylresorcinols, Bilayer, Phospholipase A_2 Inhibition

The effects of resorcinolic lipids (5-*n*-alk(en)ylresorcinols) isolated from cereal grains on the phospholipase A_2 catalyzed hydrolysis of phospholipid vesicles were examined. Studied compounds inhibited the apparent enzyme activity at the molar fraction in the membrane as low as 0.025, which is equivalent to the concentration of 3 μM. This effect was visualized by dramatic increase (over ten-fold) of the latency period of the reaction progress. This makes resorcinolic lipids one of the most potent inhibitors of phospholipase A_2 among already studied compounds. Highest inhibitory activities were shown for dienoic and monoenoic homologs of 17 carbon atoms in the aliphatic chain. Both saturation of the chain and the increase of its length reduced inhibitory properties of resorcinolic lipids. The data suggest that the compounds studied in this paper like other known amphiphilic inhibitors of phospholipase A_2 owe most of their effects to the ability to modify the quality of the substrate interface. These are the alteration of the enzyme binding, velocity of the formation and redistribution of the products. However part of the effect seems to be attributed to direct interaction and modification of enzyme properties by alk(en)ylresorcinols.

Introduction

Resorcinolic lipids, amphiphilic compounds present in numerous plant materials [1] were also demonstrated in cereal grains [2–4]. The biological role of these compounds, particularly in the nutritional effects of cereal grains and cereal grain-derived products (*e.g.* bran products) is not known. Previous studies showed that resorcinolic lipids isolated from rye grains strongly affect structure and function of biomembranes and phospholipid bilayers [5–8]. Most recent data indicate that the alk(en)ylresorcinols also protect unsaturated lipids against oxidative processes [9]. The enzymatic conversion of arachidonic acid, one of the essential unsaturated fatty acids, into biologically active eicosanoids that control numerous pathophysiological responses [*e.g.* 10] is under absolute control by phospholipases and stimulatory and inhibitory regulatory control by peroxides within the membrane. Since phospholipases act on the substrate which is present as a bilayer membrane it was interesting to examine the effect of resorcinolic lipids on the activity of these enzymes.

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In this paper the effects of various resorcinolic lipids homologs on the action of phospholipase A_2 on liposome membrane are presented. It is also shown that even fine changes of bilayer structure upon interaction with small amounts of additives can be demonstrated by following the changes of phospholipase A_2 kinetics.

Materials and Methods

The 5-*n*-alk(en)ylresorcinol homologs were isolated from rye grains using the procedure described elsewhere [11]. The concentration of the compounds was determined colorimetrically [12]. For the study 2 mM methanolic stock solutions of each homolog were used.

Phospholipase A_2 activity was studied by the continuous titration of fatty acid protons released during the hydrolysis of liposome membrane phospholipids [13]. The substrate (dimyristoylphosphatidylcholine (Avanti), 10 mg/ml) was prepared in 0.1 M KCl–10 mM $CaCl_2$, sonicated 15 min in the bath-type sonicator and kept above lipid transition temperature (56 °C). The time-course of hydrolysis of vesicles by pig pancreatic phospholipase A_2 (Sigma) was followed in the presence of Ca^{2+} (10 mM) at pH 8.0 and temperature 30 °C unless stated otherwise. For the reac-

tion 40 µl of vesicles and 1 µg of phospholipase were used. The resorcinolic lipid homologs were injected into 4 ml of reaction mixture after the substrate and allowed to interact with the vesicles for at least 1 min. The reaction was started by the injection of aqueous solution of the enzyme. The decrease of the initial value of the latency period (τ) to several minutes was achieved by the injection of aqueous solution of myristoyllysolecithin prior the enzyme according to the data of Jain and De Haas [14].

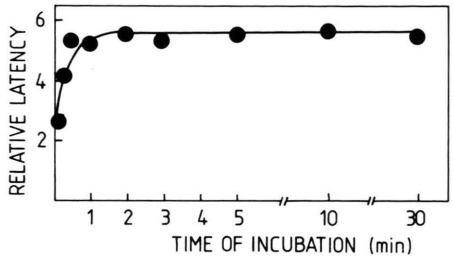

Fig. 2. The effect of incubation of substrate vesicles with 5-*n*-heptadecenylresorcinol ($X_i = 0.03$) on the latency period of the reaction progress curve for the hydrolysis of dimyristoylphosphatidylcholine vesicles by pig pancreatic phospholipase A_2 at 25 °C.

Results and Discussion

The reaction progress curves for the hydrolysis of phospholipid bilayer by phospholipase A_2 are complex, showing several already described steps [15, 16]. As shown in Fig. 1 externally added 5-*n*-heptadec(en)yl resorcinols inhibit the reaction by increase of the latency phase. This phase is reflecting the period of enzyme binding to the substrate and formation within the bilayer of the critical mole fraction of the products, necessary for the initiation of the next, steady-state step [13, 16, 17]. The effect of incubation of substrate (phospholipid vesicles) with constant amounts of studied compounds for various periods of time upon the values of the latency phase gives some information concerning the binding of alk(en)ylresorcinols to the substrate interface. This process, as shown in Fig. 2, is fast and already after 30 sec of the incubation of the vesicles with resorcinolic lipid the latency period reaches its maximal value.

Phospholipase A_2 binding to the substrate interface is sensitive to the type of phase separation induced thermally as well as with the use of various additives [18]. The most pronounced binding which results in the reduction of the latency phase appears in codispersions containing both products [13, 19] or in the presence of freshly, externally added lysophospholipids [14]. The effect of resorcinolic lipid on the latency value of the system in which the initial latency period was lowered by freshly added myristoyllysolecithin is shown in Fig. 3. Also in this system the latency period was

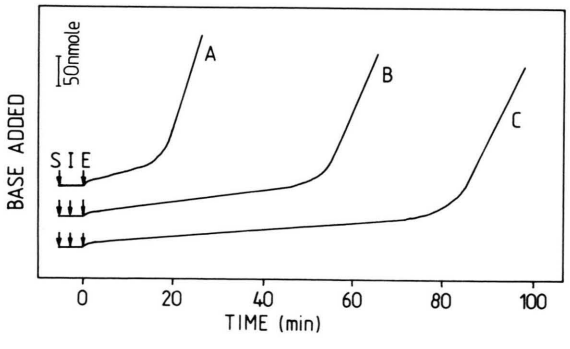

Fig. 1. Reaction progress curves for the hydrolysis of dimyristoylphosphatidylcholine vesicles by pig pancreatic phospholipase A_2 in absence (A) and presence (B, C) of 5-*n*-alk(en)ylresorcinol at 25 °C. S, E, I, injection of substrate, enzyme and inhibitor, respectively; X_I, molar fraction of inhibitor in the substrate vesicles; B, 5-*n*-heptadecylresorcinol ($X_i = 0.05$); C, 5-*n*-heptadecenylresorcinol ($X_i = 0.05$).

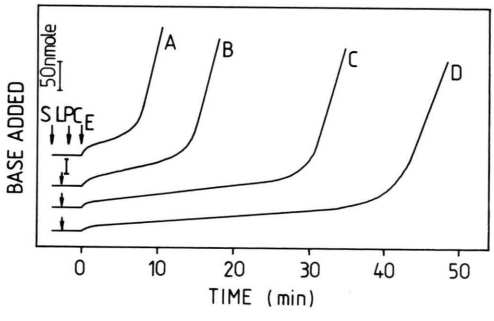

Fig. 3. The effect of 5-*n*-alk(en)ylresorcinol ($X_i = 0.027$) on latency phase of the hydrolysis of dimyristoylphosphatidylcholine vesicles by pig pancreatic phospholipase A_2 in the presence of 1-myristoyllysophosphatidylcholine ($X_{LPC} = 0.1$) at 30 °C. A, control; B, 5-*n*-heptadecylresorcinol; C, 5-*n*-heptadecenylresorcinol; D, 5-*n*-heptadecdienylresorcinol. S, E, I, as in Fig. 1; LPC, injection of lysophospholipid.

increased in the presence of resorcinolic lipid. The effect of resorcinolic lipids was observed already at the molar fractions of these additives in the bilayer as low as 0.025, which was equivalent to the concentration of 3 μM. The ability for increase of the latency was highest for dienoic and monoenoic homologs with an aliphatic chain length of 17 carbon atoms. Both, saturation of the side chain and the increase of its length reduced their inhibitory effect (Fig. 4).

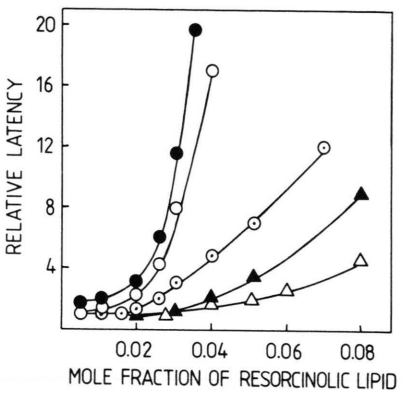

Fig. 4. The effect of various mole fractions of resorcinolic lipids on latency phase of the hydrolysis of dimyristoylphosphatidylcholine vesicles in the presence of lysophosphatidylcholine ($X_{LPC} = 0.1$) at 30 °C. ●, 5-*n*-heptadecdienylresorcinol; ○, 5-*n*-heptadecenylresorcinol; ⊙, 5-*n*-heptadecylresorcinol; ▲, 5-*n*-tricosenylresorcinol; △, 5-*n*-tricosylresorcinol.

The incorporation of resorcinolic lipids into phase boundaries of phase-separated domains will modify the phase equilibria (for some calorimetric data see ref. [6] and [20]) and can result in destabilization and disappearance of the asymmetrical packing defects. This process may reflect the increase of the latency phase due to restriction in binding of the enzyme or the reduction of its mobility and subsequent formation of enough products necessary for reaching the steady-state step of the reaction. This is in agreement with data showing the ordering effect of resorcinolic lipids on the motional freedom of membrane phospholipids [21].

The highest inhibitory effect of enoic homologs is consistent with previous studies [5–7] indicating that the presence of double bonds in aliphatic chain is an important fact determining the high membrane perturbing properties of resorcinolic lipids.

Alk(en)ylresorcinols also show the ability for some reduction of the rate of the steady-state period of the phospholipid hydrolysis (Fig. 5). The extent of this reduction is also dependent upon the chain length and unsaturation of the homolog. The effect, however, is less pronounced than the effect of the latency phase. The extent of the effect is dependent on the time-point of the addition of the inhibitor (Fig. 6). The earlier the injection of resorcinolic lipids after the initiation of the reaction, the larger the increase of the latency period. When the resorcinolic lipids were injected just at

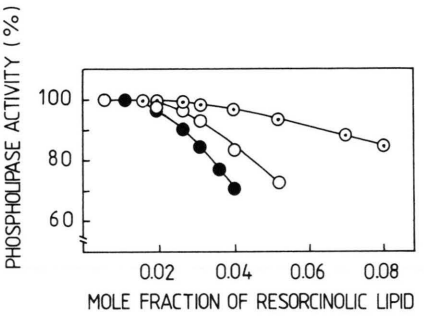

Fig. 5. The rate of steady-state phase of the hydrolysis of dimyristoylphosphatidylcholine vesicles by pig pancreatic phospholipase A_2 as a function of resorcinolic lipid concentration expressed in mole fractions. The reactions were performed at 30 °C in the presence of freshly added 1-myristoyllysophosphatidylcholine ($X_{LPC} = 0.1$). ●, 5-*n*-heptadecdienylresorcinol; ○, 5-*n*-heptadecenylresorcinol; ⊙, 5-*n*-heptadecylresorcinol.

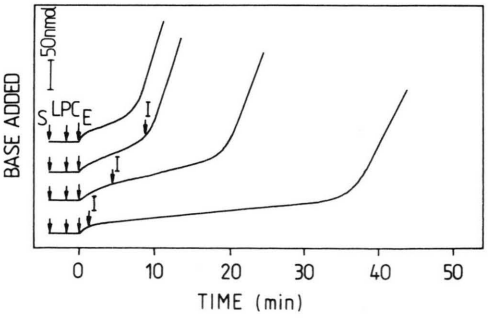

Fig. 6. The effect of the delay of injection of the inhibitor (5-*n*-heptadecdienylresorcinol, $X_i = 0.027$) into the reaction mixture on latency phase of the dimyristoylphosphatidylcholine vesicles hydrolysis by phospholipase A_2 in the presence of 1-myristoyllysophosphatidylcholine ($X_{LPC} = 0.1$) at pH 8.0 and 30 °C. Other abbreviations as in Fig. 3 legend.

the beginning of the steady-state phase only some decrease of the rate was observed. These results indicate that the presence of resorcinolic lipids in the bilayer affects the formation and possible distribution of the nascently formed products. The inhibitor molecules would affect the packing at the interface and protect the formation of the specific phase-separated regions that are responsible for the further binding and action of the enzyme. The ability of the inhibitor to destabilize the products-dependent defects is closely related to the mole fraction of products already formed in the bilayer. At higher mole fraction of resorcinolic lipid present in the membrane higher critical mole fraction of products is necessary for initiation of the steady-state phase (data not shown). When additional portion of the substrate vesicles was injected into the reaction mixture after completion of the reaction the rate of their hydrolysis was significantly reduced in the presence of resorcinolic lipid (Fig. 7). This also suggests the restrictive action of resident in the bilayer inhibitor molecules upon the mobility of enzyme and/or products and their exchange between substrate vesicles.

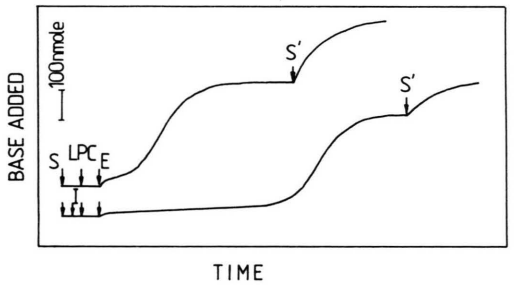

Fig. 7. The effect of 5-*n*-alkenylresorcinol presence on the rate of hydrolysis of additional portion of phospholipid vesicles injected after the first portion of the substrate was hydrolyzed. The conditions as in the legend of Fig. 6. S', injection of new portion of the substrate, other abbreviations as in Fig. 3 legend.

The effect of resorcinolic lipids upon the apparent phospholipase activity is several times stronger than the effect of other simple amphiphiles like fatty acids (data not shown and [22]). It might be related to stronger interface disturbing properties of cone-shaped, odd numbered molecules of resorcinolic lipids. It is very likely that the effect of resorcinolic lipids upon phospholipase activity is dual. First, these compounds effect the interfacial substrate properties determining binding and scooting of the enzyme (as it was shown for bilayers containing one of the products) similarly to *e.g.* fatty acids [22]. Second, the presence of resorcinolic lipids would affect the affinity of the enzyme to the substrate by the direct alk(en)ylresorcinol-protein interaction. This is very likely as resorcinolic lipids were shown to interact directly with hydrophobic domains in the protein molecules [23] with the extent several times stronger than fatty acids and other amphiphiles [24]. The higher values of the latency determined in the systems in which enzyme was preincubated with the studied inhibitors (data not shown) support this possibility. Although quantitative discrimination between participation of mentioned events in the effect of resorcinolic lipids on phospholipase activity is still to be solved the results indicate possibility of biomedical importance of the studied compounds. They could play a role in the modulation of arachidonate metabolism similarly as was shown for urushiol, an other member of the phenolic lipids family [25]. Participation of these compounds in alteration of synthesis of arachidonic acid metabolites would be of importance in modulation of numerous cellular processes *e.g.* inflammation, antimicrobial action of macrophages etc.

The presented results also show the usefulness of phospholipase kinetic studies as a system for the first instance *in vitro* screening of potential enzyme inhibitors as well as membrane alterating agents.

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