STUDIES ON PHOSPHOLIPASE A AND ITS ZYMOGEN FROM PORCINE PANCREAS

IV. THE INFLUENCE OF CHEMICAL MODIFICATION OF THE LECITHIN STRUCTURE ON SUBSTRATE PROPERTIES

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(Received February 3rd, 1972)

SUMMARY

1. A series of chemically modified lecithins were used to investigate by kinetic analyses their substrate *c.q.* inhibitor properties for porcine pancreatic phospholipase A. The substrate analogues used were systematically modified in: the stereochemical configuration, the susceptible ester bond, the phosphate moiety, the alkylchains, the glycerol backbone and in the position of the phosphorylcholine moiety.

2. The desired relationship between chemical structure and inhibitory properties requires elimination of purely physical effects of the inhibitor on the organization of the substrate molecules at the lipid–water interface.

3. Lecithins of the opposite stereochemical configuration and certain lecithin analogues with a modification of the susceptible ester bond were found to be purely competitive inhibitors. The 1-*sn*-phosphatidylcholines have K_i values identical to the K_s values of the corresponding 3-*sn*-phosphatidylcholines. The lecithin analogues with an acylamide linkage at the 2-position were found to be the most potent competitive inhibitors, while on the contrary substitution of the acylester bond by a sulfonyl ester linkage does not give rise to inhibitory properties.

4. Lecithins with a modification in the glycerol–phosphate bond and in the position of the phosphorylcholine moiety are substrates, but exhibit much lower V values and their binding constants are similar to those of the corresponding normal substrates.

5. Introduction of two methyl groups at the carbon atom adjacent to the carboxyl in the acyl chain of the potentially susceptible ester bond gives a lecithin which is not degraded by the enzyme. The presence of only one methyl branch in this position greatly diminishes the hydrolysis rate, probably due to steric hindrance.

6. Increasing the distance between the susceptible ester bond and the phosphate moiety in a lecithin by introducing a methylene group completely abolishes

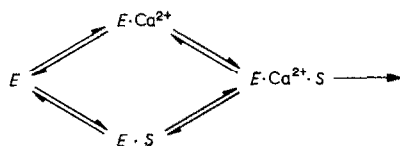* Present address: ALZA Corporation, Palo Alto, Calif., U.S.A.
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enzymatic activity. These lecithin analogues were found to be competitive inhibitors.

7. The minimal requirements for a phospholipid to be a substrate for phospholipase A, as established earlier, should be extended to include the fact that the phosphate moiety can be replaced by a phosphonate or sulfonate group.

INTRODUCTION

Reversible active-site protection or the design of active-site directed inhibitors of phospholipase A (EC 3.1.1.4) is complicated by the fact that up to now no satisfactory competitive inhibitors for the enzyme are known. To the best of our knowledge, the only attempt to prepare more specific inhibitors for this enzyme have been reported by Rosenthal and Han[1]. These authors studied the effects of lecithin and phosphatidylethanolamine analogues, containing long chain ethers, phosphonate, and phosphinate moieties, on the phospholipase action (crude venom from *Agkistrodon piscivoris*). Their assay systems containing egg-yolk lipoprotein or activated egg lecithin as substrate, were characterized by lag periods. The observed kinetics showed anomalous behaviour indicating that the inhibition may be caused more by purely physical effects on the substrate dispersion than by the chemical alterations in the substrate analogues studied. In the present study we have tried to overcome these difficulties by the use of water-soluble substrates and inhibitors. As was demonstrated recently[2], short-chain 3-*sn*-phosphatidylcholines which produce isotropic micellar solutions in water are hydrolysed effectively by the pancreatic phospholipase A in the absence of activator molecules such as bile salts or ether. The enzyme specifically requires Ca^{2+} for its action and the kinetic results with the short-chain lecithins as substrates appeared to be consistent with a random mechanism:

$$
\begin{array}{c}
\nearrow \quad E \cdot Ca^{2+} \quad \searrow \\
E \quad E \cdot Ca^{2+} \cdot S \longrightarrow \\
\searrow \quad E \cdot S \quad \nearrow
\end{array}
$$

From previous work[3] we know the minimal substrate requirements of phospholipase A: one fatty acid ester bond has to be present in a position adjacent to the alcohol–phosphate ester function and the acyl chain should occupy a well-defined stereochemical orientation in space (Fig. 1).
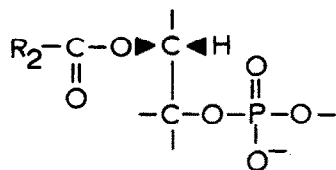
$$
\begin{array}{c}
R_2-\overset{\textstyle |}{\underset{\textstyle \underset{O}{\|}}{C}}-O \blacktriangleright C \blacktriangleleft H \quad O \\
 | \quad \quad \| \\
 -\overset{\textstyle |}{C}-O-\overset{\textstyle |}{P}-O- \\
 | \quad \quad | \\
 O^-
\end{array}
$$

Fig. 1. Minimal substrate requirements for phospholipase A₂.

Modification of this part of the lecithin structure, which is supposed to be important either for binding with phospholipase A or in the catalytic reactions, could possibly give lecithin analogues with inhibitory properties. Therefore, a number of short-chain, water-soluble lecithin analogues were prepared by chemical synthesis[4], in which the following modifications were introduced:

(A), a change in the stereochemical configuration;

(B), changes in the ester group;

(C), changes in the glycerol-phosphate ester;

(D), changes in the alkyl chain;

(E), changes in the glycerol moiety;

(F), a change in the position of the polar headgroup.

From our previous work[2] it appeared that the fine structure of the lipid–water interface is a highly important parameter for phospholipase A action. So it is evident that inhibition studies on lipolytic enzymes will be difficult to interpret if the inhibitor induces alterations in the architecture of the substrate–water interface. In order to be able to investigate quantitatively a pure inhibitory action of these compounds and to correlate this with a chemical modification, they have to fulfil at least two requirements: (a) the micellar interface of the substrate should not be changed considerably by the incorporated inhibitor molecules and (b) the added inhibitor molecules should be either completely incorporated into the substrate micelles or, if this is not the case, it should be possible to calculate the distribution of the inhibitor over the micelles and monomers. Because of our rudimentary knowledge of mixed micellar systems at a molecular level, the only approach to minimize physico-chemical effects of added inhibitor on the substrate interface, was to study inhibitors which resemble as much as possible the chemical and physicochemical properties of the substrates. Therefore, we were limited to compounds with a phosphorylcholine moiety as polar headgroup and with alkyl or acyl moieties of a similar chain length as those of the substrates. The minor chemical modifications in the lecithin structures which are needed to obtain possibly inhibitory compounds, are usually attended also by undesired small changes in the physicochemical behaviour. One of the experimentally most accessible techniques to investigate the physicochemical properties of the modified phospholipids is the determination of the critical micelle concentration. Although we have to admit that this criterion for comparison of different substrates on a molecular level is a very rough one, the identical critical micelle concentration values of, for instance, the isomeric 1,2-diheptanoyl-*sn*-glycero-3-phosphorylcholine and 1,3-diheptanoyl-glycero-2-phosphorylcholine (critical micelle concentration equals 0.6 mM in 2 M NaCl) point to a similar structure of the lipid–water interface.

Using the criterion of the critical micelle concentration we always tried to equalize as much as possible the physicochemical behaviour of the inhibitor and substrate by adjustment of the chain length of the fatty acids in the substrate.

The effects of the variously modified phospholipids on the kinetic parameters of pancreatic phospholipase A with short-chain 3-*sn*-phosphatidylcholines as substrates are reported.

MATERIALS AND METHODS

Enzyme source

Porcine pancreatic phospholipase A_2 was obtained by the activation of the pure zymogen with trypsin as described previously[5].

Substrates and inhibitors

1,2-dihexanoyl-, 1,2-diheptoanoyl- and 1,2-dioctanoyl-*sn*-glycero-3-phosphoryl-

cholines were prepared as described by Cubero Robles *et al.*[6,7]. The following lecithins and lecithin analogues, the chemical synthesis of which has been reported separately[4] were used:

A-1: 2,3-dihexanoyl-*sn*-glycerol-1-phosphorylcholine,
A-2: 2,3-diheptanoyl-*sn*-glycerol-1-phosphorylcholine,
A-3: 2,3-dioctanoyl-*sn*-glycerol-1-phosphorylcholine,
B-1: *rac*-1-heptanoyl-2-deoxy-2-heptanamidoglycero-3-phosphorylcholine,
B-2: *rac*-1-octanoyl-2-deoxy-2-octanamidoglycero-3-phosphorylcholine,
B-3: *rac*-1-heptanoyl-2-octylglycero-3-phosphorylcholine,
B-4: *rac*-1-octanoyl-2-deoxy-2-hexylglycero-3-phosphorylcholine,
B-5: 1-octanoyl-2-actanesulfonyl-*sn*-glycero-3-phosphorylcholine,
C-1: 1,2-dihexanoyl-3-deoxy-*sn*-glycero-3-phosphonylcholine,
C-2: 1,2-dioctanoyl-3-deoxy-*sn*-glycero-3-phosphonylcholine,
C-3: *rac*-1,2-diheptanoyl-3-deoxyglycero-3-sulfonic acid (as sodium salt),
D-1: 1-octanoyl-2-(2-methyl)hexanoyl-*sn*-glycero-3-phosphorylcholine,
D-2: 1-octanoyl-2-(2,2-dimethyl)pentanoyl-*sn*-glycero-3-phosphorylcholine,
D-3: 1-heptanoyl-2-(3,3-dimethyl)butyryl-*sn*-glycero-3-phosphorylcholine,
D-4: *rac*-1,2-dibenzoylglycero-3-phosphorylcholine,
E-1: *rac*-1,2-dihexanoylbutanetriol-4-phosphorylcholine,
E-2: 1-palmitoyl-2-deoxyglycero-3-phosphorylcholine,
E-3: 1-palmitoylglycol-2-phosphorylcholine,
E-4: 1-palmitoyl-*sn*-glycero-3-phosphorylcholine,
F-1: *rac*-1,3-diheptanoylglycero-2-phosphorylcholine,
F-2: 2,2-(dioctanoylhydroxymethyl)propanol-1-phosphorylcholine.

All phospholipids were chromatographically pure (thin-layer chromatography). Optical rotations and element analysis are given in the publication dealing with the chemical synthesis of these lecithin analogues[4]. All substrates and substrate analogues, except dioctanoyllecithin (see ref. 2), are water-soluble and give optically clear solutions.

Assays

Enzymic hydrolysis of the synthetic lecithins and the effects of the various substrate analogues were measured by continuous titration of the liberated fatty acids at pH 7.0 at 40 °C with a Radiometer pH-stat equipment (consisting of Titrator TTT-1, Titrigraph SBR-2, and Syringe burette SBU-1). The assay system was standardized to 4.0 ml total volume and contained 0.5 mM Tris–HCl, 1 mM $CaCl_2$, and NaCl in various amounts.

The lecithins and lecithin analogues, dissolved in distilled water, were used in various amounts, assuming instantaneous intermixing of the components. Lyophilized enzyme, dissolved in distilled water was added in aliquots of 10–100 μl. Titration with 0.04 M NaOH were carried out under nitrogen and with rapid stirring. Initial rates of hydrolysis were measured under conditions where a good proportionality between enzyme concentration and activity existed. Specific activity of the enzyme is expressed as the number of μmoles of fatty acid released per min per mg of protein (dry weight). The titration efficiency of hexanoic, heptanoic and octanoic acid at pH 7.0 was taken as 100% (ref. 2).

Critical micelle concentration determination

Critical micelle concentrations were determined by measuring the spectral shift induced by the incorporation of a dye (Rhodamine 6G) into phospholipid micelles. The gradual increase of phospholipid concentration gives a maximal change in absorption at the critical micelle concentration. The inflection points of the curves obtained by plotting the change in absorbance at 542 nm *versus* the lipid concentration were taken as the critical micelle concentration values. The critical micelle concentrations were usually measured in 0.1 M NaCl (being the salt concentration in the assays) and a dye concentration of 20 mg/l. Occasionally the determinations were performed in 1 M or 2 M NaCl. This method is essentially the same as used by Becher[8]. Incidentally the light-scattering and surface tension techniques were applied to check the values obtained by the spectrophotometric method.

RESULTS AND DISCUSSION

Recently we reported on the kinetic properties of pancreatic phospholipase A with water-soluble short-chain 3-*sn*-phosphatidylcholines as substrates[2]. It was shown that micellar solutions of these lecithins are hydrolysed with a high rate by the enzyme but that the same substrate in molecularly dispersed form is hardly attacked. Fig. 2 (Curve a) demonstrates that with these lecithins Michaelis curves can be obtained at substrate concentrations above the critical micelle concentration, which describe the progressive adsorption of the enzyme to the micellar surface. Below the critical micelle concentration, however, where presumably mainly monomers are present in solution, only a weak enzyme activity could be found, indicating a preferential attack by the enzyme of substrate molecules organized in certain lipid-water interfaces. Curve b in Fig. 2 represents the change in absorbance at 542 nm of solutions containing increasing amounts of substrate in the presence of an organic dye (Rhodamine 6G). In this way the critical micelle concentration region of the substrate could be approached.
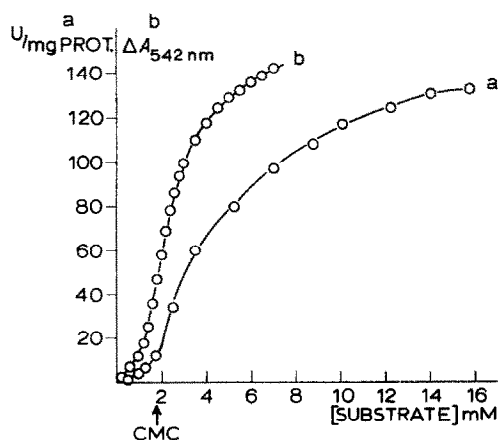

Fig. 2. (a) Michaelis curve of the enzymatic hydrolysis of diheptanoyllecithin with phospholipase A. Assays were done at pH 7.0 in 0.1 M NaCl. For further details, see Methods. (b) Change in absorbance at 542 nm of a Rhodamine 6G solution with increasing concentration of diheptanoyllecithin, measured at pH 7.0, in 0.1 M NaCl. CMC, critical micelle concentration.

also the weak enzymatic activity found below the critical micelle concentration might be attributed. These observations support our idea that monomeric substrate molecules cannot be effectively hydrolysed by this enzyme. Therefore, in order to obtain values for the Michaelis constant K_m, data as shown in Fig. 2 (Curve a) are converted into Lineweaver–Burk plots after a correction has been made for the amount of substrate present as monomers. In this way apparent K_m values are expressed as concentration of substrate totally and exclusively present in micellar form.

In the next sections the properties of the various classes of lecithin analogues are described. In case compounds were hydrolysed by phospholipase A, only their substrate properties were investigated and compared with the normal substrates. When an enzymatic hydrolysis was not observed, the inhibitory properties were studied in the presence of dihexanoyl-, diheptanoyl- or dioctanoyllecithin as substrate. For a detailed description of the properties of these water-soluble short-chain lecithin as substrates for phospholipase A, reference is made to a previous publication[2].

A. Lecithins with an opposite stereochemical configuration (Fig. 3)

The absolute stereospecificity of the enzyme for 1,2-diacyl-3-sn-phosphoglycerides suggested immediately an investigation of the isomeric 2,3-diacyl-sn-phosphoglycerides (D-lecithins*) as possible inhibitory compounds. As shown in Fig. 4, the D-isomer of dioctanoyllecithin (A-3) behaves as a competitive inhibitor for the enzyme in assays with L-dioctanoyllecithin as substrate.

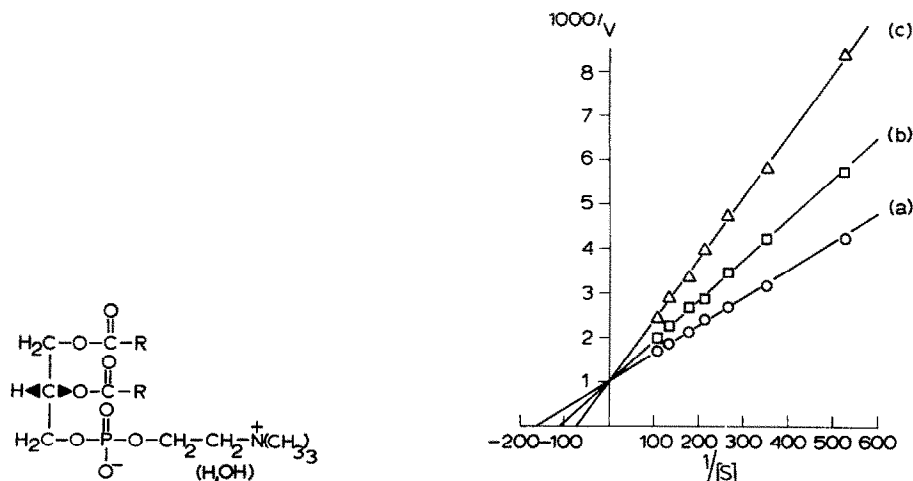Because this lecithin is characterized by a rather low critical micelle concentra-

Fig. 3. Chemical structure of 1-sn-phosphatidylcholine (D-lecithin). A_1, 2,3-dihexanoyl-sn-glycero-1-phosphorylcholine; A_2, 2,3-diheptanoyl-sn-glycero-1-phosphorylcholine; A_3, 2,3-dioctanoyl-sn-glycero-1-phosphorylcholine.

Fig. 4. Lineweaver–Burk plot of the hydrolysis of dioctanoyllecithin by phospholipase A in 0.1 M NaCl (Curve a). For other details see Methods. (b) Inhibition with 1.9 mM 2,3-dioctanoyl-sn-glycero-1-phosphoryl choline (D-lecithin). (c) Inhibition with 4.75 mM of the same D-lecithin.

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* For the sake of shortness we will use on the following pages the D/L nomenclature in stead of the system of stereospecific numbering.

tion (about 0.19 mM in 0.1 M NaCl) no corrections were necessary for the amount of D- and L-lecithin present as monomers. From Fig. 4 the following constants were calculated: $K_s = 6.1$ mM and $K_i = 5.4$ mM.

As might be expected, using the enantiomeric pairs of lecithins with shorter acylchains (C_7 and C_6 fatty acids) not such a direct proof of pure competitive behaviour could be obtained. These lecithins have critical micelle concentrations which are roughly 7 times (diheptanoyllecithin) and 60 times (dihexanoyllecithin) higher than that of the dioctanoyllecithin. This means that at low substrate concentrations in the absence of the inhibitory D-compounds most of the substrate is present in monomeric form and only a very weak activity can be detected. Addition of the physicochemically identical D-isomer to such systems produces an increasing number of mixed micelles, providing in this way a larger micellar lipid–water interface capable of enzyme adsorption. Notwithstanding the inhibitory properties of the D-analogue, the increased area of the lipid–water interface of the mixed micelles can result in a higher rate of breakdown of the L-isomer, because more L-monomer now was shifted to the micellar form. In these cases, the D-lecithin displays an activating behaviour instead of inhibition!

For such short-chain enantiomeric lecithins the competitive behaviour of the D-compounds can be demonstrated indirectly as shown in Fig. 5. Use is made here of the fully identical physicochemical properties of the enantiomeric L- and D-lecithins. By keeping the total lipid–water interface available for the enzyme constant (L+D), the activity of a fixed amount of enzyme can be measured as a function of the percentage of the interface occupied by the substrate (L-isomer). The linear relationship between activity and percentage of substrate in the micellar interface as shown in Fig. 5, can only be explained if the enantiomeric D-lecithin (A-1) possesses a fully
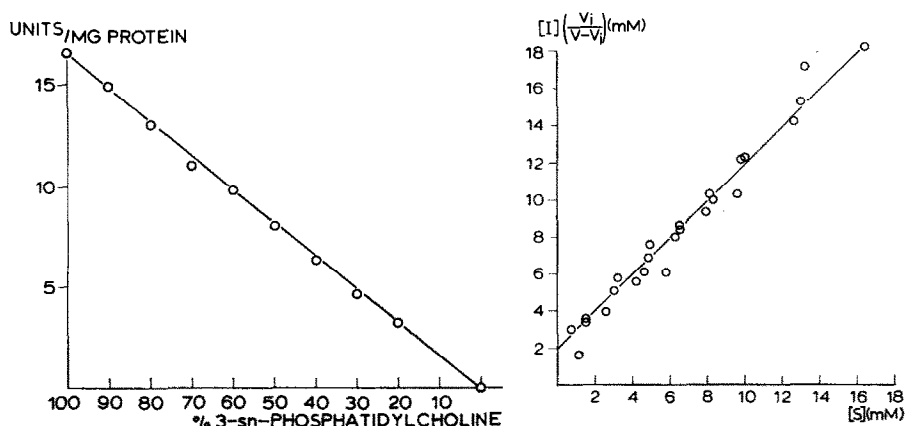

Fig. 5. Hydrolysis of mixtures of the D-and L-isomer of dihexanoyllecithin. Total concentration of the two compounds was kept constant (21 mM). The assays were performed at pH 7.0 in 1 M NaCl. For other details see Methods. The activity is plotted against the percentage of the L-isomer present in total lipid mixture.

Fig. 6. Hunter and Downs[9] plot of the inhibition of phospholipase A by the D-isomer of diheptanoyl lecithin with the corresponding L-isomer as substrate. Assays were done at pH 7.0 in 0.1 M NaCl and 10 mM $CaCl_2$. Substrate and inhibitor concentrations were corrected for the amounts present as monomers. The monomer concentration of each of the components is calculated by multiplication of the critical micelle concentration and the molar fraction of each of the components.

identical affinity constant for the enzyme as the L-isomer ($K_i = K_s$). Similar plots were also obtained for the D- and L-isomers of diheptanoyl- and dioctanoyllecithin.

Finally, Fig. 6 shows a Hunter and Downs[9] plot of the inhibition of the enzyme by D-diheptanoyllecithin (A-2) with the L-isomer as substrate. In such plots the slope of the curve equals K_i/K_s. For the enantiomeric lecithins this ratio equals 1.0 (Fig. 6), whereas the intersection with the ordinate gives the inhibition constant K_i (2 mM).

In summary, the results with the enantiomeric lecithins clearly show that D-lecithins are competitive inhibitors possessing identical affinity constants to the enzyme as the corresponding L-isomers. The stereochemical specificity of the enzyme with respect to the configuration of its substrate is an expression of catalytic specificity and not of binding specificity.

B. Lecithin analogues in which the susceptible 2-acylester bond is modified

Fig. 7 gives the structural formulae of the lecithin analogues modified in the ester moiety at the 2-position*.

In agreement with the minimal substrate requirements of the enzyme these four types of lecithin structures, modified in the susceptible 2-acyl ester bond, were found to be completely inert against phospholipase A attack. In view of the results with the short-chain D-lecithins (A-1 and A-2) with respect to their physicochemical
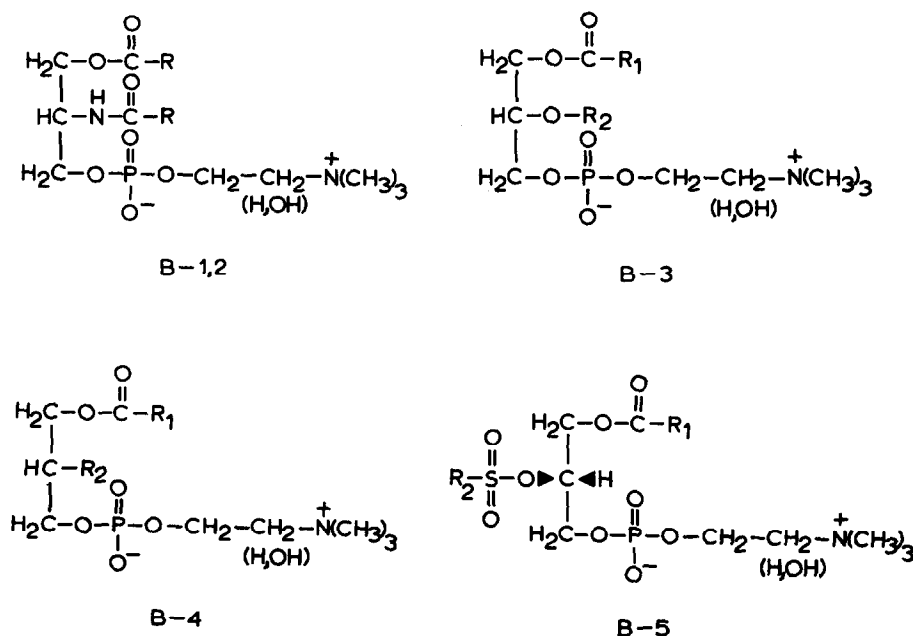

Fig. 7. Chemical structures of lecithin analogues modified in the ester moiety of the 2-position of glycerol. B-1, rac-1-heptanoyl-2-deoxy-2-heptanamido-glycero-3-phosphorylcholine; B-2, rac-1-octanoyl-2-deoxy-2-octanamido-glycero-3-phosphorylcholine; B-3, rac-1-heptanoyl-2-octylglycero-3-phosphorylcholine; B-4, rac-1-octanoyl-2-deoxy-2-hexylglycero-3-phosphorylcholine; B-5, 1-octanoyl-2-octanesulfonyl-sn-glycero-3-phosphoryl-choline.

* Short-hand notation of these compounds used in this section: B-1: diheptanoylamidelecithin, B-2: dioctanoylamidelecithin, B-3: etherlecithin, B-4: alkyllecithin, B-5: sulfonyllecithin.

properties, it was anticipated that similar difficulties would arise with other lecithin analogues which possess a relatively high critical micelle concentration. For instance, in 0.1 M NaCl the enzymic hydrolysis of diheptanoyllecithin was not inhibited by small quantities of the diheptanoylamidelecithin (B-1). On the other hand, a relatively strong inhibition was observed with the same amount of the dioctanoylamidelecithin (B-2) using again diheptanoyllecithin as substrate.

In order to take into account the physicochemical differences between substrates and inhibitors, the determination of their critical micelle concentration values was undertaken (see Table I).

TABLE I

CRITICAL MICELLE CONCENTRATIONS OF SUBSTRATES AND SOME INHIBITORS

Critical micelle concentrations were determined as described under Methods. The salt concentrations are given below. The measurements were performed at pH 7.0 at room temperature.

Substrate	*Critical micelle concentration (mM)*		
	0.1 M NaCl	*1 M NaCl*	*2 M NaCl*
Dihexanoyllecithin	13.8	7.4	4.0
Diheptanoyllecithin	1.8	0.9	0.6
Dioctanoyllecithin	0.19	—	—
Inhibitors			
Diheptanoylamidelecithin (B-1)	3.0	1.35	—
Dioctanoylamidelecithin (B-2)	0.57	—	—
Etherlecithin (B-3)	0.50	—	—
Alkyllecithin (B-4)	1.3	0.53	—
Sulfonyllecithin (B-5)	0.80	—	—

From Table I one can see that the chemical change of an ester function into an amide linkage considerably raises the critical micelle concentration (both compounds with equal chain length). Therefore, using diheptanoyllecithin as substrate and small quantities of the corresponding amidelecithin (B-1) as inhibitor, implies that most of
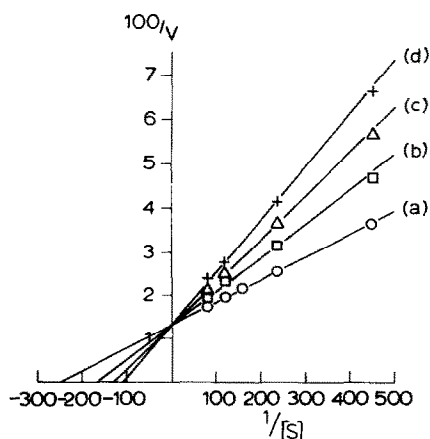

Fig. 8. Lineweaver–Burk plots showing the linear competitive inhibition of the hydrolysis of diheptanoyllecithin by phospholipase A with dioctanoylamidelecithin as inhibitor. Assays were done at pH 7.0 in 0.1 M NaCl. For further details see Methods. (a) No inhibitor added; (b) with 0.24 mM inhibitor; (c) with 0.48 mM inhibitor; (d) with 0.72 mM inhibitor.

the amide lecithin molecules is not incorporated into the micelles, but is present in monomeric form. If the enzyme has a much lower affinity to monomers, it will mainly adsorb to pure substrate micelles and similar reaction rates will be found.

However, if we use relatively small amounts of the dioctanoylamidelecithin (B-2) as inhibitor and again diheptanoyllecithin as substrate, the much lower critical micelle concentration of B-2 will force most of the inhibitor molecules into the substrate micelles and consequently its inhibitory properties will emerge. Fig. 8 gives an illustration in a double reciprocal plot of this purely competitive inhibition. Plotting the same data in different ways, as described by Dixon and Webb[10], also pointed to pure competitive inhibition.

As could be expected, if we use dioctanoyllecithin as substrate (with a much lower critical micelle concentration than the diheptanoyl derivative) and the dioctanoylamide lecithin (B-2) as inhibitor, no difficulties were encountered. No corrections had to be made for the critical micelle concentration and pure competitive inhibition was found. In Table II the various kinetic parameters are listed.

In order to demonstrate the inhibitory properties of the diheptanoylamidelecithin (B-1) we had to lower its critical micelle concentration artificially by the addition of 1 M NaCl in the assay system. In this way most of the inhibitor molecules are incorporated into substrate micelles and pure competitive inhibition was observed with similar binding constants as the dioctanoyl derivative (B-2).

The two other lecithin analogues, viz. the ether lecithin B-3, and the alkyl lecithin B-4 were also shown to inhibit the enzyme in a competitive way (Table II).

As could be expected, especially the alkyllecithin (B-4) having a rather high critical micelle concentration (1.3 mM) required the presence of 1 M NaCl to demonstrate inhibition*. From Table II one can conclude that, whereas the alkyl and ether lecithins are only weak competitive inhibitors, with binding constants comparable to those of the substrate, the amide lecithins (B-1 and B-2) show a much stronger inhibition. The enzyme has an affinity for these compounds which is at least 5–10 times higher than for the corresponding normal lecithins.

Unexpectedly, the sulfonyllecithin (B-5) in mixtures with diheptanoyl- or dioctanoyllecithin as substrate could not be shown to possess inhibitory properties. Although the chemical change of an ester function into a sulfonyl ester linkage again considerably raises the critical micelle concentration (for the dioctanoyl derivatives from 0.19 mM to 0.80 mM, compare Table I), even sulfonyllecithin concentrations well above its critical micelle concentration do not inhibit the hydrolysis of micellar diheptanoyl- and dioctanoyllecithin.

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* Even in the presence of high salt concentrations, the critical micelle concentration values are not always negligible. In order to eliminate the deviations of the apparent inhibitor concentration from the actual inhibitor concentration in the micelles an approximated calculation was made based on the following considerations[11]. The micelle structure probably varies continuously from that of one pure component to that of the other. Both substrate and inhibitor contribute to the actual mixed critical micelle concentration. If the critical micelle concentrations of both components are not very different, ideal mixing can be expected. The mixed critical micelle concentration then is represented by the weight average of the individual critical micelle concentrations, taking into account the total concentrations of both components. The amount of monomers of both components will approximately be proportional to their individual critical micelle concentrations. Knowing the monomer concentrations of both components, the real substrate and inhibitor concentrations, exclusively present in the micelles can be calculated.

TABLE II

KINETIC CONSTANTS OF SUBSTRATES AND COMPETITIVE INHIBITORS

Assays were done at pH 7.0 (0.5 mM Tris), with 1 mM $CaCl_2$ at 40 °C. Values for V (μmoles/mg per min), K_s (mM) and K_i (mM) were obtained from double reciprocal plots $1/v$ *versus* $1/[S]$. K_s is expressed in substrate concentration present in the form of micelles (which means that the critical micelle concentration values are subtracted from every total substrate concentration to obtain the actual substrate concentration, being exclusively micelles). In some cases B-1, B-3 and B-4, it was necessary to make corrections for the amount of inhibitor present as monomers. For details see text.

Substrate	Inhibitor	NaCl (M)	K_s (mM)	K_i (mM)	V_{max}
Diheptanoyllecithin	Dioctanoylamidelecithin (B-2)	0.1	4.2	0.54	45
Dioctanoyllecithin	Dioctanoylamidelecithin (B-2)	0.1	3.3	0.6	833
Dioctanoyllecithin	Dioctanoylamidelecithin (B-2)	2	1.9	0.09	1560
Diheptanoyllecithin	Diheptanoylamidelecithin (B-1)	1	3.8	0.8	96
Dioctanoyllecithin	Alkyllecithin (B-4)	1	3.3	2.1	1040
Diheptanoyllecithin	Alkyllecithin (B-4)	1	4.0	3.3	97
Dioctanoyllecithin	Ether lecithin (B-3)	1	3.3	5.2	1040
Dioctanoyllecithin	Sulfonyllecithin (B-5)	0.1	3.3	——	833

C. Lecithin analogues modified in the carbon–oxygen–phosphate linkage

Fig. 9 gives the structural formulae of a lecithin with a direct C-P bond (C-1 and C-2) and that of a sulfonic acid analogue of phosphatidic acid (C-3).

In deviation from the previously derived minimal substrate requirements of the enzyme it turned out that the direct carbon–phosphorus bond does not abolish the substrate properties. The substrate properties of two optically active phosphonoleci-
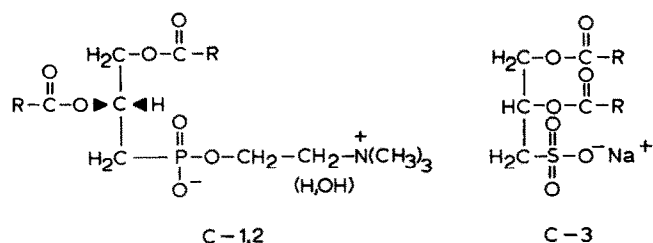

Fig. 9. Chemical structures of lecithin analogues modified in the carbon–oxygen–phosphorus linkage. C-1, 1,2-dihexanoyl-3-deoxy-*sn*-glycero-3-phosphonylcholine; C-2, 1,2-dioctanoyl-3-deoxy-*sn*-glycero-3-phosphonylcholine; C-3, sodium salt of *rac*-1,2-diheptanoyl-3-deoxyglycero-3-sulfonic acid.

TABLE III

COMPARISON OF KINETIC CONSTANTS OF NORMAL LECITHINS AND PHOSPHONO LECITHINS

Assay conditions: see Table II. NaCl concentration as indicated. K_s (mM) and V (μmoles/mg per min) were obtained from Lineweaver–Burk plots. For the dihexanoylderivatives K_s is expressed in substrate concentration present as micelles. Critical micelle concentration values were determined as described in Methods.

Substrate	NaCl (M)	K_s (mM)	V	Critical micelle concentration (mM)
Dihexanoyllecithin	2	8.7	45	4.0
Dihexanoylphosphonolecithin (C-1)	2	5.6	6.7	4.0
Dioctanoyllecithin	0.1	3.3	833	0.19
Dioctanoylphosphonolecithin (C-2)	0.1	2.8	43	0.195

thins containing either two C_6 or two C_8 acyl chains were investigated and compared under similar experimental conditions with the corresponding normal lecithins. As shown in Table III, the dihexanoylphosphonolecithin (C-1) and the dioctanoyl-phosphonolecithin (C-2) have affinities for the enzyme which are close to those of the corresponding normal lecithins, but the phosphono linkage considerably diminishes the maximal velocities of the enzymic hydrolysis.

As regards the sulfur containing analogue (C-3), this class of compounds will not be treated in detail. Because of the intrinsic structure of the sulfur-atom it is impossible to prepare a lecithin analogue in which phosphorus is replaced by sulfur and which still possesses a negative ionization. The synthesized lipid C-3, which lacks the zwitterionic structure, undoubtedly produces a lipid–water interface, which, however, will be different from that of the "neutral" lecithin molecules. Moreover, the affinity of such a sulfur-containing acidic lipid for Ca^{2+} might completely change the mechanism of enzyme action proposed for the lecithin as substrate.

On the other hand, we want to mention this sulfonic acid containing lipid (C-3) because here again it turned out that the definition of the minimal substrate requirements of the enzyme needs some extension. This compound is in fact a good substrate for the enzyme. Comparison with deheptanoyllecithin shows that under similar assay conditions (pH 7.0, 1 mM $CaCl_2$, 0.1 M NaCl), the sulfur analogue is hydrolysed in a stereospecific way five times more rapidly that the lecithin with the same fatty acid esters. The critical micelle concentration of the sulfurcontaining lipid is substantially lower than that of the lecithin (0.4 mM and 1.8 mM respectively).

D. Lecithins containing branched acylchains in position 2 of the glycerol moiety

The members of this class of lecithin analogues (Fig. 10) have in common that they all possess the minimal substrate requirements and consequently should be considered as possible substrates for the enzyme. On the other hand, the introduction of one or more bulky methyl groups close to the ester bond to be split, may give rise to
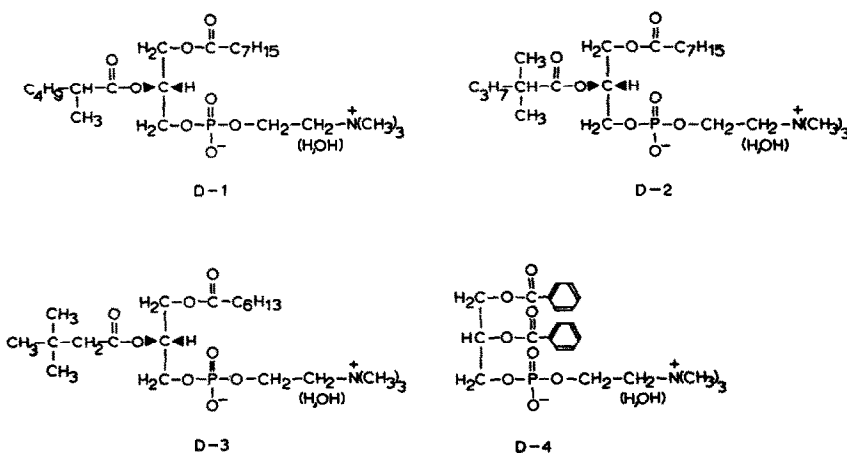

Fig. 10. Chemical structures of lecithins with branched acylchains in the 2-position of the glycerol moiety. D-1, 1-octanoyl-2-(2-methyl)hexanoyl-sn-glycero-3-phosphorylcholine; D-2, 1-octanoyl-2-(2,2-dimethyl) pentanoyl-sn-glycero-3-phosphorylcholine; D-3, 1-heptanoyl-2-(3,3-dimethyl)-butyryl-sn-glycero-3-phosphorylcholine; D-4, rac-1,2-dibenzoylglycerol-3-phosphorylcholine.

TABLE IV

KINETIC PARAMETERS OF 3-*sn*-PHOSPHATIDYLCHOLINES WITH MODIFIED ACYL-CHAINS

Assays were performed as given in Table II. NaCl concentrations as indicated. K_s (mM) and V (μmoles/mg per min) were obtained from double reciprocal plots ($1/v$ *vs.* $1/[S]$). K_s is expressed as micellar substrate concentration. Critical micelle concentration values were determined as described in Methods. The V values given in parentheses are approximative values expected for the straight-chain lecithins with the same acyl chain length under similar conditions. Because the straight-chain lecithins containing mixed fatty acids were not available, the V values given represent the arithmetic mean of the maximal velocities found experimentally under the same conditions for lecithins containing two identical fatty acids *e.g.*

V (heptanoyl, octanoyllecithin) $\approx \frac{1}{2}$ [V(diheptanoyllecithin) $+ V$ (dioctanoyllecithin)] $\approx$ $\frac{1}{2}(100 + 1040) = 570$.

Substrate	NaCl (M)	K_s (mM)	V	Critical micelle concentration (mM)
2-Methylacyllecithin (D-1)	1.0	4.5	29 (570)	0.49
2,2-Dimethylacyllecithin (D-2)	1.0	—	0 (570)	0.68
3,3-Dimethylacyllecithin (D-3)	1.0	3.3	84 (60)	0.97
Benzoyl lecithin (D-4)	3.0	—	0	> 20

structures in which the formation or breakdown of the Michaelis complex is sterically hindered. Because of the limited amounts of material and the rather elevated critical micelle concentration values, the kinetic parameters of the enzyme were determined in 1 M NaCl solutions of these lecithins. In Table IV the kinetic parameters and critical micelle concentration values of compounds D-1 to D-4 are listed. Comparison of Table IV with Table I shows that the introduction of a (2-methyl) fatty acid chain in the lecithin molecule does not greatly change its physicochemical behaviour as far as the critical micelle concentration is concerned. Also the apparent affinity constants K_s seem to be very close to those of lecithins containing straight-chain fatty acid esters of the same chain length (*cf* Table II). On the other hand, one observes a dramatic fall in enzyme activity going from straight chain compounds to lecithins with acyl chains, branched in the 2-position. Whereas the (2-methylacyl)-lecithin (D-1) still can be considered as a (bad) substrate, the (2,2-dimethyllecithin (D-2) is not hydrolysed at all, even in the presence of high enzyme concentrations and long incubation periods. The latter lecithin is a pure competitive inhibitor for the enzyme and its apparent inhibition constant K_i is similar to the substrate affinity constant K_s (about 3 mM in 1 M NaCl). Apparently the decomposition of the Michaelis complex (and probably the rate-limiting acylation step) is very sensitive either to steric hindrance produced by branching of the acylchain adjacent to the susceptible ester bond or to an inactivation of the susceptible ester bond by the electron-donating methyl group(s) ($+I$-effect). The assumption that steric hindrance or a $+I$-effect caused by the branching in the 2-position of the acyl chain is the main reason for the observed fall in maximal velocities, is suggested by the fact that even a double CH_3-branch in the 3-position (compound D-3) does not lower the V (Table IV). Finally we did not succeed in obtaining any hydrolysis of the dibenzoyllecithin (D-4) notwithstanding the high salt and enzyme concentrations used.

From a physicochemical point of view, the phenyl ring can be considered as equivalent to a straight alkyl chain of 3.5 carbon atoms[12]. This means that the micellar properties of the dibenzoyllecithin (D-4) should be comparable to a mixed-acid lecithin with a C-4 and C-5 fatty acid ester. If this is true, lecithin concentrations up

to 20 mM in 3 M NaCl are still far from the critical micelle concentration. Experimentally, micelle formation could be not detected up to 20 mM solutions. Mainly (or exclusively) monomers will be present and scarcely any hydrolysis should occur. On the other hand, in the presence of very high enzyme concentrations and upon prolonged incubation periods, some degradation of monomers of dibutyryllecithin can be observed. Therefore, we have to assume that the total inertness of the dibenzoyl-lecithin (D-4) as substrate for phospholipase A is caused by at least two effects: (a) the high critical micelle concentration of this lecithin which counteracts the formation of an organized lipid–water interface and (b) steric hindrance of the phenyl ring close to the susceptible ester bond.

E. Lecithin analogues modified in the glycerol moiety (Fig. 11)

In agreement with the established minimal substrate requirements of phospholipase A, the introduction of a methylene group between the susceptible secondary ester function and the carbon–oxygen–phosphate linkage completely abolishes all substrate properties. The lecithin analogue derived from racemic 1,2,4-trihydroxy-
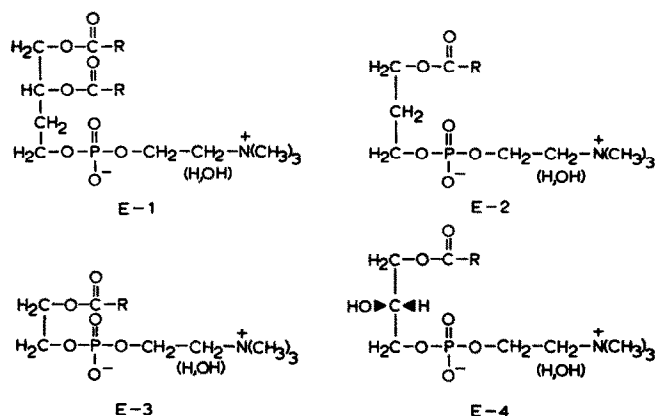

Fig. 11. Chemical structures of lecithin analogues modified in the glycerol moiety. E1, *rac*-1,2-dihexanoylbutanetriol-4-phosphorylcholine; E-2, 1-palmitoyl-2-deoxyglycero-3-phosphorylcholine; E-3, 1-palmitoylglycol-2-phosphorylcholine; E-4, 1-palmitoyl-*sn*-glycero-3-phosphorylcholine.

butane (E-1) having two hexanoic acid esters, possesses a critical micelle concentration of about 9.3 mM in 0.1 M NaCl. Therefore, high salt concentrations had to be used to investigate its possible inhibitory properties. In 2 M NaCl this lecithin analogue E-1 turned out to be a weak competitive inhibitor when tested with dihexanoyl lecithin as substrate (Fig. 12). The affinity to the enzyme (K_i 7.2 mM) is quite similar to that of the corresponding substrate (K_s 8.7 mM).

As regards the analogues E-2 and E-3, these compounds contain only one fatty acid chain, and will be compared therefore with 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (lysolecithin, E-4). All three compounds E-2, E-3 and E-4 are freely water-soluble and all possess a low critical micelle concentration in 0.1 M NaCl of about 0.04 mM. In analogy with a long-chain lysolecithin also E-2 and E-3 probably form spherical micelles containing 150–200 monomers*. The 1-palmitoyllysolecithin (E-4) being a product of phospholipase A hydrolysis of a lecithin, and its deoxy derivative E-2 are not hydrolysed by phospholipase A. The glycol analogue E-3, which in principle
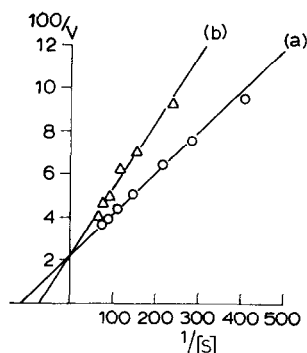
Fig. 12. Double reciprocal plots ($1/v$ versus $1/[s]$) demonstrating the competitive inhibition of phospholipase A hydrolysis of dihexanoyllecithin by *rac*-1,2 dihexanoylbutanetriol-4-phosphorylcholine. Assays were carried out at pH 7.0 in 2 M NaCl. For further details see Methods. Both substrate and inhibitor concentrations are corrected for the amounts present as monomers. For the method of calculation, see Section B. (a), without inhibitor; (b), with 4.8 mM inhibitor; $K_s = 8.7$ mM and $K_i = 7.2$ mM. $V = 45$ (units/mg per min).

can be degraded by the enzyme, usually very slowly in the presence of high enzyme concentrations and bile salts[3], cannot serve as substrate in our normal assay without the addition of a detergent**. The possible inhibitory effect of 1-palmitoyllysolecithin (E-4), 1-palmitoyldeoxylysolecithin (E-2) and palmitoylglycollecithin (E-3) was investigated using dioctanoyllecithin as substrate in 2 M NaCl solution. All three compounds, if present in low concentrations, appeared to increase the hydrolysis rate of the substrate. Higher amounts of the lecithin analogues showed no or very little inhibitory effect on the hydrolysis of dioctanoyllecithin. The clearing effect of these surface-active agents on the coacervate droplets of dioctanoyllecithin indicates that mixed micelles are formed. Apparently the arrangement of the substrate molecules in these micelles is slightly changed resulting in a somewhat higher rate of hydrolysis by the enzyme. A similar activating effect of long-chain lysolecithins on the hydrolysis of dihexanoyllecithin has been reported by Roholt and Schlamowitz[15]. Short-chain lysolecithins, for instance, 1-octanoyl-*sn*-glycero-3-phosphorylcholine and lower homologues which are highly water-soluble and hence not incorporated into the substrate micelles, have no effect on the rate of hydrolysis. However, the fact that the palmitoyl derivatives E-2, E-3 and E-4 fail to inhibit the hydrolysis of dioctanoyllecithin appreciably, should not be interpreted in terms of a lack of affinity to the enzyme. The overall effect which is observed, *i.e.*, a little activation might have been produced by two factors: (a) an inhibitory effect caused by the presence of a substance which binds

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* Lysolecithin derived from egg-yolk lecithin has been reported to have a micellar weight around 95000 (in water)[13]. Light scattering studies[14] indicated a spherical shape of the micelles with a diameter of about 56 Å. This implies a very close packing of the monomers (181) in the micelle with an area per molecule of about 55 Å².

** That the very close packing of the molecules in the micelle is at least partially responsible for the very bad substrate properties of long-chain glycol lecithins as compound E-3, is supported by the observation that addition of certain "spacing" molecules enhances the enzymatic breakdown. Even the addition of the D-isomer of dioctanoyllecithin (A-3), which was shown to be a competitive inhibitor of the enzyme, accelerates the hydrolysis of the glycollecithin E-3. This indicates that the activating effect of the D-lecithin, because of its "spacing power" is more important than its inhibitory action. It should be noted that the D-isomer of diheptanoyllecithin (A-2) was not able to accelerate the enzymatic hydrolysis of the glycollecithin E-3.

to the enzyme, but which is not hydrolysed and (b) an activating effect related to an improved mixed micellar interface.

Unfortunately, when we started the preparation of these compounds, our knowledge about the physicochemical parameters of the modified phospholipids was even more restricted than now, and the choice of the fatty acid chain length (16 C-atoms) in the monoacyl derivatives was quite arbitrary. Only if the chemical and physicochemical nature of the substrate and inhibitor are very similar, one may hope that inhibition in the classical sense (*i.e.*, in terms of chemical structure) is not masked or influenced in some way by physicochemical effects.

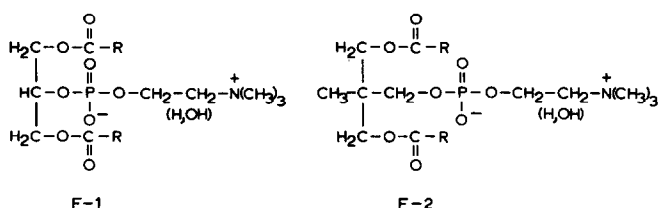*F. Lecithin analogues with the phosphorylcholine moiety in the 2-position of the glycerol moiety*

F-1   F-2

Fig. 13. Chemical structures of lecithin analogues modified in the position of the phosphoryl-choline moiety. F-1, *rac*-1,3-diheptanoylglycero-2-phosphorylcholine; F-2, 2,2-(dioctanoylhydro-xymethyl)propanol-1-phosphorylcholine.

In Fig. 13 the structural formulae are given of some lecithin analogues in which the position of the phosphorylcholine moiety is different from that in the normal sub-strate. Note that compound F-2 is symmetrical around the 2-carbon atom of the propanol moiety and can be considered as a member of Class E as well.

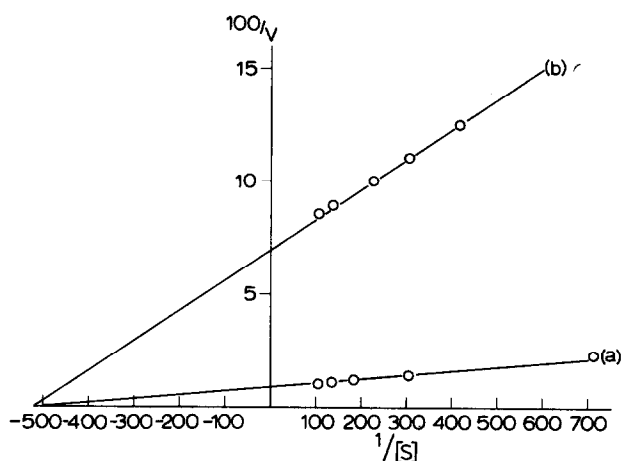In previous work it was shown that the structural isomer of normal substrates

Fig. 14. Lineweaver–Burk plots of the hydrolysis of the diheptanoyl derivatives of 3-*sn*-phosphati-dylcholine (a) and of 2-phosphatidylcholine (b) by phospholipase A. Assays were performed at pH 7.0 in 2 M NaCl. K_s values are given in concentration of lecithin present in micellar form. K_s for both lecithins: 1.9 mM.

(*i.e.*, compound F-1, a so-called β-lecithin) can be degraded by phospholipase A. The enzyme hydrolyses such lecithins in a fully stereospecific way, yielding one molecule of optically active lysolecithin and one molecule of fatty acid[16]. However, at that time the lack of a good assay system made it impossible to compare the kinetic constants of the enzyme with the two isomeric lecithins as substrate.

As shown in Fig. 14, large differences exist in the rate of enzymatic breakdown of such positional isomers. The V value of the enzyme in the hydrolysis of lecithin analogue F-1 (β-lecithin) is only 13% of the value found for the normal diheptanoyl-lecithin (α-lecithin). The K_s values on the contrary, appear to be very similar for both isomers. Apparently the catalytic rate constant of the enzyme is very sensitive even to minor structural changes in the substrate, such as in positional isomers. The 2,2-(dioctanoylhydroxymethyl)propanol-1-phosphorylcholine (F-2) is not hydrolysed by the enzyme notwithstanding very high enzyme concentrations and prolonged incubation periods. This analogue inhibits the enzymic hydrolysis of dioctanoyllecithin, however, the inhibition kinetics are not purely competitive. As both the substrate and the inhibitor (containing two octanoyl chains) produce coacervate structures[2] in water in stead of clear micellar solutions, from a physico-chemical point of view the system is too complicated to investigate quantitatively.

CONCLUSIONS

Investigations on the binding site and catalytic site of phospholipase A require a thorough knowledge of structure and properties of enzyme inhibitors. A large number of chemically modified phospholipids have been studied in the past for their substrate properties[3]. Although the minimal structural requirements for enzyme action could be determined, these studies were hampered by the lack of a quantitative assay procedure. In the present study small modifications were systematically introduced in the lecithin structure and the resulting influence on enzyme activity was determined quantitatively. The assay system is based on the use of short-chain lecithins as substrates and automatic titration of the enzymically liberated fatty acids. Such an assay system is actually the most simple one; it contains only a clear aqueous solution of substrate, calcium ions and enzyme. However, recently Entressangles and Desnuelle showed that even in such a simple assay system enzyme activity is not only a function of the usual parameters as substrate concentration and pH[17]. It appeared that the physicochemical state of the lipid molecules has a great influence on the activity of the enzyme, *e.g.*, for a given substrate in a clear aqueous solution the enzyme is scarcely active if the substrate is present as monomers (below the critical micelle concentration) and activity sharply rises in a Michaelis way at concentrations above the critical micelle concentration. Even at concentrations far above the critical micelle concentration where the substrate is mainly present as micelles, any addition which modifies the nature of the lipid–water interface has a pronounced effect on enzyme activity. This indicates that a lipolytic enzyme such as phospholipase A is extremely sensitive to the overall parameters of an organized lipid–water interface (*e.g.*, the interfacial free energy, the organized water layer surrounding the interface, the electrical double layer, *etc.*) or that the interface determines in a specificway the molecular conformation of every substrate molecule and hence facilitates or impedes the interaction with the enzyme. An indication for the latter possibility, *viz.* a molecular conformation of

each substrate molecule "induced" by the interface, was recently obtained in our laboratory by monolayer techniques (G. Zografi, R. Verger and G. H. de Haas, unpublished). These studies showed a pronounced optimum of phospholipase A activity at a certain surface pressure of the monolayer. It is tempting to correlate the maximal rate of hydrolysis with a defined orientation of the substrate molecules at the interface being optimal for enzyme–substrate interaction. In "bulk" studies, however, every substrate spontaneously forms a given interface, determined by chemical structure and conditions as pH and ionic strength. As a consequence of this, one is not allowed to compare the binding constants K_s found for different substrates, because of possible small differences in the architecture of the various lipid–water interfaces.

One of the most important results of the present study is the detection of two classes of linear competitive inhibitors of phospholipase A.

(a) *1-sn-Phosphatidylcholines (D-lecithins)*. These inhibitors are characterized by a rather weak affinity to the enzyme. However, their main interest is the fully identical binding to the enzyme as compared with the corresponding 3-*sn*-phosphatidylcholines. Any investigation on the formation of the Michaelis complex can be performed with these D-isomers without perturbation caused by product formation.

(b) *1-Acyl-2-acylamidolecithins*. Up to now this class of inhibitors possesses the highest affinity towards the enzyme and makes them very valuable for active-site protection and the design of bifunctional irreversible inhibitors.

Not all modifications in the lecithin structure completely abolished enzymatic hydrolysis by phospholipase A. In most cases, however, a much lower V value was obtained. The observation that the phosphono-lecithins (C-1, C-2) are hydrolysed at a much lower rate and that a lecithin in which the distance between the susceptible ester bond and the negative ionization is increased (compound E-1) is not a substrate at all, indicates that the geometry of the substrate is an important factor for the catalytic reaction steps.

In lecithin and derivatives which meet the minimal structural requirements, the position and the absence or presence of a second fatty acid ester moiety appear to have a large effect on the rate of hydrolysis of the susceptible ester bond (*cf.* the slow hydrolysis of 2-phosphatidylcholines, glycollecithin and 2-acyllysolecithin[18]. In analogy to similar observations with the hydrolysis of various glycerides by pancreatic lipase, this phenomenon might be interpreted as an activation by a neighboring electrophilic ester group (Brocherkoff[19]).

From this study one might conclude that the binding site of the enzyme apparently has a much broader specificity than the catalytic site. With the exception of the amide- and sulfonylesterlecithin, most of the structural modifications introduced in the lecithin molecule give rise to lecithin analogues having affinity constants (K_s or K_i) to the enzyme which are of the same order of magnitude as the normal lecithin. However, it should be emphasized once more that these affinity constants are apparent values, which probably include besides the true Michaelis constant also a contribution of an adsorption equilibrium preceding the formation of the Michaelis complex:

Enzyme+interface $\rightleftharpoons$ enzyme interface
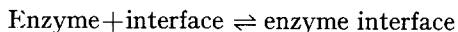
Studies are in progress to confirm the described kinetic results by direct enzyme–substrate binding experiments with the aid of ultraviolet difference-spectroscopy, fluorescence spectroscopy and Sephadex elution.

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