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EFFECT OF A DIAZAFLUORANTHEN DERIVATIVE ON PHOSPHOLIPASES A STUDY AT THE AIR-WATER INTERFACE

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SUMMARY

AC-3579 (2-*N*-methylpiperazinomethyl-1,3-diazafluoranthen 1-oxide) produces in rat hepatocytes a hypertrophy of the endoplasmic reticulum.

Two possibilities that can explain this phenomenon are (1) that AC-3579 inactivates the phospholipases, and (2) that an AC-3579-lipid interaction hinders the enzymic activity.

To demonstrate these hypotheses, a physicochemical model of biological membrane, the lipid-water interface, has been used. Dipalmitoyl DL-α-phosphatidylcholine was spread at the air-water interface, the enzymes (phospholipase A or phospholipase C) dissolved in the aqueous phase.

The enzymic reaction was first studied with and without AC-3579 dissolved in the aqueous phase; no enzymic inactivation was observed. However an AC-3579-lipid complex completely inhibited the enzymic reaction in the case of phospholipase A.

An explanation is given in terms of steric hindrance to the enzyme-substrate complex formation.

INTRODUCTION

Recent studies have shown that various drugs may produce an abnormal accumulation of lipid material in cells of many tissues (lung, liver, spleen, etc.) [1, 2]. These lipidoses are due either to an inhibition of the phospholipid degradation or to a stimulation of their biosynthesis.

Hildebrand et al. [3] showed that AC-3579, a diazafluoranthen derivative (2-*N*-methylpiperazinomethyl-1,3-diazafluoranthen 1-oxide) (Fig. 1) caused a hypertrophy of the endoplasmic reticulum in rat hepatocytes by accumulating phospholipid material. The incorporation of labelled precursors ([^{14}C]ethanolamine, [^{32}P]-orthophosphate) was not significantly modified in treated rats and controls [4]. This result demonstrated an unaltered lipid biosynthesis. Consequently the accumulation of the phospholipids in the liver must be due to a modification of the phospholipid breakdown.

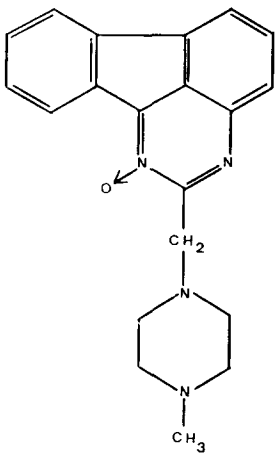

Fig. 1. AC-3579: 2-*N*-methylpiperazinomethyl-1,3-diazafluoranthen 1-oxide.

Two possibilities were proposed to explain this phenomenon. (1) AC-3579 inactivates the phospholipases. (2) An AC-3579 - lipid interaction hinders the enzymic activity.

In order to explain the mechanism of action of the drug, a physicochemical model of biological membrane, the lipid-water interface, has been used. The phospholipids were spread in the close-packed state which would mimic the packing conditions in the biological membrane. The enzymes (phospholipases A and C) were injected in the aqueous phase.

The enzymic reaction was first studied with and without AC-3579 dissolved in the aqueous phase (hypothesis 1). If no enzyme inactivation occurs, the existence of an AC-3579-lipid complex will be considered and the nature of the interactions will be detailed. A modification of the enzymic process due to the complex will then be investigated (hypothesis 2).

MATERIALS AND METHODS

Materials

Phosphatidylserine and dipalmitoyl DL-α-phosphatidylcholine were purchased from Mann Research Laboratories. Phospholipids were spread from a chloroform solution using an Agla Microliter Syringe Unit.

The phospholipase A (*Vipera russelli*), enzymic activity 5 units/mg, and the phospholipase C (*Clostridium welchii*), enzymic activity 5 units/mg, are Sigma Chemical Company Products.

Buffered solutions (Tris · HCl 10^{-2} M, pH 7.4) were used to prepare the subphase. The Ca^{2+} concentration was fixed at 10^{-2} M.

AC-3579 (Christiaens Laboratories, Brussels, Belgium) was generously supplied by Dr. J. Hildebrand (Department of Medicine and Clinical Investigation, Institut J. Bordet, Brussels). AC-3579 was tritiated in pure tritium gas [5]. To increase the rate of exchange between tritium and hydrogen, the system was subjected to a silent electric discharge for 45 min. The tritiated sample was dissolved in 1-4 dioxane

and precipitated with light petroleum (40–65 °C). Its specific activity is 140 μCi/mg. The purity of the tritiated compound was checked by thin-layer chromatography and adsorption at the air-water interface. No change in surface tension at equilibrium was observed between the adsorbed tritiated and untritiated compounds.

Tritium-labeled dipalmitoyl DL-α-phosphatidylcholine was obtained as described elsewhere [6] by quaternization of the respective phosphatidylethanolamine with tritium-labeled methyl iodide (International Chemical and Nuclear Corporation). No traces of phosphatidylethanolamine or of any intermediary products were detected by infrared spectroscopy [7, 8]. The specific activity is 0.5 μCi/mg.

All the experiments were carried out at a temperature of 25 °C.

Methods

Surface pressure measurements were made on a Cahn Rg electrobalance using the Wilhelmy plate method. The surface radioactivity was measured with a gas flow counter [9, 10].

RESULTS AND DISCUSSION

Inactivation of the phospholipases due to an interaction with the drug

Dipalmitoyl DL-α-phosphatidylcholine was spread at the air-water interface at a surface concentration of 2.3 mg/m^2. Immediately after injection of the enzyme in the aqueous phase a drastic decrease of surface pressure was noticed (Figs. 2 and 3). This fall of surface pressure obtained for the phospholipases A and C was identified with the enzymic activity. Now if a mixture of AC-3579 and enzyme was injected, the rate of enzymic cleavage, in both cases, was not modified (Figs 2 and 3). It must be concluded that AC-3579 has no direct effect on the activity of the enzymes used. The hypothesis of a direct inhibition of phospholipases may consequently be excluded.

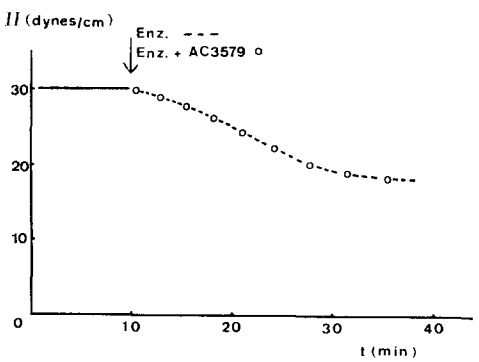

Fig. 2. Surface pressure-time relationship during the hydrolysis of dipalmitoyl DL-α-phosphatidylcholine (C_s = 2.3 mg/m^2) by phospholipase C (0.1 mg/l) without (- - -) and with (○) AC-3579 (bulk concentration, 5 mg/l) dissolved in the subphase. $pH_{support}$, 7.4; buffer, Tris · HCl, 10^{-2} M; Ca^{2+}, 10^{-2} M; temperature, 25 °C.

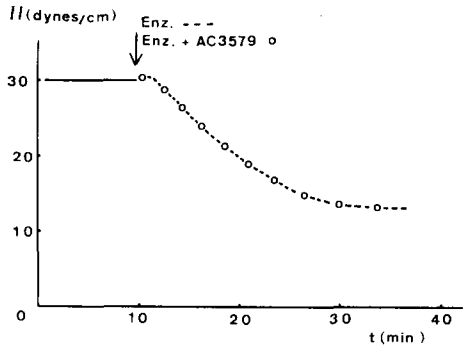

Fig. 3. Surface pressure-time relationship during the hydrolysis of dipalmitoyl DL-α-phosphatidylcholine (C_s = 2.3 mg/m^2) by phospholipase A (2 mg/l) without (- - -) and with (○) AC-3579 (bulk concentration, 5 mg/l) dissolved in the subphase. The enzymes were used under standard conditions.

Complex AC-3579-phospholipid

The phospholipids are spread at the air-water interface at the same concentration (C_S = 2.3 mg/m^2.) Tritiated AC-3579 is injected in the aqueous phase. Surface radioactivity measurements allow its adsorption to be followed.

The surface concentration of the adsorbed AC-3579 in a layer immediately adjacent to the interface was estimated from the well defined specific activity of AC-3579 and from the counter efficiency. This last value was determined for every end window by comparison with a spread tritium-labeled monolayer of known radioactivity [11]. For an AC-3579 bulk concentration of 5 mg/l, the AC-3579 surface concentration is 1.65 mg/m^2.

In Fig. 4, the AC-3579 surface concentration is plotted against the phospholipid surface concentration. As the number of phospholipid molecules increases, the

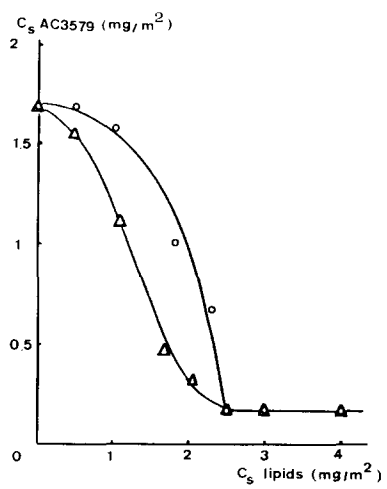

Fig. 4. AC-3579 adsorption. AC-3579 surface concentration (C_s) as a function of the phospholipid surface concentration (C_s). (○), dipalmitoyl DL-α-phosphatidylcholine; (△), phosphatidylserine. AC-3579 bulk concentration, 5 mg/l. Bulk standard conditions.

number of adsorbed molecule decreases. For a close-packed film, the adsorption is no more modified.

Moreover, the number of AC-3579 molecules adsorbed does not depend on the lipid charge. Indeed, identical results (Fig. 4) were obtained for dipalmitoyl DL-α-phosphatidylcholine with a zero net charge and for the phosphatidylserine with a negative net charge at pH 7.4. Only for diluted films, a small difference was observed. This may be a consequence of the slightly negative charge of the AC-3579 at this pH of the subphase. It can nevertheless be accepted that the AC-3579 adsorption is not modified by electrostatic interactions between the drug and the polar head of the phospholipids. Moreover the adsorption of the AC-3579 on the lipid film does not depend significantly on the pH values of the support. In the range $pH_{support} = 2$ to $pH_{support} = 10$, the AC-3579 surface concentration varies between 0.1 mg/m^2 ($pH_{support} = 2$) and 0.2 mg/m^2 ($pH_{support} = 10$). It must be pointed out that the number of AC-3579 molecules adsorbed at the lipid-water interface is less important than the number adsorbed at the air-water interface. Probably the hydrophilic groups of the lipid do not allow the approach of the AC-3579 molecules [10].

A final experiment shows that there is a strong AC-3579-lipid interaction. The AC-3579 is injected under the phospholipid film. At equilibrium, a volume of the aqueous radioactive subphase is removed and replaced with an equal volume of buffered solution. Using this procedure, the concentration of AC-3579 in the support can be modified from 20 mg/l to 0.5 mg/l. Surprisingly, no modification of the surface radioactivity was detected. This means that there are interactions between the hydrocarbon chains of the phospholipid and the aromatic rings of the drug retaining the AC-3579 molecules in the lipid film. The stoichiometry of the complex is eight molecules of phospholipid for one molecule of AC-3579. This result was calculated from the known quantities of spread phospholipid and absorbed AC-3579.

Phospholipase action on the AC-3579-phospholipid complex

The existence of an AC-3579-lipid complex would render the substrate less accessible to enzymatic action. To check this hypothesis, a lipid film is spread at the air-water interface; AC-3579 is injected in the subphase. When the stabilization of the surface pressure is reached, the enzyme is added into the aqueous phase. Fig. 5 shows that, in the case of phospholipase C, the existence of a complex does not modify the enzymic reaction. Indeed, the decrease of surface pressure observed is the same with and without AC-3579 dissolved in the aqueous phase. The conclusion is quite different in the case of phospholipase A. Indeed, the unaltered surface pressure (Fig. 5) shows that the complex completely inhibits the action of the enzyme.

To complete the study, the results obtained with the surface pressure measurements were compared with those of radioactivity. The dipalmitoyl DL-α-phosphatidylcholine tritiated on the choline group is spread at the air-water interface. The increase in bulk radioactivity observed in adding the enzyme was used as a measure of enzymic activity. Table I gives the percentage of radioactivity (compared with the initial surface radioactivity) in the subphase after 80 min of enzymic hydrolysis. Again the inhibition of the phospholipase A action by the AC-3579-lipid complex is demonstrated.

This inhibition is clearly not a consequence of a modification of the ζ potential of the lipid layer. Indeed, to allow the enzymic action, this ζ potential must be nega-

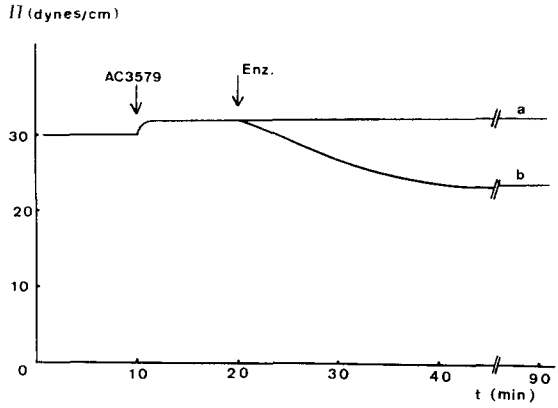

Fig. 5. Surface pressure-time relationship during the hydrolysis of dipalmitoyl DL-α-phosphatidylcholine (C_s = 2.3 mg/m^2) by phospholipase A (2 mg/l) (Curve a) and phospholipase C (0.1 mg/l) (Curve b) under standard conditions. In a first step, AC-3579 (5 mg/l) is injected in the subphase. At equilibrium, the enzyme is injected.

tive for the phospholipase A [12] and positive in the case of phospholipase C [13]. The penetration of the AC-3579 molecule, with its slightly negative charge, in the monolayer would provide an easier hydrolysis in the case of phospholipase A and an inactivation of phospholipase C. Such results are not observed.

However the enzymic hydrolysis depends on the substrate concentration [14–16]. Generally, the rate of enzymic cleavage is greatly reduced at high and low surface concentrations of the substrate. One can thus suppose that a modification of the surface pressure, due to the AC-3579 adsorption, modifies the enzymic kinetics.

It was checked that an increase of 2 dyne/cm in surface pressure of the substrate does not modify the kinetics. Moreover, a change in packing of the lipid film would have the same effect for the two enzymes used; this is not observed.

It must be concluded, therefore, that the AC-3579-phospholipid interaction introduces a steric hindrance to the enzyme-substrate complex formation. This is supported by the observation that phospholipase C which hydrolysis the hydrophilic part of the phospholipid is not inhibited, whereas phospholipase A, which acts on the hydrophobic groups of the phospholipid, is inactivated.

TABLE I

RADIOACTIVITY MEASUREMENTS OF ENZYMIC ACTIVITY

Tritium-labeled dipalmitoyl DL-α-phosphatidylcholine ([^{3}H]PC) surface concentration: 2.3 mg/m^2. The enzymes were used under standard conditions.

Enzyme	Phospholipase activity (%) on		
	[^{3}H]PC	[^{3}H]PC + AC-3579 in the subphase	[^{3}H]PC – AC-3579 complex
Phospholipase C	85	85	85
Phospholipase A	67	71	0

CONCLUSIONS

In a recent paper, Hildebrand et al. [17] investigated the endogenous and exogenous phospholipase action on liver homogenates of rats treated with AC-3579. The results revealed a significant inhibition of endogenous and exogenous phospholipase A. The results obtained in the present work with a physicochemical model agree with his statement. Indeed, the existence of an AC-3579-lipid complex has no influence on phospholipase C action but inhibits completely the enzymic activity of the exogenous phospholipase A.

A possible mode of action is that the presence of an important hydrophobic group in the AC-3579 molecule allows its penetration into the lipid layer. The steric inhibition would be due to the hydrophilic group, which does not allow the formation of the enzyme – substrate complex.

Moreover, a large number of compounds known to have the property to modify the enzymic breakdown of lipids show hydrophobic and hydrophilic groups. Small variations in the molecule structure, changing these hydrophilic and hydrophobic properties, may cause them to lose their effect on the phospholipid metabolism. The question of the importance of this amphiphilic balance is now under investigations.

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