Structure-Dependent Biological Activity of Racemic 1-Substituted 2-O-Hexadecylglycero-3-phosphocholines and Analogues

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1-substituted 2-O-hexadecylglycero-3-phosphocholines and some structural analogues were tested for their biological activity on two entirely different test systems: human thrombocytes and potato virus X in tobacco plants. Structural prerequisites for a high activity in both systems are the alkyl glyceryl ether structural element, a short-chain substituent in the C-1 position, and the phosphocholine grouping. Accordingly, 1-O-methyl derivative (**2**) and 1-O-acetyl derivative (**5**) have the strongest effect on the biological activity of both test systems. The intensity of the effect of the tested compounds does not correlate with membranolytic properties measured by the lytic action on red cells.

Introduction

Glycerophospholipids with a lipophilic alkyl chain or with two different long alkyl chains are known to have membranolytic or fusogenic effects [1–3]. The so-called platelet-activating factor (PAF = 1-O-alkyl-2-O-acetyl-sn-glycero-3-phosphocholine), which has a similar structure, is produced and secreted by different types of leukocytes upon immune and non-immune challenges [4] and obviously plays an important role as mediator of allergic [4, 5] and inflammatory reactions [6, 7]. Also, PAF and its analogues are potent stimulators of blood platelet activation in different species [8–11]. The extent to which membrane effects are primarily involved in these processes is not properly understood at present; perhaps PAF and analogous compounds are bound to a specific membrane receptor [12]. Membrane properties play an important role in another biological process – the replication of viruses [13, 14]. Therefore it is of interest to know the role of glycerophosphocholines in virus multiplication. This paper deals with the structure-effect relations of synthetic racemic 1-substituted 2-O-hexadecyl-glycero-3-phosphocholines and of some structural analogues using two different biological systems (human blood platelets and viruses undergoing multiplication in tobacco plants).

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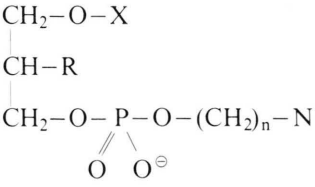
Materials and Methods

Compounds

Eight compounds (Table I) were prepared with the following general structure:



The methods of preparation of compounds **1**–**4**, **6**, and **8** were analogous to [2], [15], and [16], respectively. **5** and **7** were prepared according an analogous procedure to [17] from the corresponding lyso compounds by acetylation with acetic anhydride in the presence of absolute pyridine.

Blood collection

Blood obtained by venipuncture from healthy volunteers was collected into 0.1 vol. trisodium citrate (0.106 M) and immediately centrifuged at

Table I. Racemic 1-substituted 2-O-hexadecylglycero-3-phosphocholines and analogues.

Compound number	X	*n*	R
1	H	2	$O-C_{16}H_{33}$
2	CH_3	2	$O-C_{16}H_{33}$
3	CH_2-CH_3	2	$O-C_{16}H_{33}$
4	$CH_2-C_6H_5$	2	$O-C_{16}H_{33}$
5	$COCH_3$	2	$O-C_{16}H_{33}$
6	H	3	$O-C_{16}H_{33}$
7	$COCH_3$	3	$O-C_{16}H_{33}$
8	$COCH_3$	2	$C_{16}H_{33}$

$200 \times g$ for fifteen minutes at room temperature to give platelet-rich plasma (PRP). PRP was kept in tightly capped plastic tubes at 37 °C and was used for platelet studies which were started after one hour from blood collection.

Platelet aggregation

Platelet aggregation was measured turbidimetrically by recording changes in light transmission using an ELVI dual-channel aggregometer. Aggregation was triggered by addition of 50 µl of inducer to 0.5 ml of PRP at a cuvette temperature of 37 °C and at a constant stirring speed of 900 revolutions per minute.

Lytic properties of red cells

Erythrocytes were washed twice with phosphate-buffered saline (PBS) having a pH value of 7.4, and they were subsequently centrifuged for ten minutes at $400 \times g$. One ml of packed red cells was suspended in 100 ml of PBS and then used for the measurement of lytic properties. For an estimation of lytic properties, 0.1 ml of solutions of each compounds was added to 1 ml of a suspension of red cells. Albumin saline (2.5 g of human serum albumin and 0.15 M of sodium chloride per litre) served as control and Triton X-100 (1%) was used to induce complete lysis. After incubation for thirty minutes at a temperature of 37 °C, samples were centrifuged for two minutes at $8000 \times g$. 0.5 ml of each supernatant was transferred to 1 ml of PBS and the optical density was measured at 546 nm.

The 2-O-alkylglycerophosphocholines were dissolved in albumin saline, stored at a temperature of − 20 °C, and preheated to 37 °C before use.

Acetyl salicylic acid (ASA, Sigma Chemical Co.), creatine kinase (CK), and creatine phosphate (CP) were dissolved in saline, kept at a temperature of 4 °C, and used for a period of one week. Both CK and CP were obtained from Boehringer (Mannheim, FRG).

Virus and host plant

The virus strain used in these studies was potato virus X (PVX) H19. The systemic host plants *Nicotiana tabacum* L. "Samsun" were cultivated in an air-conditioned room (16-hours-day; 9000 lux; 20 ± 2 °C). When the tobacco was in the four-leaf stage (about 10 weeks after seeding) it was used for experiments.

Arrangements of experiments indicating antiphytoviral activity

The tobacco plants were sprayed two days before and two days after inoculation with PVX three times with 1 mM aqueous solutions of each compounds until the leaves were covered by a film of solution. Two fully developed leaves of each plant were inoculated mechanically using diluted crude sap of infected plants. Details are described elsewhere [18].

Virus assay

Serological determination of PVX content in inoculated (= primarily infected) and systematically (= secondarily) infected leaves was after 6 ± 1 days and after 12 ± 1 days from inoculation, respectively. The upper of the two inoculated leaves and the secondarily infected leaf separated by one leaf from the closest inoculated one were used for virus detection. Sap was pressed from the leaves, heated (40 °C, 10 min) and then clarified by centrifugation ($5600 \times g$). The clarified sap was diluted with physiological sodium chloride. The last dilution step where virus could be detected by precipitin drop test was noted. The average end point dilution (aED) is the mean of at least ten values. The experiment was repeated twice.

Results

Platelet stimulating activity

In experiments using 1 to 100 µM of the 2-O-hexadecylglycero-3-phosphocholines and analogues

1 to **8**, a concentration dependent platelet aggregation is obtained only with the compounds **2, 3, 5,** and **7** in human PRP. The highest platelet stimulating power shows the 1-O-acetyl derivative (**5**). 5 μM of this compound is capable to .produce maximum platelet aggregation (Fig. 1). The 1-O-methyl derivative (**2**) is shown to be only slightly less active than compound **5**. On the other side, seven to eight-fold higher concentrations of the derivative **3** and **7** are necessary for producing the same platelet response. The platelet aggregation induced by 2-O-hexadecylglycero-3-phosphocholines also takes place in the presence of acetylsalicylic acid or a mixture of creatinekinase/creatine phosphate (Fig. 2).

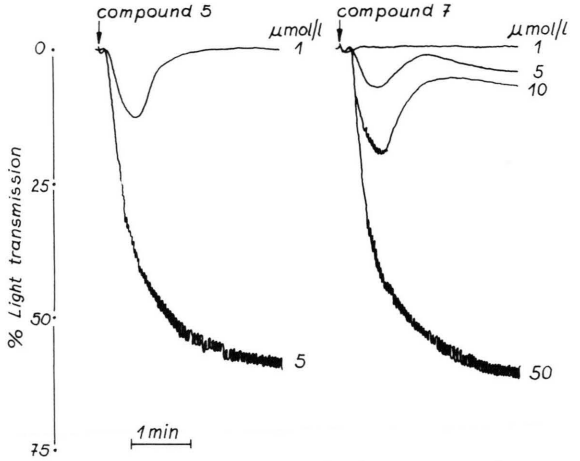


Fig. 1. Aggregation of human platelets. Aggregation was performed in human PRP as described in "Materials and Methods". Final concentrations of compounds added as indicated by arrows are given at the endings of the traces. Shown here is one out of three experiments all of which yielded similar results.

Using a concentration of 300 μM the lysophospholipids **1** and **6** trigger a small and reversible platelet aggregation. By contrast, the compounds **4** and **8** are completely inactive.

Antiphytoviral activity

The results obtained using PVX indicate a marked inhibitory activity of phospholipids **1**, **2**, and **5** (Table II). As is well known from screening for antiphytoviral effects, primarily infected leaves, in spite of having direct contact with the virus, usually are poor indicators of antiviral effects. This is also true for the viral inhibitions caused by the active agents in **1** and **2**, but is not equally true of **5**.

In the case of secondarily infected leaves, the strongest effect is produced by **2**, whereas **5** and **1** exhibit roughly the same intensity.

Membranolytic properties

The compounds **1**–**8** in concentrations from 3 to 100 μM were tested for lytic action on washed red cells (Table III). A measurable hemolysis is only

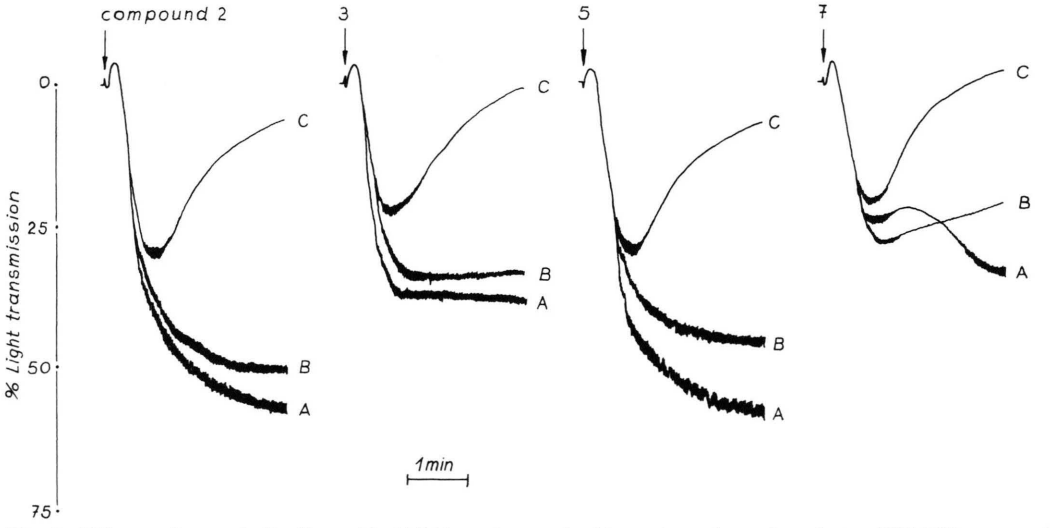


Fig. 2. Effects of acetylsalicylic acid (ASA) and creatinekinase/creatine phosphate (CK/CP) upon human platelet aggregation. Aggregation was triggered in human PRP by adding substances in a final concentration of 100 μM in the absence (A) or presence of 1 mM of ASA (B) or CK/CP (83.5 μkat/l/2 mM) (C) which was added three minutes before aggregation was triggered. The results shown here represent one out of three experiments that yielded similar results.

Table II. Effect of the compounds **1–8** on the content of potato virus X in primarily and secondarily infected tobacco leaves (*Nicotiana tabacum* L. "Samsun").

Compound	Virus content (aED)[a]					
	Primarily infected leaves			Secondarily infected leaves		
	Without treatment (control)	Compound administered	Inhibition [%]	Without treatment (control)	Compound administered	Inhibition [%]
1	4.3	4.3	± 0.0	4.4	3.1	+59.4
2	4.3	4.2	+ 6.7	4.4	2.2	+78.2
3	4.3	4.3	± 0.0	4.4	4.5	− 6.7
4	4.3	3.6	+ 38.4	4.4	3.9	+29.3
5	6.3	4.9	+ 62.1	4.7	3.2	+64.7
6	5.1	4.5	+ 34.0	5.5	5.4	+ 6.7
7	5.1	4.9	+ 12.9	5.5	5.0	+29.3
8	6.3	5.6	+ 38.4	4.7	5.2	−29.3

[a] aED = average end point dilution (see "Materials and Methods").

Table III. Lytic action of 1-substituted 2-O-hexadecylglycero-3-phosphocholines and structural analogues. Human red cells were treated with various concentrations of compounds for 30 min at 37 °C and the resulting hemolysis was quantified as described in "Materials and Methods". Average values of three experiments are documented.

Compound	% Hemolysis			
	Concentration [μM]	12.5	25	50
1		0.7	14.5	98.4
2		0.5	49.0	99.6
3		0.1	26.1	98.1
4		0.3	19.6	98.2
5		1.6	41.6	99.6
6		0.5	14.2	98.4
7		0.5	24.7	97.8
8		1.2	74.7	100.7

observed with concentrations higher than 12.5 μM. Using 50 μM or higher concentrations a complete hemolysis results with each compound. In the presence of plasma proteins, however, the membranolytic effects are greatly suppressed. Accordingly, a concentration of 100 μM of all compounds is incapable to produce any hemolysis in whole blood.

Discussion

The results provide evidence that 1-substituted 2-O-hexadecylglycero-3-phosphocholines and analogues, besides membranolytic effects, possess also platelet activating and antiphytoviral properties.

The platelet aggregation triggered by 2-O-hexadecylglycero-3-phosphocholines obviously occurs in analogy to that of PAF-acether largely independent of the metabolization of arachidonic acid and the effect of ADP which is supported by experiments with acetylsalicylic acid and creatinekinase/creatine phosphate. However, the concentration of the most effective compound **5** for triggering platelet aggregation is about tenfold higher than that of PAF-acether [19].

Structural comparison of the tested phospholipids indicates that the same structural requirements are of great importance both in the platelet activating properties and the inhibitory action to PVX, the molecular mechanisms of which are entirely different. Thus, the 1-O-acetyl derivative (**5**) and the 1-O-methyl derivative (**2**) show the highest biological activity in both test systems. Lengthening of the alkyl chain in position 1 results in an insignificant antiviral activity and a markedly decreased platelet stimulating power. Substitution of the choline head group by the 1-trimethylaminopropan-3-ol residue also causes a substantial loss of biological activities. Moreover, the 1-O-acetyl-2-hexadecyl-propanediol-3-phosphocholine (**8**) was proven to be completely inactive in both test systems although lack of the etheral oxygen is the only structural difference from the most active compound **5**. Only the lyso compound **1** out of the phospholipids tested shows a major difference in biological activity with a marked reduction of virus

content contrasting with an insignificant platelet stimulating activity.

These facts indicate that the alkyl glycerol backbone, a short chain substituent in position 1 and the phosphocholine grouping are of primary importance for antiphytoviral and platelet stimulating activity of the 2-O-hexadecylglycero-3-phosphocholines and analogues.

As a whole the reduction of virus content gives no information about the modes of the antiviral action. It is possible that the virus multiplication is inhibited but a hindered distribution of viruses in plant tissue may also be suggested. Further investigations are necessary to elucidate this problem. Since a hemolyzing action was demonstrated for all of the compounds in this class of substances, it was reasonable to assume a correlation between lysis and biological activity in the test systems. However, the results obtained clearly show that, interalia, the analogue **8** with the greatest lytic efficiency, is completely inactive. This seems to suggest that membranolytic properties of the compounds do not correlate with the biological activities observed.

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