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THE ACTION OF PHOSPHOLIPASE C AND LIPASE ON BLACK FILM BILAYER MEMBRANES*

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Phospholipase C (*C. perfringens*) added to one side of a phosphatidyl choline–phosphatidyl serine–cholesterol (0.7 : 0.3 : 1.0, w/w/w) bilayer membrane resulted in a rapid decrease in membrane resistance which leveled off after several minutes at about one-half its initial value. Pancreatic lipase added at this time resulted in a rapid increase in membrane resistance to a value somewhat higher than the initial. This effect was independent of the side to which the lipase was added, indicating that diglyceride is rapidly equilibrated across both sides of the membrane. When both phospholipase C and lipase were added at zero time to the same or opposite sides, the resistance decreased slightly and then increased to a value higher than the initial. Replacement of phosphatidyl choline by 2-hexadecoxy-3-octadecoxypropyl-phosphonylcholine, a phosphonate analog, resulted in an inhibition of the phospholipase C reaction which was equal to the percent analog. The analog, thus, seemed to act only as inert lipid and did not inhibit hydrolys of the active lipid.

I. Introduction

In a recent study of the action of phospholipase C (*C. perfringens*) on bilayer membranes [1], we observed that the addition of this enzyme to one side of a phosphatidyl choline–phosphatidyl serine–cholesterol (0.7 : 0.3 : 1.0, w/w/w) bilayer membrane resulted in a rapid decrease in membrane resistance. The maximal rate of resistance decrease was proportional to the amount of enzyme added. The effects of membrane composition, calcium ion concentration and pH on the rate of resistance decrease were reported. Since the product of this enzyme reaction, diglyceride, should accumulate in the bilayer, a study of lipase action on these phospholipase C-treated membranes was undertaken. The results of this study are presented here.

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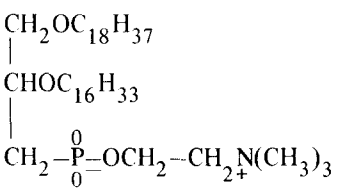
We also report on the inhibition of phospholipase C action by 2-hexadecoxy-3-octadecoxypropylphosphonylcholine (1), a phosponate analog of phosphatidyl choline. Since the inhibition



(1)

of phospholipase C by this analog in aqueous dispersions of phosphatidyl choline was reported by Rosenthal and Pousada [2], we were interested in studying the effect of this analog in the bilayer membrane system.

II. Experimental

A. Materials

Phosphatidyl serine was prepared from beef brain by the method of Folch [3]. Phosphatidyl choline was prepared from egg yolk by the method of Singleton et al. [4], and was shown to be chromatographically homogeneous by TLC. 2-Hexadecoxy-3-octadecoxypropylphosphonylcholine was a gift from Dr. A.F.Rosenthal. Cholesterol (A grade) was purchased from Calbiochem. Phospholipase C (*C. perfringens*, ~1 unit/mg) was obtained from Nutritional Biochemicals. Lipase (hog pancreatic, ~100 units/mg) was obtained from Worthington Biochemical Corporation.

B. Bilayer membrane studies

Bilayer membranes were prepared from a solution of phosphatidyl choline, phosphatidyl serine and cholesterol (0.7 : 0.3 : 1.0, w/w/w) in n-decane (1.5% w/w) (unless otherwise specified) as described by Hendrickson and Scattergood [1]. The membrane resistance was determined using Ag–AgCl electrodes by measuring the voltage drop across the membrane with 50 mV applied potential in series with a known resistance [5]. Experiments were begun after the membrane was completely black and gave stable resistance readings. The temperature was 25 ± 1°C unless otherwise stated. The electrolyte solution contained 0.1 M NaCl, 0.01 M Tris chloride buffer (pH 7.2), and 4 mM $CaCl_2$. Solutions of phospholipase C (2 mg/ml) and lipase (1.7 mg/ml) were added as indicated to the inner cup (volume = 13 ml). Both compartments were stirred by magnetic stirrers at 740 rpm.

C. Analysis of the products of phospholipase C and lipase action in lipid dispersions

The incubation mixture consisted of 0.2 ml of a sonicated dispersion of lipids (3.0 mg/ml phosphatidyl choline, 1.6 mg/ml phosphatidyl serine, 5.5 mg/ml cholesterol), 2.5 ml pH 7.2 Tris chloride buffer (0.01 M Tris, 0.1 M NaCl, 4 mM $CaCl_2$), 0.1 ml phospholipase C (2 mg/ml) or 0.1 ml phospholipase C and 0.1 ml lipase (1.7 mg/ml), and water to 5 ml. The mixture was incubated at 37°C for 30 min with occasional shaking. Chloroform–methanol (2 : 1, v/v) was then added, the mixture was shaken and centrifuged, and the lower chloroform layer concentrated to 1 ml for TLC analysis. A control (no enzyme) was also incubated under the same conditions.

TLC analysis of the lipid products was performed on silica gel HR plates using a $CHCl_3$: CH_3OH : HOAc : H_2O (25 : 15 : 2 : 1, v/v/v/v) solvent for phospholipids and diethyl ether for neutral lipids. Spots were visualized by exposure to iodine vapor.

III. Results and Discussion

A. Phospholipase C/lipase action on bilayer membranes

The addition of phospholipase C (*C. perfringens*) to one side of a phosphatidyl choline-phosphatidyl serine-cholesterol membrane (0.7 : 0.3 : 1.0, w/w/w) resulted in a rapid decrease in membrane resistance which leveled off after several minutes as reported previously [1]. If pancreatic lipase was added at this time to the same side of the membrane to which the phospholipase C was added, the membrane resistance rapidly increased to a value somewhat higher than the initial value. These resistance

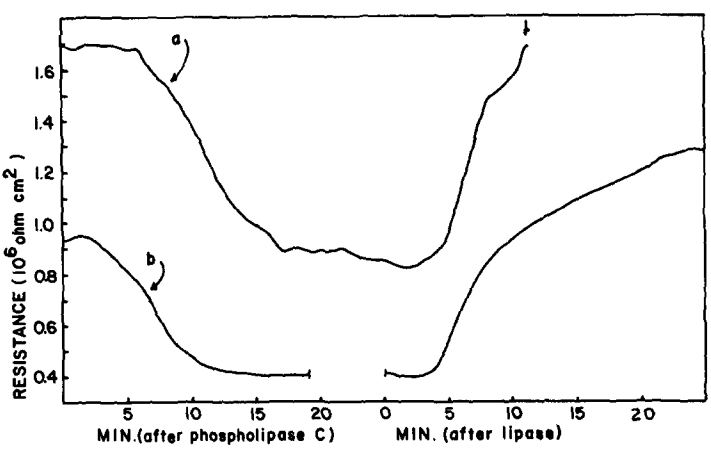


Fig. 1. Action of Phospholipase C and Lipase on Bilayer Membranes. Phospholipase C added first, then lipase added on same side (a) or opposite side (b). Conditions described in text. Arrow indicates where membrane broke.

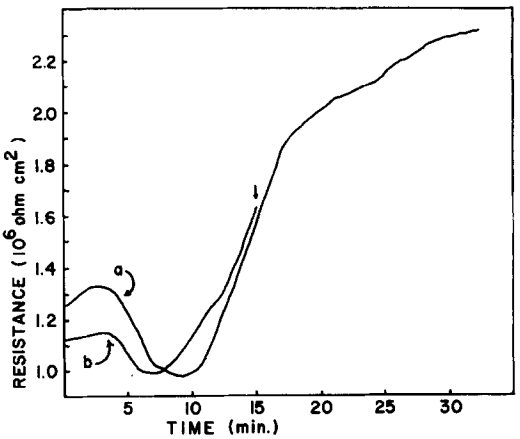


Fig. 2. Action of Phospholipase C and Lipase on Bilayer Membranes. Both enzymes added at zero time to same side (a) or opposite sides (b). Conditions described in text. Arrow indicates where membrane broke.

changes are shown in fig. 1a. If the lipase was added to the opposite side of the membrane to which the phospholipase C was added the same results occurred as shown in fig. 1b. In fact, within experimental error, little difference in the resistance change was observed when the lipase was added on the same or the opposite side to which the phospholipase C had been added. Lipase added alone (without phospholipase C) caused no change in membrane resistance or stability. When phospholipase C and lipase were added together at zero time, a small decrease in membrane resistance was observed followed by a somewhat larger increase as shown in fig. 2. Again, no significant differences were observed if the two enzymes were added on the same or opposite sides. The maximum resistance changes of bilayer membranes in several sets of experiments are shown in table 1. It can be seen that the rate of resistance increase after the addition of lipase is somewhat proportional to the amount of lipase added, similar to the dose response of phospholipase C reported previously [1].

These results indicate that diglyceride does indeed accumulate in the bilayer after phospholipase C treatment since subsequent treatment with lipase results in a dramatic change in membrane resistance, and treatment of the membrane with lipase alone has no effect on membrane resistance or stability. The diglyceride which accumulates in the membrane must rapidly distribute itself across both sides since the side to which the lipase is added makes no difference in the change of membrane resistance. Although Kornberg and McConnell [6] have reported a half-time of several hours for the transverse (flip-flop) migration of phosphatidyl choline molecules in lipid bilayers, the uncharged glycerol moity of diglyceride can conceivably pass through the hydrophobic region with a much lower energy barrier. The possibility that lipase can penetrate the bilayer and cause hydrolysis of diglyceride on the opposite side may also explain these results, although this possibility seems unlikely.

Table 1
PHospholipase C/lipase action on bilayer membranes[1].

Initial resistance	Phospholipase C	Maximum resistance change	Lipase	Maximum resistance change
(10^6 Ω cm^2)	μl (2 mg/ml)	(10^6 Ω cm^2/min)	μl (1.7 mg/ml)	(10^6 Ω cm^2/min)
Phospholipase C added – when resistance stabilized, lipase added to same side				
1.70	5	−0.0875	10	+0.186
Phospholipase C added – when resistance stabilized, lipase added to opposite side				
0.93	5	−0.0750	10	+0.109
1.28	5	−0.0792	15	+0.166
1.68	5	−0.107	20	+0.326
Both enzymes added to same side at zero time				
0.98	5	−0.0531	10	+0.0306
1.36	5	−0.0506	10	+0.0419
1.25	10	−0.0822	10	+0.136
Both enzymes added to opposite sides at zero time				
1.05	5	−0.0250	10	+0.0455
1.31	10	−0.0567	19	+0.0987
1.13	10	−0.0583	10	+0.0962

[1] Conditions as described in text.

B. Products of phospholipase C/lipase action on lipid dispersions

Mixtures of phosphatidyl choline, phosphatidyl serine and cholesterol were incubated in aqueous dispersion with phospholipase C and lipase under conditions similar to those of the bilayer studies, and the products were analyzed by TLC. A control (no enzyme) showed spots for only the original lipids. Incubation with phospholipase C produced diglyceride as the only lipid product. Most of the phosphatidyl choline was hydrolized while the phosphatidyl serine spot appeared as intense as in the control. Incubation with both phospholipase C and lipase produced mostly monoglyceride with a small amount of diglyceride. The phosphatidyl choline was completely hydrolyzed while the phosphatidyl serine spot was as intense as in the control. These results indicate that only the known reactions of phospholipase C and lipase are occurring under conditions similar to those of the bilayer studies.

C. Inhibition by Phosphonate Analog

Inhibition of the phospholipase C reaction by the phosphonate analog (1) is shown in table 2. These experiments were performed at 38°C to aid in thinning of

Table 2
Inhibition of phospholipase C by phosphonate Analog[1].

	Maximum resistance change (10^5 Ω cm^2 min^{-1})		
% Analog [3]	5 μl Enzyme	10 μl Enzyme	% Inhition
0	−2.6 ± 0.5 (4)[2]	−5.1 ± 0.3 (3)	—
28		−3.4 ± 0.6 (3)	33
57	−2.4 ± 1.5 (5)		8
78	−0.75 ± 0.1 (2)		71
100		−0.34 ± 0.31 (2)	93

[1] Phosphatidyl choline, phosphatidyl serine, cholesterol (0.7 : 0.3 : 1, w/w/w) bilayer incubated with phospholipase C as described in text. T = 38°C.
[2] Standard deviation (number of experiments).
[3] Phosphatidyl choline replaced by 2-hexadecoxy-3-octadecoxypropyl-phosphonylcholine.

the membrane at high analog concentrations. Except for the experiments with 57% analog which were quite variable, the percent inhibition seemed to follow the percent analog present. Thus, the analog appears to act only as inert substrate and does not inhibit hydrolysis of the active substrate. This is contrary to the inhibition of phospholipase C by the analog in aqueous dispersions where Rosenthal and Pousada [2] found over 90% inhibition with 50% analog. Inhibition in aqueous dispersions could be due to the multibilayer structure of the micelles. Complete hydrolysis of the phosphatidyl choline in this system would have to involve reorganization of the micelle structure and/or penetration of the enzyme into the inner bilayers. These processes could be inhibited by the presence of analog. In the black film bilayer system there is a possibility that the composition of the bilayer is not the same as that of the original lipid solution. Since there is no way to evaluate this uncertainty, these studies are in no way definitive.

IV. Conclusions

Lesslauer et al. [7] have reported on the effects of phospholipase A on black film bilayer membranes. Membranes were formed from lecithin analogs which were resistant to enzyme hydrolysis. The resistance of these membranes was lowered by factors of 100 and 1000 in solutions of 10^{-5} M pre-phospholipase A and phospholipase A respectively, as compared to the value in 10^{-2} M $CaCl_2$ in the absence of protein. They attributed this lowering of resistance to either the adsorption of protein molecules at the membrane surface or the penetration of a few protein molecules into the hydrocarbon region. In the studies reported here, the concentrations of enzymes used were less than 10^{-8} M. Since the addition of lipase alone had no effect on membrane resistance, and the subsequent addition of phospholipase C resulted in a slight decrease followed by an increase in membrane resistance (see fig. 2)

rather than the decrease observed when phospholipase C was added alone, it would appear that the resistance changes reported here are due to enzyme catalyzed hydrolysis of the phospholipid rather than simply adsorption or penetration of enzyme. This conclusion is also supported by the inhibition studies with the phosphonate analog.

The limitations of black film bilayer studies are such that analysis of lipid composition and determination of surface properties are not practical. Therefore, one can only speculate concerning a mechanism for these resistance changes. If the lipids are homogeneously distributed throughout the bilayer, the choline phosphate groups of phosphatidyl choline could act to screen the negative charge repulsion between the phosphatidyl serine molecules. The removal of these choline phosphate groups by phospholipase C action could then result in an expansion of the bilayer film as this screening is removed, and an increase in ion conductance. Removal of the diglyceride by lipase action could allow the chelation of calcium ions between adjacent phosphatidyl serine molecules. This would result in a decrease in charge repulsion, a contraction of the film, and a decrease in ion conductance. This mechanism is, of course, very hypothetical and perhaps quite oversimplified. We propose to test features of this mechanism in monolayer studies with synthetic lipids and purified enzymes.

These results indicate that phospholipase action on one side of a bilayer membrane could be an effective way of modifying membrane permeability. Such a mechanism could be involved in the control of ion permeability in the nerve axon or synaptic membrane. We have proposed a model for the involvement of disphosphoinositide-triphosphoinositide interconversion in the control of Na^+/K^+ permeability [8]. Durell [9] and others [10–12] have suggested a model for the control of ion permeability in the synaptic membrane involving the action of phosphoinositide phosphodiesterase, a phospholipase C specific for phosphoinositides. This enzyme is very active in brain tissues [13]. De Scarnati and Arnaiz [14] have reported a stimulation of this enzyme by acetyl choline in nerve ending membranes; however, Schacht and Agranoff [15] have presented evidence which contradicts this reported stimulation. Thus, the importance of this mechanism has not been substantiated. However, in view of these implications, further study of the action of phospholipases on bilayer membranes should prove interesting in understanding these possible mechanisms.

Acknowledgements

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