THE ACTION OF PHOSPHOLIPASE C ON BLACK FILM BILAYER MEMBRANES

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Summary: The effects of phospholipase C (Clostridium perfringens) on the
electrical and visual properties of black film bilayer membranes are reported.
Addition of this enzyme to one side of a phosphatidyl choline : phosphatidyl
serine : cholesterol (0.71 : 0.29 : 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. The maximal rate of resistance decrease
was proportional to the amount of enzyme added. A maximal effect was observed
with phosphatidyl choline : phosphatidyl serine ratios between 1:1 to 9:1. No
effect was observed in the absence of Ca^{2+} and an optimal effect was observed
at 2 m$\underline{M}$ Ca^{2+}. A pH optimum of 7.2 was observed. These studies demonstrate
the possibility of forming asymmetric membranes by limited phospholipase
action on one side of a bilayer membrane. Such a system can be used to study
the mechanism of enzyme action on a substrate which is part of a bilayer mem-
brane and the possible role of asymmetric membranes in membrane processes such
as the control of ion permeability.

Introduction: Phospholipase C, along with other phospholipases, has been

widely used to probe the structure and functions of biological membranes

(Roelofsen, et al., 1971; Lenard and Singer, 1968; McIlwain and Rapport, 1971).

Although the action of this enzyme has been studied with intact biological

membranes and phospholipid dispersions or vesicles, very little has been re-

ported on the action of this enzyme on black film bilayer membranes. The

possibility of forming asymmetric membranes by limited phospholipase action on

one side of a black film bilayer membrane, and the possible involvement of

such asymmetric membranes in membrane processes such as the control of Na^+/K^+

permeability in the nerve axonal membrane (Hendrickson and Reinertsen, 1971;

Ohki and Papahadjopoulos, 1970; Ohki, 1971) prompted a study of phospholipase C

action on black film bilayer membranes. The effects of phospholipase C from

Clostridium perfringens on the electrical and visual properties of black film

membranes are reported here. Although this crude enzyme has been shown to

contain phosphodiesterase activities specific for sphingomyelin as well as phosphoglycerides (Pastan, et al., 1968), the term phospholipase C is used for convenience and does not exclude the possible involvement of more than one enzyme.

Experimental: Phosphatidyl serine was prepared from beef brain by the method of Folch (Lees, 1957). Phosphatidyl choline was prepared from egg yolk by the method of Singleton, et al., (1965) and was shown to be chromatographically homogeneous by TLC. Phospholipase C (C. perfringens) was obtained from Nutritional Biochemicals.

Bilayer membranes were formed across a 1.5 mm diameter hole in a teflon cup in an apparatus similar to that described by Huemoeller and Tien (1970). The thickness of teflon at the hole was 0.3 mm. Both compartments were stirred with magnetic stirrers. The electrolyte solution consisted of 100 mM NaCl, 10 mM CaCl$_2$, and 10 mM tris buffer, pH 7.2 unless specified otherwise. All the experiments were carried out at a temperature of 27°±1°. Resistance and capacitance were measured with Ag-AgCl electrodes. The membrane resistance was calculated from the voltage drop across the membrane in series with a known resistance (Huemoeller and Tien, 1970). Capacitance was measured with a General Radio 1650-A impedance bridge. The membrane was observed with reflected light by a 40X binocular scope. Mixtures of phosphatidyl choline, phosphatidyl serine, and cholesterol were prepared in n-decane (1.5% w/w). Experiments were begun after the membrane was completely black and gave stable resistance and capacitance readings.

Results and Discussion: The addition of phospholipase C to one side of a phosphatidyl choline-phosphatidyl serine-cholesterol membrane (0.71 : 0.29 : 1.0, w/w/w) resulted in a rapid decrease in membrane resistance which leveled off after several minutes at about one-half its initial value as shown in Figure 1. The membrane resistance was measured continuously by applying 50 mv to the membrane and series resistor and recording the voltage drop across the
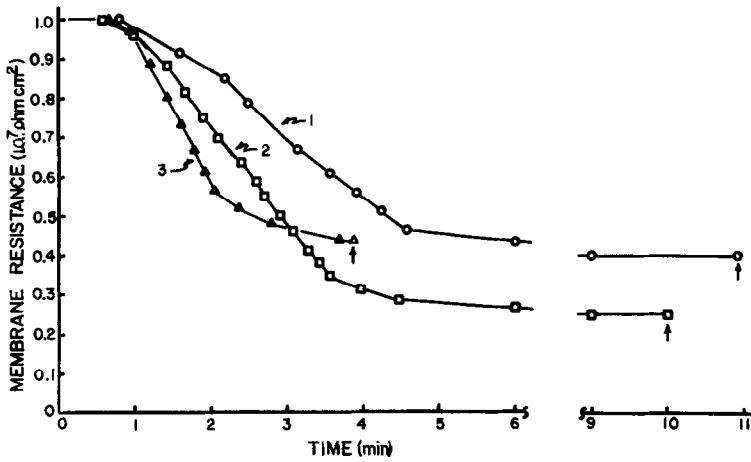
Figure 1.   Membrane resistance versus time after addition of phospholipase C.
Concentration of enzyme: (1) 0.44 µg/ml; (2) 0.88 µg/ml;
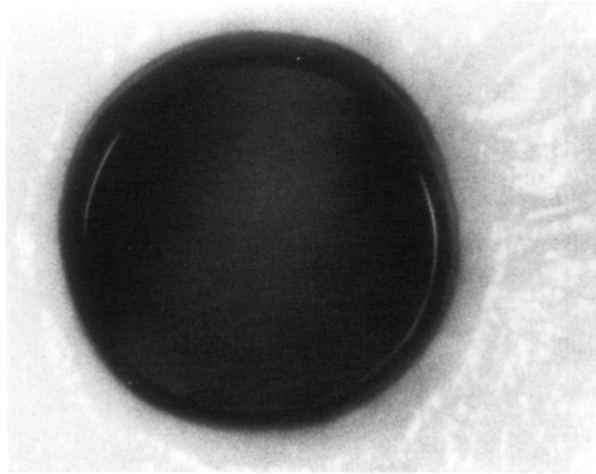(3) 1.76 µg/ml. Stirring rate: 740 rpm.   ↑ - indicates time
membrane broke.

Figure 2.   Membrane before addition of enzyme, capacitance = 0.42 µF/cm^2.

membrane. With high concentrations of enzyme the membrane broke as the resis-

tance was leveling off, while with low concentrations of enzyme the membrane

did not break, but the resistance, after decreasing and leveling off, increased

to a value sometimes slightly greater than its initial value. The capacitance

increased, as the resistance decreased, to a value about 30% greater than the

initial value. At low enzyme levels, after the resistance had decreased and
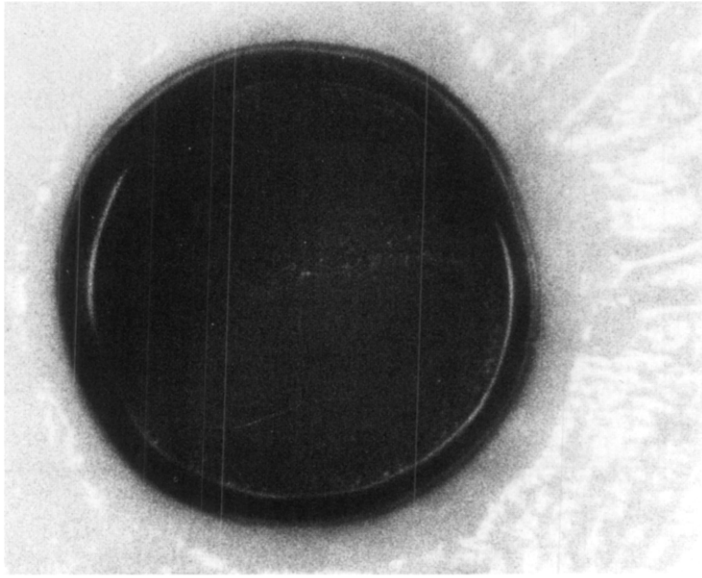
Figure 3.   Membrane 15 minutes after addition of enzyme,
            capacitance = 0.51 µF/cm^2.
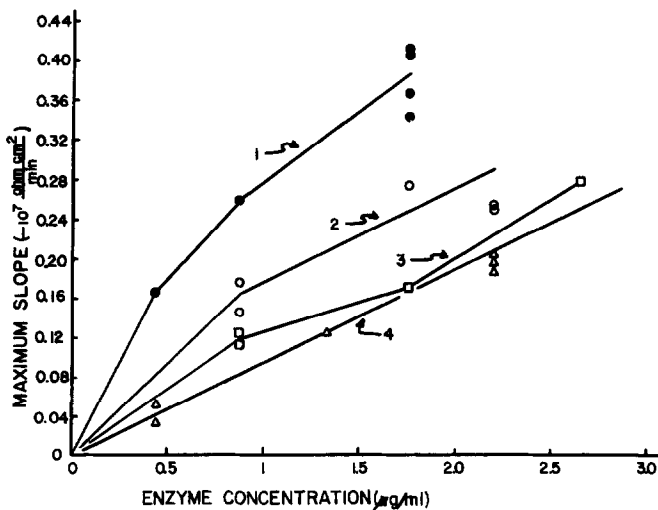

Figure 4.   Rate of resistance decrease versus enzyme concentration.
            (1)   1 week old lipid solution, stirring rate: 740 rpm.
            (2)   1 week old lipid solution, stirring rate: 580 rpm.
            (3)   5 week old lipid solution, stirring rate: 740 rpm.
            (4)   10 week old lipid solution, stirring rate: 580 rpm.

leveled off, the capacitance then decreased (as the resistance slowly in-

creased) to a value sometimes less than its initial value. Several minutes

after addition of the enzyme many small light spots appeared on the membrane

which appeared to coalesce and become larger and more spread out in time. Figures 2 and 3 show the membrane before and 15 minutes after addition of the enzyme. It can be seen that the capacitance increase from 0.42 to 0.51 $\mu F/cm^2$ is not due to an increase in membrane area.

The maximum rate of resistance decrease was proportional to the amount of enzyme added as shown in Figure 4. The slope of this curve was also a function of the age of the lipid solution and the rate of stirring. In subsequent experiments these factors were kept as constant as possible.

The effect of lipid composition on the rate of resistance decrease is shown in Figure 5. A maximum effect is seen between phosphatidyl choline : phosphatidyl serine ratios of about 1:1 to 9:1 (w/w). With no phosphatidyl serine the membranes were not as stable and there was little decrease in resistance after addition of the enzyme. Also, the appearance and capacitance of the membranes did not change significantly after addition of the enzyme. The phosphatidyl choline was apparently still hydrolyzed, however, since the membranes all broke within 12 minutes after addition of the enzyme, but did not break in the absence of Ca^{2+}. With phosphatidyl serine, but no phosphatidyl choline, there was no change in resistance, capacitance, or appearance, nor did the membranes break after addition of enzyme.
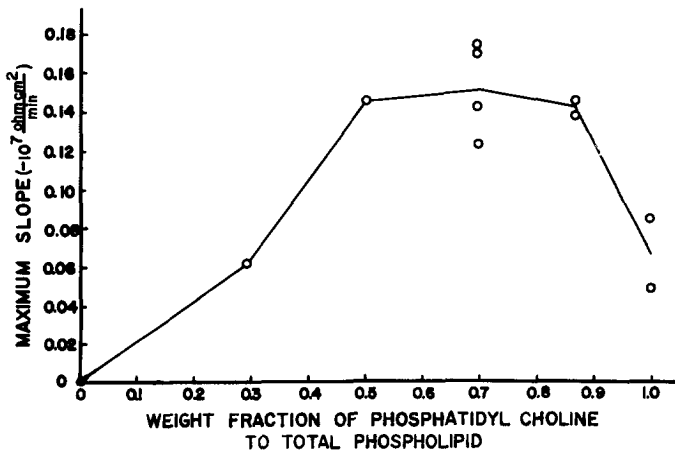

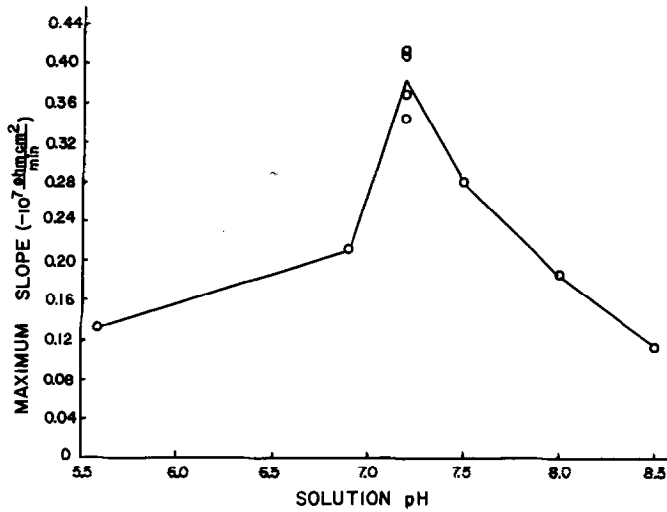Figure 5. Rate of resistance decrease versus lipid composition (enzyme concentration: 1.76 μg/ml, stirring rate: 740 rpm).

Figure 6.   Rate of resistance decrease versus pH (enzyme concentration:
1.76 µg/ml, stirring rate: 740 rpm; Acetate buffer used at
pH = 5.6).

The enzyme gave an optimum response at pH 7.2 as shown in Figure 6.
This is similar to the pH optimum reported by MacFarland and Knight (1941) for
the C. welchii enzyme and Bangham and Dawson (1962) for the C. perfringens
enzyme with micellar dispersions of phosphatidyl choline. Ca^{2+} was necessary
for enzyme activity since in its absence no effect on the membrane was ob-
served. A Ca^{2+} concentration of 2 mM was required for optimum activity as is
shown in Figure 7. A second plateau of lower activity was seen between 0.2
and 1.0 mM Ca^{2+}. The optimal Ca^{2+} concentration of 2 mM is similar to that
observed in micellar dispersions by MacFarland and Knight (1941) and Bangham
and Dawson (1962). The sharp increase in activity between 1 and 2 mM Ca^{2+} may
be due to a reorganization of lipids in the membrane. Butler, et al., (1970)
observed an effect of Ca^{2+} on the ESR signal of spin-labeled myelin lipid
vesicles between 1 and 10 mM Ca^{2+}, which they interpreted as an organizational
change of lipids in the membrane. Papahadjopoulos (1968) observed a change in
the surface pressure and surface potential of phosphatidyl serine monolayers
at about this same Ca^{2+} concentration.

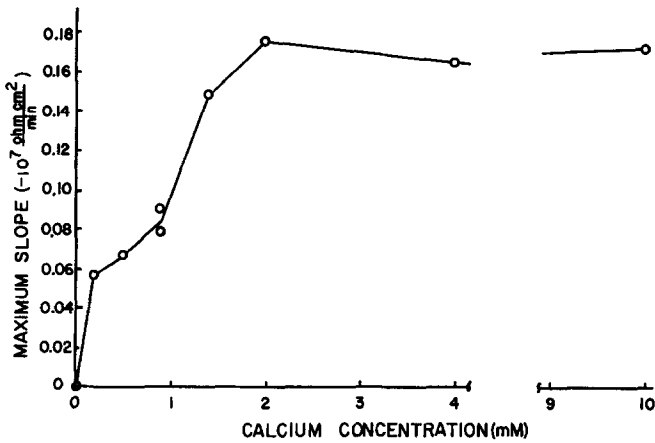The exact nature of the resistance and capacitance changes is not

Figure 7.   Rate of resistance decrease versus calcium concentration (enzyme
concentration: 1.76 µg/ml, stirring rate: 740 rpm).

known. These results indicate that phosphatidyl serine is not hydrolyzed to

any great extent. McIlwain and Rapport (1971) report hydrolysis of only 14%

of phosphatidyl serine in myelin vesicles versus 92% of phosphatidyl choline

after incubation with phospholipase C (C. perfringens). Hydrolysis of phos-

phatidyl choline may cause an increase in inter-phosphatidyl serine repulsion

resulting in an expansion of the film and a decrease in resistance. A decrease

in the ζ potential to more negative values may result from the accumulation of

diglyceride and reduced Ca^{2+} binding. This may account for the cessation of

enzyme activity at a certain point as evidenced by the leveling off of

resistance. Addition of a second portion of enzyme on the same side of the

membrane, after the resistance had leveled off, had no further effect on the

membrane. However, addition of enzyme, then, to the opposite side resulted in

breakage of the membrane. This would indicate that there is no rapid redis-

tribution of lipids from one side of the membrane to the other. This also

indicates that the enzyme does not penetrate the lipid bilayer as was suggested

by McIlwain and Rapport (1971) in their study of phospholipase C action on

myelin vesicles. Their results can be explained by migration of diglyceride

into the interior of the micelle and its replacement by phosphatidyl choline

migrating from the interior (Bangham and Dawson, 1962), or by lysis and reorganization of the micelle bilayer structure. The spots which appear on the membranes could be caused by the accumulation of diglyceride which probably remains with the membrane (Lenard and Singer, 1968, Ottolenghi and Bowman, 1970).

Although Ohki (1971) has postulated that asymmetric bilayer membranes which differ in surface charge from one side to the other should exhibit a transmembrane potential, no such membrane potential could be measured with these enzyme-treated membranes. Phospholipase C treatment, however, does not change the net charge on phosphatidyl choline. Changes in lipid packing and calcium binding brought about by loss of choline phosphate may not sufficiently alter the surface charge to give a measurable transmembrane potential. There was no rectification of current in the membrane since the polarity of the applied voltage used to measure the membrane resistance had no effect on the resistance measured.

These studies demonstrate the possibility of forming asymmetric membranes by limited enzyme action on one side of a black film bilayer membrane. This system can be used to study the mechanism of enzyme action on a substrate which is part of a lipid bilayer and also the possible role of phospholipase action in membrane function such as the control of Na^+/K^+ permeability in the nerve axonal membrane. Studies are now being conducted on the effects of other phospholipases on black film bilayer membranes.

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