### Title

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### Journal

Biophysical Journal, 37(1)

### ISSN

0006-3495



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### Publication Date

1982

### DOI

10.1016/s0006-3495(82)84653-5

Peer reviewed

# PHOSPHOLIPASE $A_2$ ACTION ON MEMBRANE SURFACES

## EFFECT OF IONIC STRENGTH, TRITON X-100, AND MELITTIN

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Phospholipase $A_2$ has been used extensively as a probe for the distribution of phospholipids in membrane surfaces. The implicit assumption has been that the activity and specificity of the enzyme are dependent on the distribution of the phospholipids across the membrane. However, to achieve significant hydrolysis of intact erythrocytes, nonphysiological conditions and/or high concentrations of enzyme are necessary (1). In previous studies using low concentrations of a highly purified homogeneous preparation of phospholipase $A_2$ from cobra venom (*Naja naja naja*) and metabolically competent human erythrocytes, we found that under initial rate conditions the enzyme is barely active toward the outer surface of intact erythrocytes, but that the ghosting procedure results in an enormous activation of the enzyme toward phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (1, 2). With phospholipid mixtures in Triton X-100 mixed micelles, we have found that phospholipase $A_2$ activation toward PE is dependent on the direct binding of specific phosphorylcholine-containing activator lipids to the enzyme (3, 4).

For a valid interpretation of the distribution of membrane phospholipids using phospholipase $A_2$, keeping in mind possible phospholipid activation of the enzyme, we must assess the cause(s) of the low activity of phospholipase $A_2$ toward intact erythrocytes, and whether activation toward the erythrocyte results from prehemolytic subtle alterations in the outer surface of the membrane or from posthemolytic exposure of the lipids at the inner membrane surface. We have now examined the action of cobra venom phospholipase $A_2$ toward minimally perturbed human erythrocytes to see if hydrolysis can be activated in the absence of hemolysis.

### MATERIALS AND METHODS

Fresh, defibrinated human erythrocytes were prepared in varying ionic strength solutions of NaCl in buffer (20 mM Hepes, pH 7.4 and 5 mM D-glucose) (1). Duplicate incubation mixtures were prepared in a total volume of 2.0 ml and included 0.5 ml packed erythrocytes (1 μmol phospholipid), the same buffer, 10 mM $CaCl_2$, and an ionic strength of 306 mosmol (measured) unless indicated otherwise. Samples were incubated for 1 min at 37°C in a shaking water bath prior to the addition of 2 μg enzyme purified as in previous studies (1). The reaction was quenched after 10 min with the addition of 0.3 ml 0.5 M EDTA and 0.5 ml methanol. Following organic extraction, total and individual phospholipid concentrations were determined by two-dimensional thin-layer chromatography and phosphorus analysis described previously (1). Where indicated, Triton X-100 (Rohm and Haas Co., Philadelphia, PA) or melittin (Sigma Chemical Co., St. Louis, MO) first treated with *p*-bromophenacyl bromide (BPB) to inactivate any residual bee venom phospholipase $A_2$ (4) were included in the incubations. Hemolysis was determined on duplicate samples (not treated with EDTA and methanol) by the method of Anderson et al. (5). For the measurement of total hemoglobin, Triton X-100 was added to the samples to give a final concentration of 9 mM during preincubation. Triton X-100 up to 18 mM does not interfere with hemoglobin absorbance measurements at 540 nm.

### RESULTS AND DISCUSSION

Table I shows that a two-fold decrease in ionic strength, reported to effect shape changes (discocyte to spherocyte) but not to exceed the critical hemolytic volume (CHV) (6), resulted in a significant activation of phospholipase $A_2$ toward PE and PC, with PC the preferred substrate.

The action of phospholipase $A_2$ toward erythrocytes as

TABLE I
ACTION OF COBRA VENOM PHOSPHOLIPASE $A_2$ TOWARD HUMAN ERYTHROCYTES AT VARIOUS OSMOTIC STRENGTHS

| Osmotic strength (mosmol) | Phospholipid (mol fraction) | | | | | | Total phospholipid hydrolysis (%) | Total hemolysis (%) |
|---|---|---|---|---|---|---|---|---|
| | PE | LPE | PC | LPC | PS | SM | | |
| 306 | 0.37 ± 0.02 | 0.00 ± 0.00 | 0.31 ± 0.04 | 0.01 ± 0.00 | 0.14 ± 0.02 | 0.16 ± 0.01 | 1 | 0 |
| 188 | 0.38 ± 0.04 | 0.02 ± 0.00 | 0.27 ± 0.06 | 0.06 ± 0.01 | 0.14 ± 0.01 | 0.14 ± 0.01 | 8 | 1 |
| 108 | 0.31 ± 0.01 | 0.10 ± 0.01 | 0.26 ± 0.01 | 0.02 ± 0.00 | 0.15 ± 0.01 | 0.16 ± 0.01 | 12 | * |

The numbers quoted are the mean and error from the mean for duplicate determinations. Lyso PE (LPE) and Lyso PC (LPC) products are indicated; phosphatidylserine (PS) and sphingomyelin (SM) are not hydrolyzed.
*Hemolysis prior to incubation experiments had occurred yielding red ghosts.


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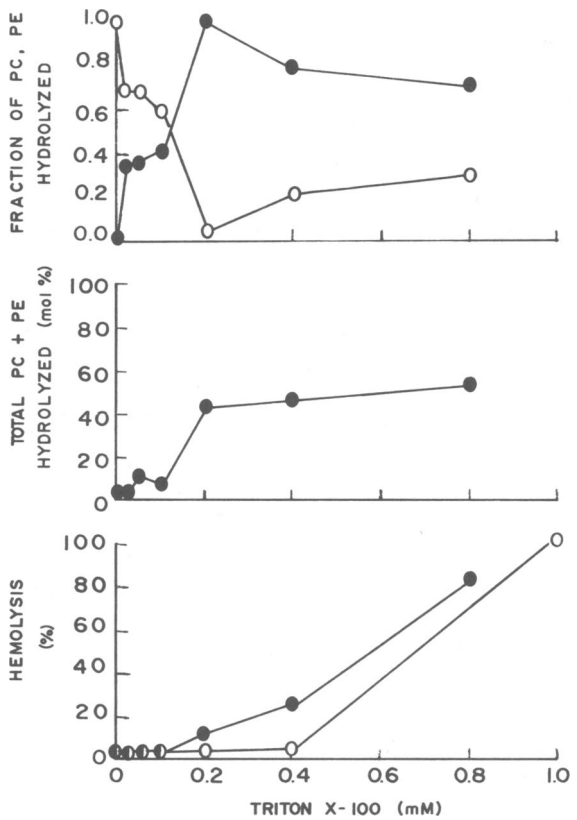


FIGURE 1 The effect of Triton X-100 on erythrocyte hemolysis with (●) and without (○) enzyme present (bottom); phospholipase $A_2$ hydrolysis of total PE and PC (middle); fraction of total phospholipid hydrolyzed which is PE (●) and PC (○) (top). Sphingomyelin is not a substrate of the enzyme and phosphatidylserine is not hydrolyzed significantly under the assay conditions. At low concentrations of Triton X-100 hemolysis is enhanced by enzyme presence and hydrolysis.

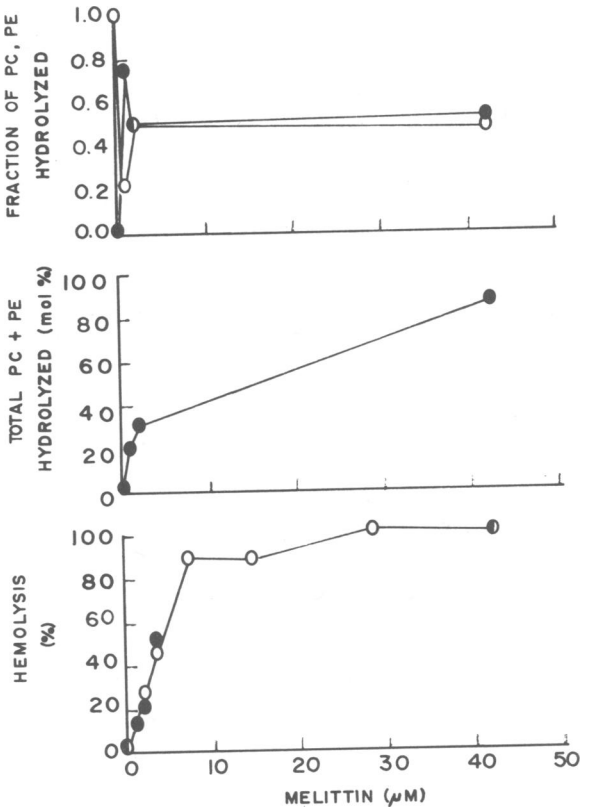


FIGURE 2 The effect of melittin on erythrocyte hemolysis with (●) and without (○) enzyme present (bottom); phospholipase $A_2$ hydrolysis of total PE plus PC (middle); fraction of total phospholipid hydrolyzed which is PE (●) and PC (○) (top). Sphingomyelin is not a substrate of the enzyme and phosphatidylserine is not hydrolyzed significantly under the assay conditions. Hemolysis profiles for melittin show that enzyme presence does not result in further dectable hemolysis.

a function of Triton X-100 concentration is shown in Fig. 1. Triton X-100 at 0.2 mM, which is just below its critical micelle concentration (c.m.c.), produces a large activation of the enzyme toward PE which cannot be accounted for totally by hemolysis; 10% hemolysis should have exposed a total of ~ 0.07 μmol combined PC and PE, yet four times this amount of lipid (0.28 μmol) is hydrolyzed of which over 90% is PE. Increasing surfactant concentration further, which results in almost total hemolysis, did not lead to a further dramatic activation of the enzyme and PE remained the preferred substrate.

In Fig. 2, melittin is observed to be lytic at all concentrations tested and phospholipase $A_2$ hydrolysis appears to parallel hemolysis. At extremely low concentrations of melittin enzyme hydrolysis of PE is preferred; but with higher concentrations of the basic peptide the specificity of the enzyme becomes about equal toward both substrates, PE and PC.

To the extent that hemolysis is an all-or-none phenomenon of hemoglobin release from erythrocytes beyond the CHV (6), under prehemolytic conditions the majority of the erythrocytes are intact and their outer surface is available for probing by the phospholipase $A_2$. The preliminary observations reported here raise the interesting possibility that a large part of the apparent activation of phospholipase $A_2$ hydrolysis is directed toward the outer erythrocyte membrane surface, and results at least in part from prehemolytic subtle alterations in the outer membrane surface produced by lowering the ionic strength or by introducing small amounts of Triton X-100. The activation of the enzyme toward PE when ionic strength is decreased further, which results in hemolysis, and activation at prehemolytic concentrations of the nonionic surfactant Triton X-100, suggest the intriguing possibility that these treatments perturb the membrane and lead to an increased exposure of PE for enzyme hydrolysis at the outer surface of the membrane. Haest and Deuticke (7), using sulfhydryl reagents to perturb the erythrocyte membrane, also report increased exposure of PE to phospholipase $A_2$ hydrolysis at the outer membrane surface.

This work was supported by grant GM-20501 from the National Institutes of Health.

*Received for publication 1 May 1981.*

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# SPECIFICITY IN PROTEIN-MEMBRANE ASSOCIATIONS: THE INTERACTION OF GANGLIOSIDES WITH *ESCHERICHIA COLI* HEAT-LABILE ENTEROTOXIN AND CHOLERAGEN


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Gangliosides, which are complex glycospingolipids, have been implicated in the receptor-mediated effects of numerous agents, including enterotoxins and glycoprotein hormones (6). The nonpolar portion of these compounds, consisting of an *N*-acyl fatty acid derivative of sphingosine, is believed to serve as an anchor in the plasma membrane, whereas the carbohydrate moiety, which is exposed to the extracellular space, is believed to be responsible for receptor specificity. One of the more fully characterized ganglioside-protein interactions is that between ganglioside $G_{M1}$ (galactosy-*N*-acetylgalactosaminyl-[*N*-acetyl-neuraminyl]-galactosylglucosylceramide) and the enterotoxin of *Vibrio cholerae* that is responsible for the clinical characteristics of cholera (3). Choleratoxin or choleragen (CT) is composed of two types of subunits held together by noncovalent interactions. The *A* component, which is believed to be composed of two polypeptide chains joined by a single disulfide bond, activates adenylate cyclase through a NAD-dependent ADP-ribosylation reaction (9). The *B* component, of which there are 4–6 molecules in the intact toxin, is responsible for the specific interaction between the toxin and $G_{M1}$ (3, 10). Thus the initial event in the action of CT on cells is the binding of the *B* component to the oligosaccharide moiety of $G_{M1}$. The remaining events are not known with certainty; the $A_1$ subunit is currently believed to be inserted into the membrane bilayer (presumably after reduction of the disulfide bond that links it to $A_2$) and ultimately ADP-ribosylates a regulatory protein in the cyclase system.

The effects of a heat-labile enterotoxin of *Escherichia coli* (LT) on cells resemble in many ways those of CT. LT also appears to be structurally similar to CT in that it contains two types of subunits with molecular weights similar to those found for CT (11). The catalytic *A* subunit of LT is however believed to be a single polypeptide chain (5, 7) rather than a disulfide linked dimer as found for CT (1, 2, 3). In view of the similar structure and mode of action of these two enterotoxins we have investigated the interaction between LT and $G_{M1}$.

## RESULTS AND DISCUSSION

Incorporation of purified $G_{M1}$ into $G_{M1}$-deficient C6 glioma cells increased the subsequent binding of $^{125}$I-LT or $^{125}$I-CT. This increase was blocked for both toxins by prior treatment of $G_{M1}$-treated cells with unlabeled CT. Treatment of the cells with $G_{D1a}$ (*N*-acetylneuraminylgalactosyl-*N*-acetylgalatosaminyl-[*N*-acetylneuraminyl]-galactosylglucosylceramide, $G_{M2}$ (*N*-acetylgalactosaminyl-[*N*-acetylneuraminyl]-galactosylglucosylceramide) or $G_{D1b}$ (galactosyl-*N*-acetylgalactosaminyl-[*N*-acetyl-neuraminyl-*N*-acetylneuraminyl]-galactosylglucosyl-



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Volume 37 January 1982 168–169