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REGULATORY EFFECT OF SPHINGOLIPIDS ON SECRETORY PHOSPHOLIPASE A_2. COMPARATIVE STUDY USING SUV AND GUV

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Abstract

The regulation of secretory phospholipase A_2 ($sPLA_2$) activity is of great interest due to the role of this enzyme in maintenance of the membrane structure, lipid mediator production and participation in cell signalling. The present study is an attempt to elucidate the role of sphingolipids in this regulation. $sPLA_2$ activity has been investigated using as substrate egg yolk phosphatidylcholine (EYPC) presented in two forms – giant and small unilamellar vesicles (GUV and SUV) mixed with two different sphingolipids – sphingomyelin (SM) and ceramide (CER). The results showed that in the two substrate forms $sPLA_2$ activity was inhibited almost completely by SM and was reversed to its initial value upon substitution of even just 2 mol% of SM with CER. The role of the phospholipid membrane organization in modulation of $sPLA_2$ is discussed.

Key words: ceramide, GUV, phospholipase A_2, sphingomyelin, SUV

Introduction. Phospholipases A_2 (PLA_2) comprise a family of enzyme hydrolysing the ester bond on *sn*-2 position in the glycerophospholipid molecules. The secretory PLA_2 ($sPLA_2$) present several types of enzyme proteins with a molecular mass of about 14 kDa and have been isolated from various tissues and extracellular fluids of humans and mammals, as well as from snake, lizard, turtle, and bee venom [1]. The activity of these $sPLA_2$ is Ca^{2+}-dependent and does not exhibit any specificity for the glycerophospholipid substrate molecular species [2].

In our previous papers [3,4] we illustrated the influence of the substrate composition and properties on $sPLA_2$ activity. We established that the addition of SM to glycerophospholipid substrate leads to a gradual inhibition of the enzyme activity [3]. This inhibition was explained by the affinity of $sPLA_2$ towards SM due to which the formed complex extruded the enzyme from its interaction with the substrate [4]. In experiments with human recombinant type II $sPLA_2$ we showed that the substitution of a part of SM in liposomes prepared of PE/PS/SM (40:10:50 mol/mol) by CER (from 2 to 20 mol%) not only abolished the inhibitory effect of SM, but also induced its marked reactivation [5].

In this paper we present the results obtained in our attempt to visualize the activity of $sPLA_2$ from bee venom on giant unilamellar vesicles (GUV) prepared of pure egg yolk phosphatidylcholine (EYPC), as well as of its mixture with SM and SM/CER. The results were coordinated with the data obtained from investigation of PLA_2 activity on small unilamellar vesicles (SUV) using standard biochemical procedure.

Materials and methods. Egg yolk L-α-phosphatidylcholine (PC), egg yolk sphingomyelin (SM), and the phospholipase A_2 (PLA_2) from bee venom were purchased from Sigma. The fluorescent lipid analogue C12-NBD-PC (PC*) was from Avanti Polar Lipids. Giant unilamellar vesicles (GUVs) were prepared by electroformation [6,7]. A Zeiss Axiovert 135 microscope equipped with Narishige MMN-1 plus MMO-22 micromanipulator and Hamamatsu B/W chilled CCD camera (C5985-10) connected to an image recording and processing system were used. The microinjection of PLA_2 (0.83 mg/ml (activity $\sim$ 1 U/μl) in 0.5 mM HEPES buffer, pH 7.4, and 10 mM $CaCl_2$) was carried out with an Eppendorf Transjector 5246.

The activity of bee venom $sPLA_2$ (3 m-units) [4] was assayed in vitro using 400 μM glycerophospholipid liposomal substrate suspended in 100 mM Tris-HCl (pH 8.6), 5 mM $CaCl_2$ and fatty acid-free BSA (0.1 mg/ml). The mixture (final volume of 250 μl) was incubated at 37 °C for 15 min with rotational shaking. The fatty acids released by the enzyme were extracted and methylated using diazomethane. The fatty acid methyl esters were separated and quantified by gas chromatography as described in [4].

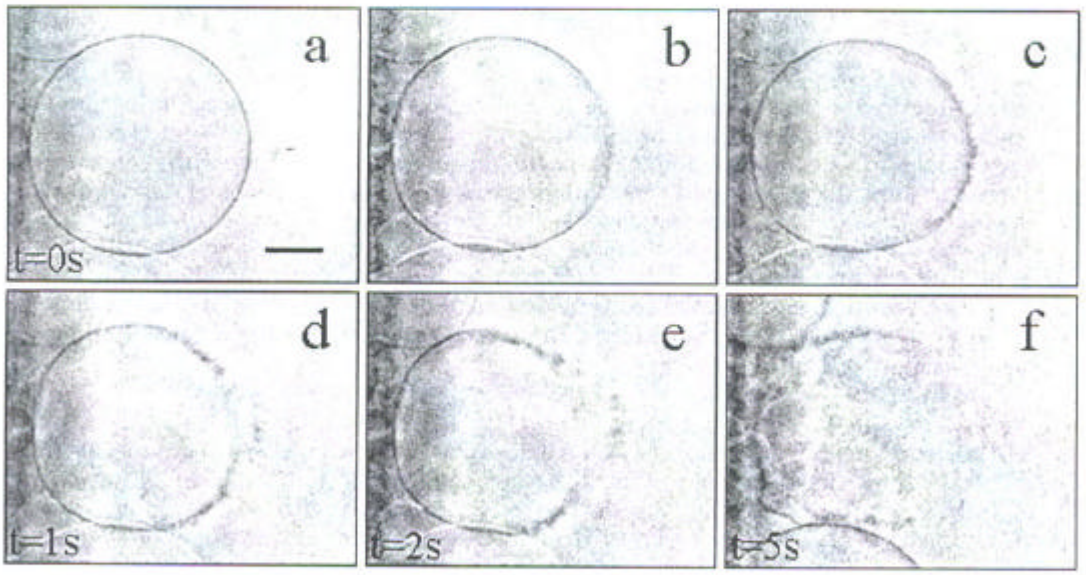

Fig. 1. PLA_2 attack on GUV composed of pure EYPC. Bar = 20 μm

Results. The activity of $sPLA_2$ on GUV prepared of pure EYPC is shown in Fig. 1. As it is evident from Fig. 1, almost immediately of the injection of $sPLA_2$ at a distance of about 15 μm from GUV (a) begins a rapid substrate hydrolysis expressed by disturbance of the membrane integrity (b-d) and only after 5 s a total liposome disintegration (f) and formation of micelles and small vesicles were observed as a result of hydrolysis of a large part of the substrate. When GUV were prepared of EYPC mixed with SM at a ratio 1:1 (mol/mol) well rounded vesicles with no phase separation in micrometric scale (as monitored by light microscopy) were observed which was

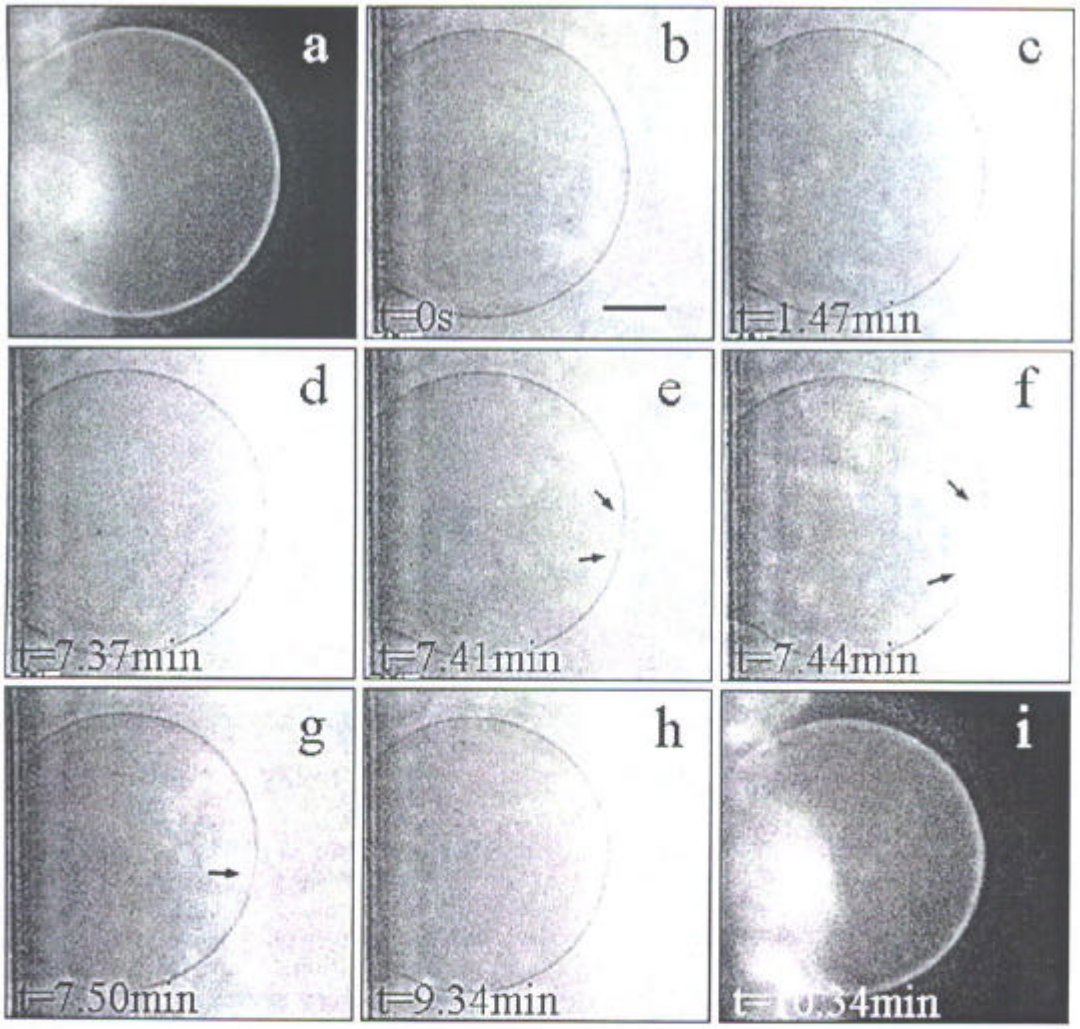

Fig. 2. PLA_2 attack on GUV composed of EYPC and SM at equimolar ratio

determined by incorporation of C12-NBD-PC fluorescent label (Fig. 2, a). Treatment of these vesicles with $sPLA_2$ did not induce any transformations in the liposome shape. Only after a several-time injection of the enzyme (d-f) weak signs of alterations of the liposome contour and slight shrinking of the liposomes indicating a slight hydrolysis were observed. This process, however, was rapidly terminated and the liposome retained its initial shape (h,i). When a small part of SM (2 mol%) in these vesicles was replaced by another sphingolipid such as CER, upon $sPLA_2$ injection (Fig. 3, b) the beginning of a rapid substrate hydrolysis was observed, expressed by a hole opening at the membrane bilayer and a sharp decrease of the liposome diameter (c-i). Of course, in this case the reaction did not proceed with the same rate as in the case with GUV of pure EYPC, but yet an almost complete substrate hydrolysis was observed for about 28 s (i).

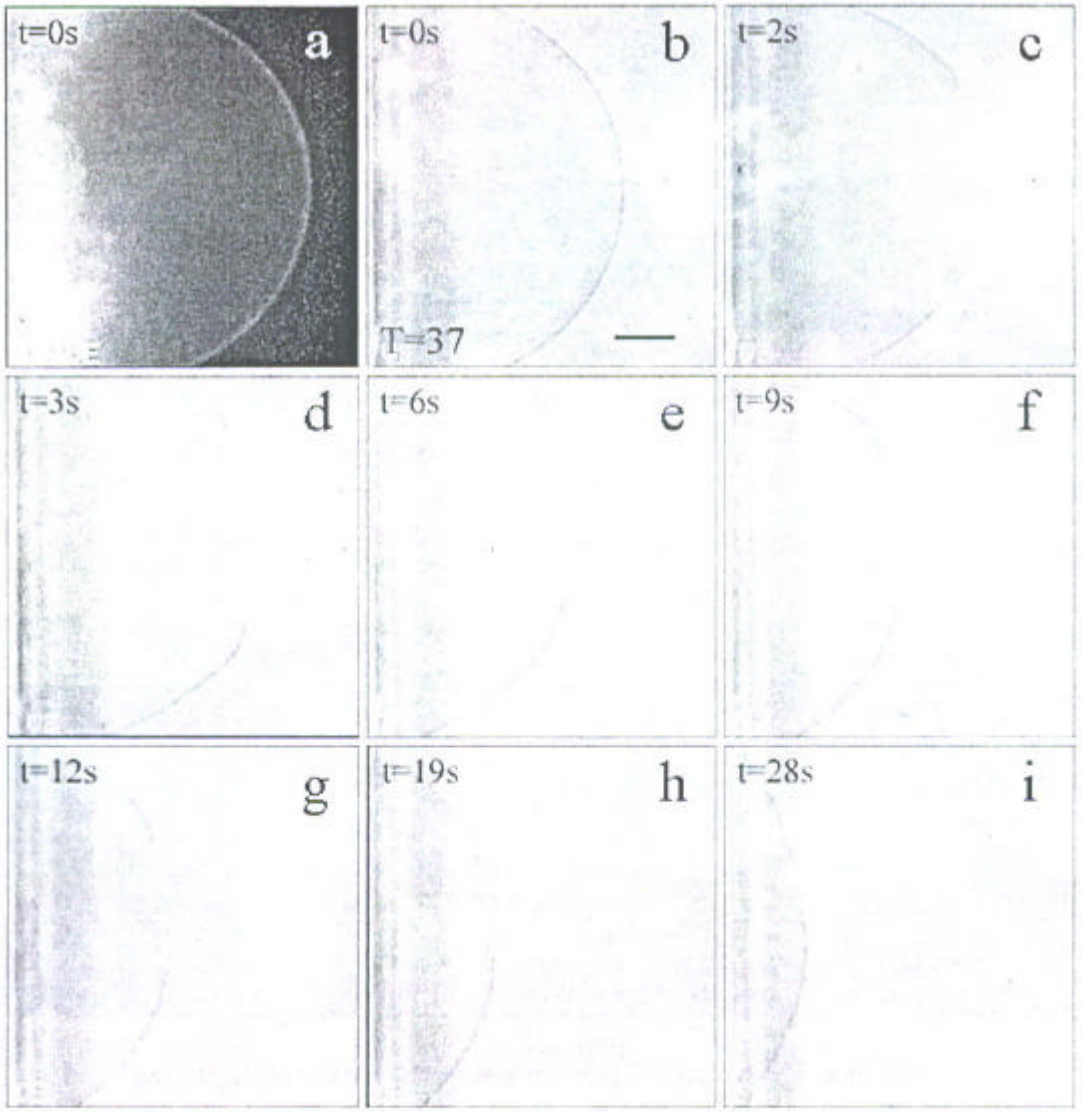

Fig. 3. PLA_2 attack on GUV composed of EYPC/SM/CER (50:48:2 mol/mol)

Similar investigations were performed with SUV having the same phospholipid composition. These liposomes were incubated in the presence of bee venom $sPLA_2$, the enzyme activity being measured by determination of the liberated fatty acids. The activity of $sPLA_2$ hydrolysing SUV containing pure EYPC was considered as 100% (Fig. 4). By elevation of the SM level incorporated into these liposomes, the activity gradually decreased. At a ratio EYPC/SM 1:1 (mol/mol) the enzyme activity was inhibited almost completely (96% inhibition). The replacement of SM by 2, 5 and 10 mol% CER induced enzyme reactivation from 10 to 200%. Only 2 mol% CER were sufficient to cause a complete reactivation of $sPLA_2$ to the one observed with SUV prepared of pure EYPC.

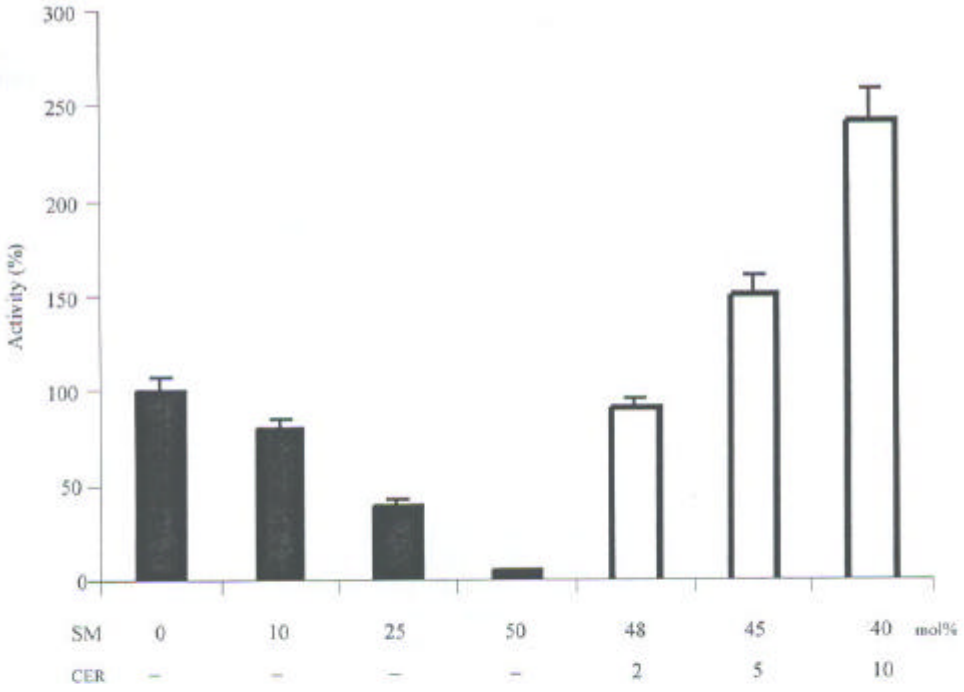

Fig. 4. PLA_2 activity on SUV composed of EYPC and different concentrations of SM and CER

Discussion. The results obtained in this study showed that EYPC acts as very suitable substrate for type III $sPLA_2$, no matter what is the vesicular form – SUV or GUV. The images presented in Fig. 1 illustrate well the so-called "scooting mode" of enzyme activity. The enzyme attack began from the spot where the enzyme was injected (b) and was rapidly spread on the vesicle surface (c-e) to reach a burst in the hydrolysis of the larger part of EYPC (f). In SUV and GUV prepared of a mixture of EYPC and SM at a molar ratio 1:1 an almost complete enzyme inhibition was observed (Fig. 2 and 4). As it is evident from Fig. 4, the inhibitory effect of SM was exerted even at its 10 mol% participation in SUV, which increased progressively upon elevation of SM concentration. In our previous investigations on the activity of human recombinant type II $sPLA_2$ we observed a similar effect of SM, which was shown to be due to the specific binding of this PLA_2 to SM [4]. Probably in the present studies bee venom PLA_2 was bound in a similar way to SM in liposomes prepared of a mixture of EYPC and SM. Depending on SM concentration a larger or smaller part of the enzyme molecules remained free to participate in the hydrolytic reaction. Apparently, at a equimolar ratio of EYPC/SM an almost complete elimination of the enzyme from the reaction was reached, no matter whether SUV or GUV was used (Fig. 2 and 4).

It is well known that due to its specific structure, CER favours phase separation in the phospholipid membranes. HOLOPAINEN et al. [8] showed that CER in concentrations above 5 mol% forms phase-separated microdomains in bilayers of DOPC. Due to its high degree of saturation of the ceramide acyl-chain, CER have a high transition temperature which favours the gel-phase separation at physiological temperatures. All this facilitates the formation of membrane defects which stimulate PLA_2 activity [9]. This fact could explain the observed effect of partial substitution of SM in vesicles

containing EYPC/SM (1:1) by definite concentrations of CER (Fig. 3 and 4). Most probably, since EYPC comprises several different molecular species of PC, the addition of only 2 mol% CER could be sufficient for formation of separate microdomains, favouring $sPLA_2$ activity at the interphase $L\alpha/L\beta$ which we observed to occur in mixture EYPC/SM/CER (50:48:2 mol/mol) (not shown) and which we used in the present study to test the activity of $sPLA_2$ (Fig. 3 and 4).

The visualization of $sPLA_2$ activity towards GUV provides useful and novel information about the mechanism of action of this enzyme. This method turned out to be extremely helpful in investigation of heterogeneous lipid mixtures which form liquid-ordered microdomains of type "rafts", which allowed the observation for the first time and the explanation of the mechanism of the budding and fission processes in GUV and made possible to find certain relevance to analogous phenomena in living cells (to be published).

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