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A model for the molecular mechanism of interfacial activation of phospholipase A_2 supporting the substrate theory

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Changes occurring in the activity of porcine pancreatic phospholipase A_2 upon formation of mixed micelles of sodium cholate and the fluorescent phosphocholines 1,2-di [6-(pyren-1-yl)butanoyl]-*sn*-glycero-3-phosphocholine or 1-[6-(pyren-1-yl)butanoyl]-2-[6-(pyren-1-yl)hexanoyl]-*sn*-glycero-3-phosphocholine were studied. A 2-fold enhancement was observed in the activity of phospholipase A_2 towards both pyrene phospholipids upon exceeding the critical micellar concentration of the system. Changes in the pyrene excimer/monomer fluorescence emission intensity ratio coincide with the enhancement of phospholipase A_2 activity at the critical micellar concentration. Due to the different effects of micellization on the alignment of the pyrene in the two fluorescent probes conformational changes could be assessed. A model describing possible conformations of these pyrene phospholipid molecules below and above the critical micellar concentration is presented and correlated with the interfacial activation of phospholipase A_2.

Phospholipase A_2; Pyrene phospholipid; Mixed micelle; Cholate; Enzyme activation

1. INTRODUCTION

Phospholipase A_2 (EC 3.1.1.4, PLA_2) catalyzes the hydrolysis of the *sn*-2 fatty acyl ester bond of *sn*-3 phospholipids [1]. PLA_2s are most abundantly present in pancreas and in venoms of snakes [2]. Pancreatic PLA_2 is secreted as a zymogen having a fully functioning active site whereas snake venom PLA_2s have not been found to have a proenzyme form [2]. A characteristic feature in the kinetics of PLA_2 is their activation at the critical micellar concentration (CMC) of the substrate (review [2]). Substrate concentration vs enzyme activity curves show a break at the CMC of the substrate illustrating this phenomenon and even 10^4-times higher activities are observed towards substrates present in lipid-water interfaces than towards the same phospholipids in monomeric solutions. However, the zymogen of pancreatic PLA_2 is not activated at interfaces [3]. The most feasible explanations for the interfacial activation are: (i) the substrate theory which assumes substrate molecules to be in interfaces in a conformation different from that in monomolecular solutions and resulting in an enhanced PLA_2 activity [4–7] or alternatively, assumes the dehydration of interfacial substrate to be responsible for the interfacial activation [8–11] and (ii) the enzyme theory which assumes a conformational change in the enzyme molecule upon adsorption to the interface [3,12,13]. These models are not mutually exclusive. Despite extensive studies no definitive agreement exists on the molecular mechanism of this phenomenon (reviews [2,14–17]). However, it is generally agreed that the 'quality' of the interface plays an important role in determining the activity of PLA_2 [2,18].

We have presented evidence that the activities of

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Abbreviations: PLA_2, phospholipase A_2; diPBPC, 1,2-di[6-(pyren-1-yl)butanoyl]-*sn*-glycero-3-phosphocholine; PBPHPC, 1-[6-(pyren-1-yl)butanoyl]-2-[6-(pyren-1-yl)-hexanoyl]-*sn*-glycero-3-phosphocholine; I_e, intensity of pyrene excimer fluorescence emission; I_m, intensity of pyrene monomer fluorescence emission; CMC, critical micellar concentration

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phospholipases A_1 and A_2 can be reciprocally regulated by changing the physical state of the substrate and proposed a model for conformational changes occurring in phospholipid molecules [19,20]. In our earlier study, however, liposomal and monolayer membranes were used to characterize electrostatically [21] induced alterations in substrate phospholipids and in PLA_2 activities [19,20]. Therefore, the direct relevance of these results to the elucidation of the mechanism underlying PLA_2 activation at the CMC is questionable as changes in the degree of substrate deprotonation were involved [21]. We have also shown that PLA_1 and PLA_2 activities can be regulated by 'vertical compression' of the substrate monolayer altering the conformation of the substrate phospholipids [22]. Recently we have observed that PLA_2 activity towards phospholipid monolayers can be triggered by an electric field across the substrate monolayer [23].

Here, constant submicellar concentrations of dipyrenephosphocholines were maintained while varying the concentration of sodium cholate from zero to values above the CMC. This allows one to observe changes in fluorescence of the pyrene phospholipids upon formation of mixed sodium cholate-phospholipid micelles thus reflecting changes in the conformation of pyrene phospholipids and to correlate these changes to PLA_2 activation at the CMC.

2. EXPERIMENTAL

The fluorescent lipids diPBPC and PBPHPC were purchased from KSV Chemical Corp. (Helsinki). Porcine pancreatic PLA_2 was from Sigma and revealed a single Coomassie brilliant blue-stained band upon polyacrylamide gel electrophoresis in the presence of SDS [24].

Pyrene excimer (I_e) and monomer (I_m) fluorescence emission intensities were measured with an SLM 4800S spectrofluorometer using averaging of 100 signals. The excitation wavelength was 343 nm and emission wavelengths were 396 and 480 nm for I_m and I_e, respectively. Emission intensities were corrected for background due to sodium cholate.

PLA_2 activities were determined with a Kontron SFM 23 spectrofluorometer as described [19,25,26]. The substrate was prepared by drying 10 μg pyrenephosphocholine with a gentle stream of nitrogen. Thereafter the lipid was dissolved in 1.0 ml of 20 mM Tris-HCl, pH 8.0, buffer by brief sonication. 100 μl of the substrate solution was added in 1.9 ml of 20 mM Tris-HCl/4 mM $CaCl_2$, pH 8.0, buffer containing the indicated amounts of sodium cholate. The addition of the enzyme started the reaction. The reaction was followed by recording the increment of the fluorescence emission intensity of pyrene monomer at 400 nm due to enzyme action. The excitation wavelength was 343 nm. The enzyme reaction was linear for at least 5 min and the activities were calculated from the linear portion of the curves.

CMCs were determined by measuring the surface tension of the air-water interface using the Wilhelmy plate method as a function of the subphase lipid or cholate concentration using the KSV 2200 Surface Barostat system (KSV Chemical Corp.).

3. RESULTS

The CMCs of diPBPC and PBPHPC were 5.1 and 3.2 μM, respectively, and thus clearly above the concentration of 0.63 μM for diPBPC and 0.61 μM for PBPHPC used in the present study. The CMC of diPBPC agrees with that reported earlier [27]. The CMCs of sodium cholate in the presence of 0.63 μM diPBPC and with 0.61 μM PBPHPC were 4.2 and 3.9 mM, respectively, in agreement with earlier observations on the CMC of sodium cholate in a different solvent [28].

The activity of PLA_2 towards 0.63 μM diPBPC as a function of sodium cholate concentration exhibited a break at approx. 4.0 mM cholate in good agreement with the CMC of sodium cholate obtained with the monolayer technique (fig.1, left). At 4.0 mM sodium cholate PLA_2 activity increased approx. 2-fold. The I_e/I_m of 0.63 μM diPBPC ex-

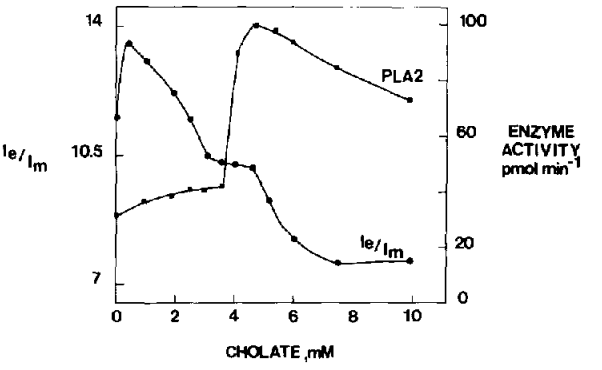

Fig.1. (Left) Activity of phospholipase A_2 towards diPBPC and pyrene I_e/I_m of diPBPC as a function of sodium cholate concentration. (Right) Activity of phospholipase A_2 towards PBPHPC and I_e/I_m of PBPHPC as function of sodium cholate concentration. Reaction mixture contained 1.25 nmol diPBPC or 1.21 nmol PBPHPC in 2.0 ml of 20 mM Tris-HCl 4.0 mM $CaCl_2$, pH 8.0, buffer and the indicated amounts of sodium cholate. In enzyme assays the reaction mixture contained in addition to the above mixture 0.1 μg (7 pmol) porcine pancreatic phospholipase A_2 in 20 μl of 20 mM Tris-HCl, pH 8.0. Temperature, 37°C.

hibited very high values between 9.5 and 10.5 when the cholate concentration varied between 0 and 3 mM (fig.1, left). When the cholate concentration was increased from 3.5 to 5.0 mM I_e/I_m decreased rapidly and levelled off to a value of 3.7 at 10 mM cholate.

Fig.1 (right) illustrates I_e/I_m and PLA_2 activity towards 0.61 μM PBPHPC as a function of sodium cholate concentration. As in the case of diPBPC PLA_2 was activated approx. 2-fold when the cholate concentration exceeded 4.0 mM. Cholate concentrations above 6.0 mM caused PLA_2 activity to decrease. Without the addition of cholate I_e/I_m had a value of 11.5 and the highest value of I_e/I_m, 13.5, was observed with 0.5 mM cholate. When the cholate concentration was increased further, up to 3.5 mM, I_e/I_m decreased steadily to 10.0. A plateau in I_e/I_m was observed between 3.5 and 4.5 mM cholate. Above 4.5 mM cholate I_e/I_m decreased again and at 10 mM cholate the I_e/I_m value was 7.5.

4. DISCUSSION

On the basis of results from NMR studies the conformation of phospholipid molecules in micelles has been suggested to differ from that in monomers [29–31]. However, contradictory results have also been reported [32,33]. Phospholipids have been suggested to adopt the so-called 'kinked' conformation in micelles [29–31] and X-ray crystallography studies on the conformation of phosphatidylcholine and phosphatidylglycerol have revealed that phospholipid molecules do adopt this conformation in lipid crystals [34–36]. In the kinked conformation the *sn*-2 acyl chain begins perpendicularly to the glycerol backbone and after the first two methylene segments bends to align the *sn*-1 chain and parallels the glycerol backbone. Notably, according to the X-ray diffraction data on PLA_2 the substrate should be in the kinked conformation in order to fit optimally into the active site of PLA_2 [37].

The present study was undertaken to investigate the possible conformational changes of pyrene phospholipids upon formation of sodium cholate-pyrenephosphocholine mixed micelles and the simultaneous interfacial activation of PLA_2. The pyrene phospholipid analogs can give rise to the formation of excited dimer, excimer [38,39]. Therefore, these fluorescent phospholipids can emit light at 400 and 480 nm corresponding to pyrene monomer and excimer emission, respectively. The dipyrene phospholipids diPBPC and PBPHPC were chosen due to their ability to form intramolecular excimer [39] in addition to the intermolecular excimer and due to their different pyrene fatty acyl chains at the *sn*-2 position. We previously concluded that changes in intramolecular excimer formation can reflect changes in the conformation of dipyrene phospholipids [19,20,40]. If the model of the extended and kinked conformations of these pyrene phospholipids below and above CMC, respectively, were to be operative, a decrement in the rate of intramolecular pyrene excimer formation of diPBPC above CMC would be observed due to the non-alignment of the pyrene moieties at the end of the fatty acyl chains in the kinked conformation (fig.2). If a similar conformational change takes place in PBPHPC it should result in an increase in intramolecular pyrene excimer formation due to the shift of the pyrene moiety at the end of the longer *sn*-2 hexanoyl chain to align the pyrene moiety at the end of the *sn*-1 butanoyl chain (fig.2). Simultaneously with the changes in the fluorescence of pyrene phospholipids an activation of PLA_2 should be seen.

As is clear from fig.1 a 2-fold activation of PLA_2 was observed upon formation of mixed micelles. The activation should be analogous to the interfacial activation upon phospholipid micellization [1,2,41]. Although pure PBPHPC was hydrolyzed at a 2-fold higher rate than pure diPBPC the activities of PLA_2 towards both pyrene phospholipids were approximately equal above the CMCs of the two systems, indicating that in mixed micelles the substrate properties of these lipids were similar. For both lipids the 2-fold activation of PLA_2 could thus reflect the preference of the active site of the enzyme for the kinked conformation [37]. The reason for the slight decrease in PLA_2 activity towards PBPHPC for increasing cholate concentrations above the CMC is at present unknown.

Phospholipids have been suggested to form premicellar aggregates below the CMC [42]. Changes in the very high I_e/I_m observed in the presence of submicellar cholate suggested the formation of premicellar molecular aggregates by

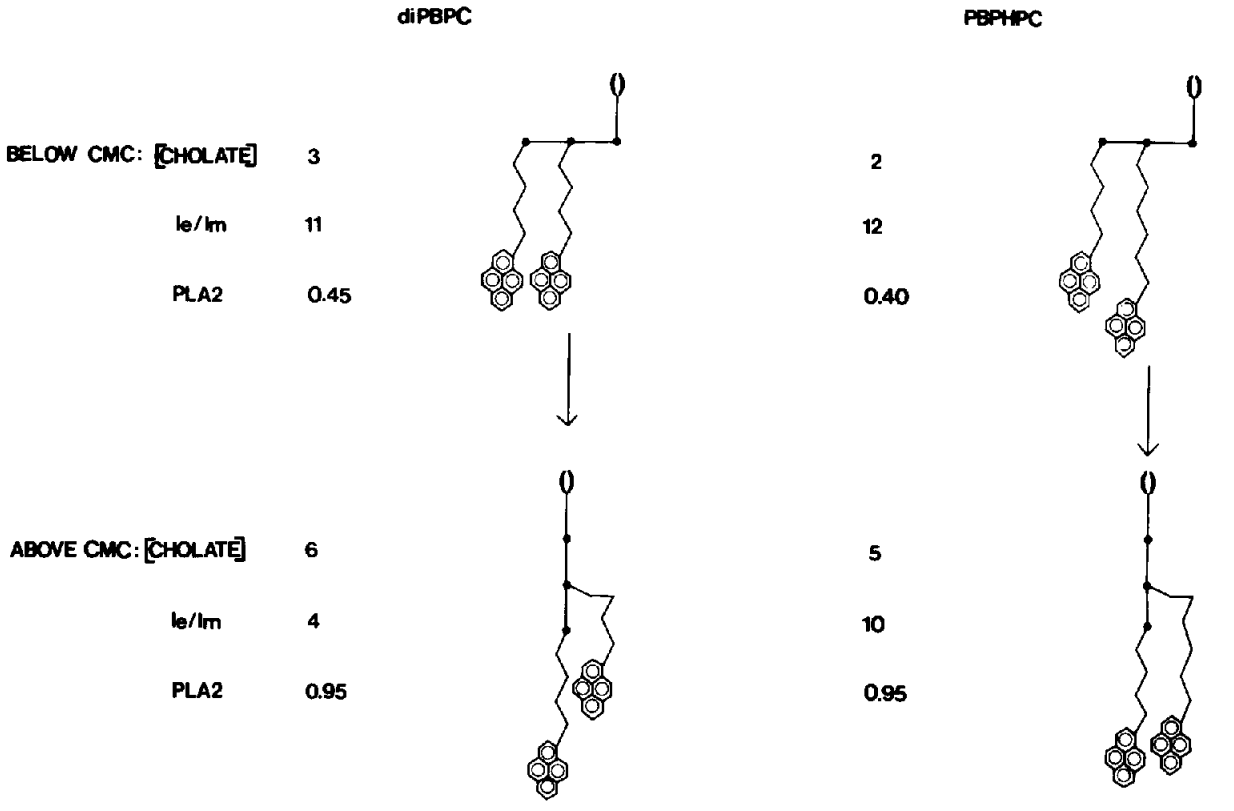

Fig.2. A model for conformational changes in diPBPC and PBPHPC upon formation of mixed micelles with cholate. Sodium cholate concentration is given in mM. Phospholipase A_2 activities are expressed as $\mu mol \cdot min^{-1} \cdot mg^{-1}$.

both pyrene phospholipids [42]. Therefore, changes in I_e/I_m are likely to be due to changes in both inter- and intramolecular excimer formation rates [19,20, 38–40]. When the premicellar pyrene phospholipid aggregates disappear at CMC and pyrenephosphocholine molecules were distributed to mixed micelles by increasing cholate concentrations a decrement in I_e/I_m should be seen. This is due to the increased distance between each pyrenephosphocholine molecule resulting in a decrement in the intermolecular pyrene excimer formation rate. Above CMC the local concentration of the pyrene phospholipid molecules was very low in the mixed micelles, the average number of pyrenephospholipid molecules in each micelle being less than unity [28].

Upon formation of mixed micelles the rapid decrease in the I_e/I_m of diPBPC would, therefore, be due to the decrement in both intra- and intermolecular excimer fluorescence (fig.2) whereas the plateau observed in the I_e/I_m of PBPHPC upon micelle formation should arise from the reciprocal effects of decreasing intermolecular and increasing intramolecular excimer formation. The observed changes in the intramolecular excimer fluorescence of both pyrenephosphocholines upon micelle formation suggest changes in the conformation of these phospholipids. In submicellar solutions phospholipids would have their acyl chains more or less parallel to each other and perpendicular to the glycerol backbone (fig.2) [29–31]. Upon formation of mixed micelles both pyrene phospholipids would adopt the kinked conformation resulting in a decrease in intramolecular excimer formation of diPBPC and an increase in that of PBPHPC, thus agreeing with the assumptions made on the basis of the model (fig.2).

These results can be taken to support the 'substrate theory' of PLA_2 activation at the CMC [4–11] as they reflect changes in the conformation

of phospholipids due to incorporation of the lipid into a mixed micelle. The observed changes in the lipid conformation influencing the action of PLA_2 also seem to be in line with our earlier model developed on the basis of results from a distinctly different experimental approach [20,21]. The present study may now justify the extension of our previous model to concern changes in the conformation of phospholipids and the mechanism of the interfacial activation of PLA_2 at the CMC. However, these results do not exclude a simultaneous conformational change in the enzyme upon adsorption into the substrate interface also contributing to the enhanced activity of PLA_2 [3, 12,13].

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