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Partial coverage of phospholipid model membranes with annexin V may completely inhibit their degradation by phospholipase A_2

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Abstract Phospholipase A_2 (PLA_2)-mediated hydrolysis of membrane phospholipids was measured by ellipsometry, and the inhibition of this process by annexin V was studied. Planar membranes, consisting of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (PC/PE/PS; 54:33:13, on molar basis), were degraded by pancreatic PLA_2, and the rate of hydrolysis was limited to about 0.7%/min. The influence of graded coverage of the membrane with annexin V was studied. The degree of PLA_2 inhibition was nonlinearly related to the amount of membrane-bound annexin V, and binding of only 12% and 54% of full membrane coverage resulted in, respectively, 50% and 93% inhibition. These findings indicate that the inhibition of PLA_2-mediated hydrolysis by annexin V cannot be simply explained by shielding of phospholipid substrates from the enzyme. Moreover, the present results leave room for a role of endogenous annexin V in regulating phospholipid turnover in the plasma membrane of parenchymal cells such as cardiomyocytes.

1. Introduction

Under normal conditions the membrane phospholipids of cardiomyocytes are subjected to a continuously balanced degradation and resynthesis cycle. However, under pathophysiological circumstances, like ischemia/reperfusion, there is a net degradation of phospholipids [1–3]. The mechanisms underlying the net decline of the membrane phospholipid pool under pathophysiological circumstances are incompletely understood. It is suggested that the phospholipid degradation rate, although enhanced, is controlled by intrinsic factors during ischemia/reperfusion [3]. One of these factors might be the binding to the membrane of annexin V, a member of a family of calcium and phospholipid binding proteins (for reviews of the annexins see [4,5]). Annexin V was found to be present at the sarcolemma of the cardiac myocyte [6]. The protein binds to membranes containing negatively charged phospholipids [7,8], such as phosphatidylserine (PS), which under normal conditions are predominantly located in the inner leaflet of the cardiac cellular membrane [9].

Antiphospholipase activity in vitro of the annexins was first reported for annexin I and later also for other annexins [4]. The inhibitory mechanism proposed involves masking of substrate (phospholipids) by annexins [10]. As such, annexins are thought to be Ca^{2+}-dependent non-specific inhibitors of phospholipases. This notion suggests that they may be involved in the phospholipid homeostasis in the heart. However, the estimated molar ratio of annexin V over the phospholipids of the inner leaflet, which is in the order of 1:1250, does not support a role for annexin V in the cardiac myocyte by simply shielding off phospholipids [6].

The aim of the present study was to investigate the potency of annexin V to inhibit phospholipase A_2 (PLA_2)-mediated hydrolysis of phospholipids at low PLA_2 activity, i.e., yielding about 1% of phospholipid hydrolysis per minute, which is in the same range as maximally observed in cardiac tissue [11]. A new ellipsometric technique for simultaneous in situ monitoring of the binding of annexin V to planar phospholipid membranes, as well as the rate of membrane degradation by PLA_2 was used [12]. This technique allows accurate control of the degree of coverage of the phospholipid surface with annexin V and continuous measurement of the membrane degradation rate.

2. Materials and methods

2.1. Materials

Dioleoyl-phosphatidylcholine (PC) and dioleoyl-phosphatidylserine (PS) were obtained from Avanti Polar Lipids (Alabaster, AL). Dioleoyl-phosphatidylethanolamine (PE) and porcine pancreatic phospholipase A_2 were purchased from Sigma (St. Louis, MO). Recombinant annexin V was prepared as described [13]. Silicon wafers (1-0-0, type n, phosphorus doped; thickness: 500 μm) were obtained from Aurel GmbH (Landsberg, Germany). All chemicals used were of the highest grade available.

2.2. Planar bilayers

Planar bilayers on silicon discs were prepared by adsorption of small unilamellar vesicles as described [14]. Briefly, vesicles were prepared by mixing PC, PE and PS in a molar ratio of 54:33:13, drying of the lipids under a stream of nitrogen, and sonication of the lipid dispersions (1 mM phospholipids) in HEPES buffer (50 mM, pH 7.4, 100 mM NaCl) for 10 min on ice at 7.5 μm peak-to-peak amplitude. Vesicles were added at a final concentration of 20 μM phospholipid to a vessel containing a rotating hydrophilic silicon disc. Adsorption of vesicles to the disc results in the formation of a continuous planar bilayer [15]. After formation of a continuous planar membrane surface on the disc (2 cm^2), non-bound vesicles were removed. The membrane-coated disc was transferred, avoiding exposure to air, to the ellipsometer cuvette filled with 5 ml of HEPES buffer containing 1 mM $CaCl_2$. In all experiments the discs were rotated at a constant angular velocity of 78 rad/s. Phospholipid concentrations were determined by phosphate analysis [16].

2.3. Ellipsometry

Changes in the adsorbed surface mass were measured directly on the rotating disc by ellipsometry at 21°C [17,18]. Light from a He-Ne laser passes a polarising prism and is reflected by the rotating silicon disc, mounted in the ellipsometer cuvette. After reflection the laser beam passes a second prism, the analyzer, before reaching a photodiode. Adsorption of biomolecules to the silicon disc results in changes of the polarization state of the reflected light. The positions

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of the polarizer and analyzer are automatically adjusted by a computer program such that the light intensity reaching the photodiode is minimized (null-ellipsometry). From the polarizer and analyzer positions the amount of adsorbed phospholipid mass can be calculated with a precision of 3–5 ng/cm^2, that is, about 1% of the mass of an adsorbed planar bilayer (445 ng/cm^2, see Section 3).

2.4. PLA_2-dependent phospholipid hydrolysis

After preparation of a planar bilayer on a silicon disc and measurement of a baseline level of surface mass, hydrolysis of membrane phospholipids was started by addition of PLA_2 (0.3–30 ng/ml). Subsequently, the cuvette was continuously flushed at 5 ml/min with the same concentrations of PLA_2. This procedure was performed in order to minimize depletion of PLA_2 from the bulk phase, for instance due to adsorption of PLA_2 to the cuvette walls. Also, the hydrolysis products appearing in the buffer were thus removed.

2.5. Measurement of annexin V binding and phospholipid hydrolysis

Annexin V binding was started by addition of annexin V at final concentrations ranging from 0.1 to 1 μg/ml. Adsorption was interrupted by removal of annexin V from the bulk phase by flushing the cuvette with 50 ml of buffer containing 1 mM $CaCl_2$. This procedure takes advantage of irreversible binding of annexin V to planar membranes, probably caused by formation of annexin V clusters on the surface [19]. It results in partial coverage of the membrane with annexin V. Throughout this report, partial coverage will be expressed as percentage of the maximal annexin V binding capacity. When a stable level of adsorbed annexin V surface mass was recorded, phospholipid hydrolysis was started by addition of PLA_2 (1 ng/ml), and the cuvette was continuously flushed with PLA_2 (1 ng/ml) at 5 ml/min. After 2000 s, EDTA (2 mM final concentration) was added to remove annexin V from the membrane and to inhibit PLA_2 activity.

3. Results

3.1. Phospholipid hydrolysis of planar bilayers by PLA_2

Exposure of rotating hydrophilic silicon discs to phospholipid vesicles composed of PC/PE/PS (54:33:13, mol/mol/mol) resulted in adsorption of vesicles and formation of a stable membrane, as shown by the initial part of the curves in Fig. 1, i.e., prior to addition of PLA_2. The adsorbed lipid mass was 445 ± 15 ng/cm^2 (mean ± SD) in the various experiments. For clarity, the initial surface mass was normalized to 100% for each experiment. After recording the baseline level of lipid mass, phospholipid hydrolysis was started by addition of porcine pancreatic PLA_2 (varying from 0.3 to 30 ng/ml). The observed desorption rates, reflecting phospholipid hydrolysis and release of hydrolytic products from the membrane, increased with the PLA_2 concentration used. Desorption rates ranged from about 0.5%/min with 0.3 ng/ml PLA_2 to about 4%/min with 30 ng/ml PLA_2. Product desorption rates were not linear with the PLA_2 concentration, as indicated by a plot of the initial rates against PLA_2 concentration (Fig. 1, insert). In order to keep the rate of phospholipid hydrolysis within the (patho)-physiological range of maximally 1%/min (see Section 4), a fixed concentration of 1 ng/ml PLA_2 was used.

3.2. Phospholipid hydrolysis of membranes partially covered with annexin V

PC/PE/PS membranes were partially covered with annexin V by addition of annexin V (0.1–1 μg/ml) to the cuvette, monitoring of annexin V binding, and flushing of the cuvette with buffer when the desired amount of protein had adsorbed. Maximal annexin V coverage observed in the presence of 1 mM Ca^{2+} was about 190 ng/cm^2, which is in agreement with previous results [8]. As shown in Fig. 2, membranes were prepared with 0, 5, 12, 27, 54 and 100% of maximal annexin V adsorption. Hydrolysis of membrane phospholipids was started by addition of PLA_2 (1 ng/ml). At the end of the experiments EDTA was added, resulting in instantaneous desorption of annexin V and complete inhibition of PLA_2 activity. In Fig. 2, the amount of immediately desorbing annexin V, after addition of EDTA, was about equal to the amount of initially adsorbed annexin V, because either the degraded fraction of the membrane or the amount of adsorbed annexin V was small. The resulting partial annexin V adsorptions to the membrane (with the number of experiments) were: 0 ($n = 10$), 9 ($n = 4$), 23 ($n = 3$), 50 ($n = 3$), 100 ($n = 2$) and 190 ($n = 1$) ng/cm^2. Standard errors of the mean are presented for the four lower coverages with annexin V. It is apparent from Fig. 2 that increased surface coverage with annexin V resulted in decreased phospholipid hydrolysis rates.

Fig. 3 more directly illustrates the relation between observed mean phospholipid hydrolysis rates and annexin V surface coverage. Hydrolysis rates were calculated from the adsorbed phospholipid surface mass, measured before addition of annexin V, minus the amount after 2000 s of PLA_2 action,

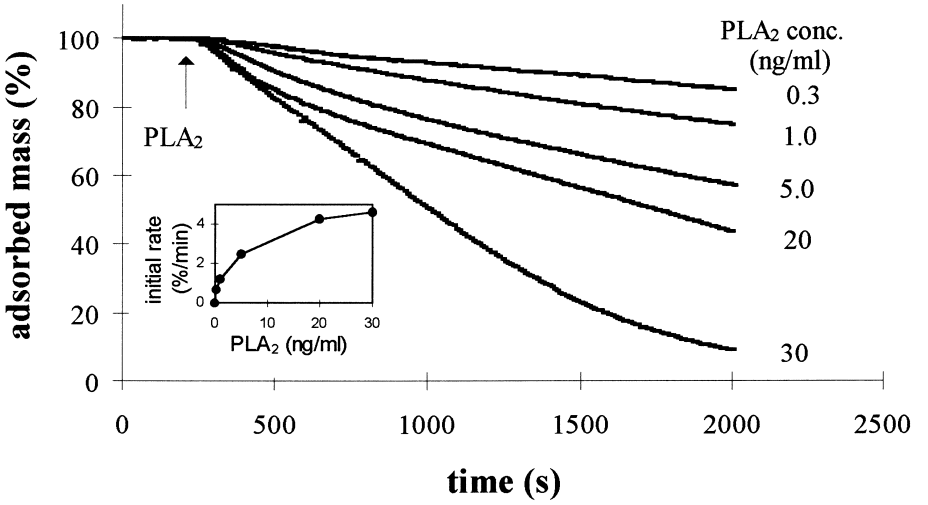

Fig. 1. Phospholipid hydrolysis of PC/PE/PS (54:33:13, mo/mol/mol) membranes by PLA_2. PLA_2 was added (arrow) at concentrations as indicated in the figure.

as inferred from the remaining phospholipid mass after annexin V desorption and PLA_2 inhibition by addition of EDTA. Already at low surface coverages significant inhibition of the phospholipid hydrolysis rate was observed. For instance, about 22% inhibition of PLA_2 activity occurred when the membrane was covered for only 5% with annexin V, 50% inhibition for only 12% coverage and almost complete inhibition for only 50% coverage.

4. Discussion

In the present study we examined the effect of annexin V on PLA_2-dependent degradation of planar model membranes. Ellipsometry was used to measure membrane coverage with annexin V and PLA_2-dependent phospholipid hydrolysis at the same time. Previously, we have shown that the degradation curves reflect desorption of degradation products, while unaffected phospholipids remain adsorbed [12]. Adsorption of PLA_2 to the membrane cannot be observed, because for the low PLA_2 concentration of 1 ng/ml, even when the PLA_2 adsorption rate would be transport-limited, the PLA_2 adsorption rate would still be less than a few percent of product desorption rates [12]. Thus, even with the highest PLA_2 concentration used, desorption rates adequately reflect true desorption of phospholipid hydrolysis products.

4.1. Choice of planar model membranes

Planar membrane surfaces were used and these are more comparable with the sarcolemma than vesicles. For instance, they allow formation of irreversibly binding annexin clusters, and thereby could decrease the lateral mobility of membrane-bound PLA_2, while such clustering is hampered by high surface curvature in phospholipid vesicles [19].

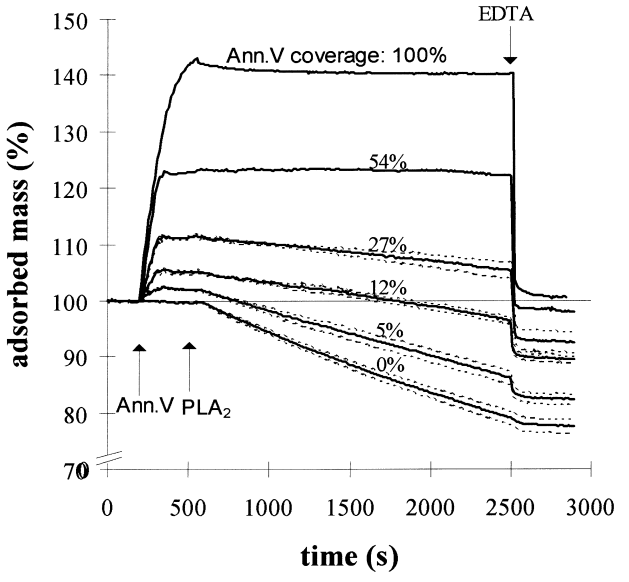

Fig. 2. Effect of annexin V surface coverage on PLA_2-dependent phospholipid hydrolysis. Membranes were partially covered with annexin V. The degree of coverage is indicated in the figure. Phospholipid hydrolysis was started after 500 s by addition of PLA_2 (1 ng/ml). After 2500 s, EDTA was added to remove annexin V from the membrane and to inhibit PLA_2 activity. Solid curves present averages and dashed lines indicate the standard errors of the mean.

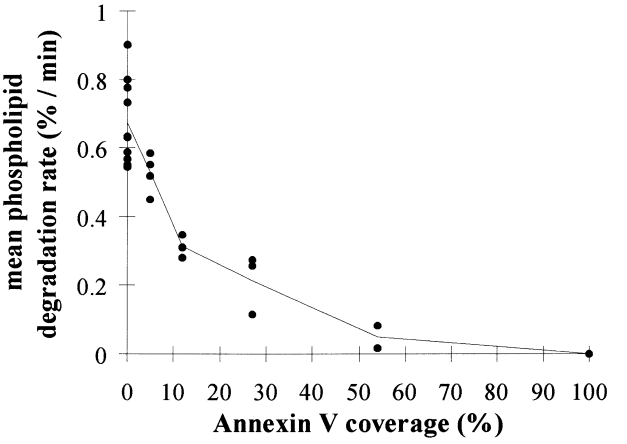

Fig. 3. Phospholipid hydrolysis rates as function of annexin V surface coverage. The mean phospholipid hydrolysis rate of all individual experiments, summarized in Fig. 2, are plotted. Degradation rates are expressed as percentage of the original amount of phospholipid adsorbed on the rotating disc.

4.2. Choice of the phospholipid composition of the membrane

Whereas the outer leaflet of the sarcolemma mainly consists of PC and sphingomyelin, the inner leaflet mainly contains PC, PE, PS and a small fraction of phosphatidylinositol (PI). The composition used in the present study, of PC/PE/PS at molar ratios of 54:33:13, roughly resembles the composition of the sarcolemmal inner leaflet [9]. It should be noted, however, that the artificial membrane did not contain naturally occurring membrane proteins as does the sarcolemma, and thus on this point is far from physiological. Possible consequences of this are discussed below.

4.3. Choice for pancreatic PLA_2 as model enzyme

Three different phospholipases A_2 have been detected in cardiac tissue: group II PLA_2, high molecular weight PLA_2, and plasmalogen-specific PLA_2 [20]. In ischemia-induced membrane damage of the heart, group II PLA_2 is suggested to be of major importance [21]. Additional evidence for this role has been provided by experiments showing that other fatty acids than arachidonic acid are also found as hydrolytic products [11]. Because high molecular weight PLA_2 is very specific for sn-2-arachidonyl-containing phospholipids, it thus seems less important for ischemia-induced phospholipid hydrolysis. In addition, mainly lysophospholipids are found as phospholipid degradation products [11], and this rules out the plasmalogen-specific PLA_2 as a likely candidate. Since purified heart-specific group II PLA_2 was not available in sufficient quantity, pancreatic PLA_2 was used in the present study because of its structural and functional resemblance to group II PLA_2 [22]. The Ca^{2+}-requirement of pancreatic PLA_2 (and of heart-specific group II PLA_2) for optimal activity largely exceeds normal intracellular levels [22]. However, ischemic conditions may result in elevated intracellular Ca^{2+} concentrations [23]. Furthermore, it has been proposed that in close vicinity to the sarcolemma Ca^{2+} concentrations are higher than in the cytoplasm, and could be in range of the Ca^{2+} concentration used in the present study (1 mM) [24,25].

4.4. Choice of the PLA_2 concentration

The average phospholipid hydrolysis rate of 0.7%/min, observed for a PLA_2 concentration of 1 ng/ml, is in the range of

degradation rates observed in (patho)physiological systems. For instance, it was shown that endogeneous hydrolytic activity in rat cardiac tissue, deliberately damaged by freezing and thawing prior to storage under anoxic conditions, is about 1% phospholipid breakdown per minute [11]. This may indicate the maximal phospholipase activity at membranes of the cardiac myocyte.

4.5. Possible mechanisms for PLA_2 inhibition by annexin V

The mechanism of phospholipase inhibition by annexins is still controversial. Specific protein–protein interactions between annexins and PLA_2 have been proposed, but evidence for such interactions is not conclusive [4]. It was observed that by increasing the phospholipid to annexin ratio, inhibition of PLA_2 was abolished [10,26]. This led to the so-called substrate depletion model, with annexin V masking the substrate phospholipids [10]. It should be noted that in most experiments the actual coverage of the phospholipid substrates with annexins was not precisely known. Our results indicate that simple masking of the membrane phospholipids is not sufficient to describe the observed inhibition. Furthermore, for higher (20–30 ng/ml) PLA_2 concentrations, substantial phospholipid hydrolysis was observed even when the membranes were maximally covered with annexin V (results not shown). Obviously, these membranes still provide binding sites for PLA_2, in agreement with the observation that coagulation factors Xa, Va and prothrombin may still bind to such maximally covered membranes [19].

Annexin V binds to PS with higher affinity then to PC and PE [8,27], and this could cause clustering of PS. Indeed, experimental evidence for annexin-induced clustering of anionic phospholipids such as phosphatidic acid [28] and phosphatidylglycerol [28,29] has been provided. The binding affinity of pancreatic PLA_2 for PC membranes is low and is markedly enhanced by the incorporation of anionic phospholipids, like PS [30]. Therefore, clustering of PS by annexin V could explain more than proportional inhibition of PLA_2.

Annexin V could also interfere with the influence of phospholipid hydrolysis products on PLA_2 activity. Although the precise role of hydrolysis products in PLA_2 action is not completely elucidated [31], their accumulation in the membrane may greatly reduce the activity of PLA_2 by product inhibition or substrate dilution [32–34]. Although in our experimental set-up the hydrolysis products desorb from the membrane, some accumulation is inevitable. From a product desorption rate of 0.7%/min, as observed with 1 ng/ml PLA_2, a product mole fraction in the membrane of about 0.03 can be calculated [12]. A similar value can be estimated from the remaining desorption after inhibition of PLA_2 by EDTA (Fig. 2, lower curve). Although modest, the non-linear dependence of the rate of hydrolysis on PLA_2 concentration (Fig. 1, inset) suggests strong product inhibition, and annexin V could increase this effect by interfering with the desorption of degradation products from the membrane. However, no effect of annexin V binding was observed on the product desorption rates from PS/PE/PC membranes containing 10 mole % of lysoPC and oleic acid (result not shown).

It has been reported that the annexins reduce the lateral mobility of phospholipids [26,35,36], even of lipid components with low affinity for annexins such as PC [35,36]. In the latter studies it was concluded that fluid–fluid phase separation occurred upon annexin binding to PG/PC membranes. Reduced lateral mobility of PC may thus be the result of a decrease in free space in which the lipid can diffuse. Similarly, annexin V may decrease the rate of lateral diffusion of phospholipid hydrolysis products away from their production site, thereby promoting local product inhibition. This effect could be enhanced by simultaneous reduction of the lateral mobility of PLA_2 molecules by annexin V clusters.

4.6. Is annexin V a candidate for regulating cellular PLA_2 in vivo?

The adsorbed mass of phospholipids on silicon discs is 445 ng/cm^2, that is about 0.55 nmol phospholipids per cm^2. We observed 50% inhibition of PLA_2 activity at a coverage of the phospholipid membrane with 23 ng/cm^2 of annexin V (12% of the maximal annexin V coverage), that is 0.65 pmol/cm^2. Hence, the molar annexin V/phospholipid ratio is 1:850 for a bilayer and 1:425 for a monolayer. This ratio is about 3 times the estimated annexin V over sarcolemmal phospholipid ratio of 1:1250 in the rat cardiac myocyte [6]. However, a major part of the sarcolemma consists of proteins which, by bulging from the membrane, could make parts of the membrane inaccessible for membrane-directed enzymes. Also, cytoskeletal proteins are partially covering the inner phospholipid bilayer. Thus, the ratio of annexin V over freely accessible phospholipid may be considerably higher than 1:1250. Together with our finding that low phospholipid coverages by annexin V are sufficient for substantial PLA_2 inhibition, this suggests that annexin V could be of biological importance in the regulation of phospholipid degradation at the sarcolemma of the cardiac myocyte.

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