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Polyamine-Phospholipid Interaction Probed by the Accessibility of the Phospholipid *sn*-2 Ester Bond to the Action of Phospholipase A2

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Summary. Conditions were used where the action of porcine pancreatic phospholipase A2 on phospholipids can be followed in the absence of added calcium and the catalytic activity is supported by the calcium brought with the nanomolar enzyme. Therefore, alterations in the enzyme velocity resulting from the presence of spermine or spermidine could be specifically studied using 1-palmitoyl-2-(pyren-1-yl)hexanoyl-sn-glycero-3-phosphocholine (PPHPC) and 1-palmitoyl-2-(pyren-1-yl)hexanoyl-sn-glycero-3-phosphoglycerol (PPHPG) as substrates. Both spermine and spermidine activated the hydrolysis of PPHPG fourfold at polyamine/phospholipid molar ratios of approximately 1 : 1 and 12 : 1, respectively. Double-reciprocal plots of enzyme activity *vs.* PPHPG concentration revealed the enhancement to be due to increased apparent V_{max} while the apparent K_m was slightly increased. In the presence of 4 mM $CaCl_2$ inhibition by polyamines of PPHPG hydrolysis by phospholipase A2 was observed. Using synthetic diamines we could further demonstrate that two primary amino groups are required for the activation. In the absence of exogenous $CaCl_2$ polyamines inhibited the hydrolysis of PPHPC by phospholipase A2. The presence of 4 mM $CaCl_2$ reversed this inhibition and a twofold activation was observed at 10 μM spermine. The results obtained indicate that the activation of PLA2 by spermine and spermidine is produced at the level of the substrate, PPHPG. This implies the formation of complexes of phosphatidylglycerol and polyamines with defined stoichiometries.

Key Words polyamine-phospholipid interaction · polyamine · phosphatidylglycerol · phospholipase A2 (pancreatic)

Introduction

Polyamines are present in most living cells where they function as regulators in several cellular processes [3, 34]. Rapidly growing cells contain increased polyamine concentrations concomitant with high rates of DNA, RNA and protein synthesis [16, 26]. The molecular mechanisms of action of these strongly basic compounds have remained by and large unresolved. One obvious site of action is at cellular membranes containing acidic phospholipids. Polyamines associate with phosphatidylserine [2, 14] and phosphatidylglyerol [7] and they have been proposed to regulate cellular membrane fusion [14]. Polyamines also bind to membranes of microorganisms and mammalian subcellular fractions [16, 26] and stabilize these against lysis and swelling [15, 21, 33]. Inhibition by polyamines of some membrane-associated enzymes such as snake venom phospholipase A2 (PLA2, EC 3.1.1.4) has been reported [15, 21, 30, 33]. On the other hand, phospholipase C from human amnion was only insignificantly affected by these compounds [29].

Phospholipases A are valuable tools in studies on phospholipid membrane properties. The expression of their catalytic activity depends on phospholipid phase transition [12, 24, 25], state of aggregation [11, 27], surface pressure in phospholipid monolayers [39, 42] as well as conformational changes in the glycerol backbone region [6, 17, 35, 41]. In addition, the identity of the polar head group and acyl chain composition of substrate phospholipids [4, 5, 36] are important determinants.

We investigated the effects of polyamines on phospholipid membranes using the well-characterized porcine pancreatic PLA2 as an enzyme probe [40] and pyrene-labeled phospholipids as substrates. Phospholipid analogs with covalently linked pyrene moiety have been widely used in studies on model biomembrane properties including diffusion, organization and conformation of phospholipids in liposomes [9, 10, 17, 18, 22, 28, 31, 32, 35]. Photophysics of pyrene-labeled phospholipids provide also a means for extremely sensitive continuous measurement of PLA activity requiring no product separation [13, 35, 37].

Our results indicate that polyamines form complexes of defined stoichiometries with phosphatidylglycerol as evidenced by changes in the activity of PLA2.

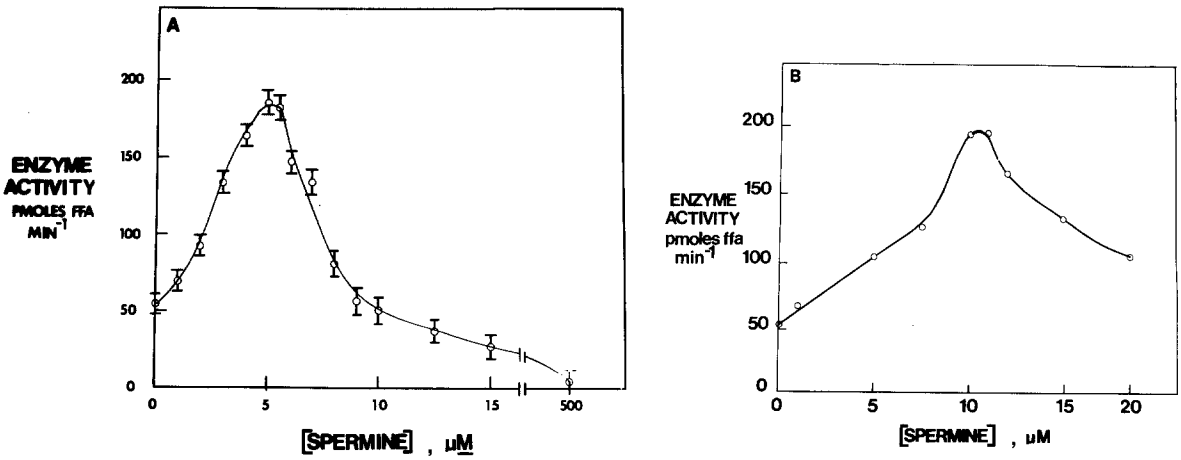

Fig. 1. Activation of PLA2 catalyzed hydrolysis of PPHPG by spermine. Reaction mixture contained (*A*) 6.1 μM and (*B*) 12.2 μM of 1-palmitoyl-2-(pyren-1-yl)hexanoyl-sn-glycero-3-phosphoglycerol in 20 mM Tris-HCl buffer pH 8.0 and the indicated polyamine concentrations. 40 ng of enzyme was used to start the reaction

Abbreviations

Porcine pancreatic phospholipase A2 (PLA2); 1-palmitoyl-2-(pyren-1-yl)hexanoyl-sn-glycero-3-phosphocholine (PPHPC); 1-palmitoyl-2-(pyren-1-yl)hexanoyl - sn - glycero - 3 - phosphoglycerol (PPHPG); free fatty acid (ffa); phosphatidylcholine (PC); phosphatidylglycerol (PG).

Materials and Methods

Reagents

1-Palmitoyl-2-(pyren-1-yl)hexanoyl-sn-glycero-3-phosphocholine (PPHPC), Na-salt of 1-palmitoyl-2-(pyren-1-yl)hexanoyl-sn-glycero-3-phosphoglycerol (PPHPG) and (pyren-1-yl)hexanoic acid were purchased from KSV-Chemicals Oy (Valimotie 7, SF-00380 Helsinki, Finland). According to the manufacturer's specifications the fluorescent lipids are prepared by total organic synthesis not involving the use of enzymes. They are essentially free of calcium. Spermine, spermidine, 1,2-diaminopropane, 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane and 1,6-diaminohexane were analytical grade from EGA-Chemie (Steinheim, West Germany). All other reagents including porcine pancreatic phospholipase A2 were from Sigma. The specific activity of this PLA2 preparation was approximately 600 μmol free fatty acids min^{-1} mg^{-1} using the egg yolk assay [11]. It appeared as a single Coomassie Brilliant blue-stained band upon gel electrophoresis performed in the presence of sodium dodecyl sulfate [20]. By atomic absorption the content of Ca^{2+} in the enzyme preparation was 625 nmol per mg of protein.

Phospholipase A2 Assay

Two alternative light-emitting pathways are available for the relaxation of an excited state pyrene [8]. Monomeric excited pyrene returns to ground state with an emission maximum at 393 nm. When the local concentration of pyrene is high an excited monomer can collide with a ground state pyrene to form an excited dimer, excimer, which emits quanta with maximum around 480 nm.

When PPHPC or PPHPG molecules are dispersed in water the pyrene excimer emission exceeds several-fold the monomer emission thus indicating a high local concentration of pyrene in the liquid phase. Hydrolysis of these fluorescent phospholipid analogs by PLA2 generates (pyren-1-yl)hexanoate which subsequently transfers into the aqueous phase. By following the time course of the increment in the pyrene monomer emission the activity of PLA2 can be determined [13, 37].

Substrates were prepared by transferring 246 nmol of the fluorescent phosphatidylcholine or 250 nmol of the corresponding phosphatidylglycerol in toluene/ethanol (1 : 1, vol/vol) stock solution into a conical glass test tube. After the removal of the solvents under a gentle stream of nitrogen the lipid was dissolved in 400 μl of ethanol. Into 2.0 ml of 20 mM Tris-HCl buffer pH 8.0 in a four-window disposable plastic cuvette 20 μl of the lipid-ethanol solution was rapidly squirted with a 100-μl Hamilton syringe to yield 6.1 μM PPHPG or 6.0 μM PPHPC [1]. Polyamines were included in the reaction mixture in the indicated final concentrations prior to the addition of the phospholipids. Unless otherwise stated the experiments were performed in the absence of added calcium. The concentration of calcium in the buffers was approximately 15 nM. Taking into account the amount of calcium brought with the enzyme, the final assay concentration of Ca^{2+} was at the most 53 nM.

Enzyme reactions were monitored with a Kontron SFM 23 spectrofluorometer equipped with fixed 10-nm slits for both exciting and emitting light with the emission output fed into a chart recorder. Magnetically stirred four-window plastic cuvettes were thermostated to 37°C with a circulating waterbath. Prior to starting the reaction by enzyme addition the contents of the cuvette were allowed to equilibrate for 5 min. The reaction was followed by recording the fluorescence emission intensity at 400 nm with continuous excitation of the reaction mixture at 343 nm. The assay was calibrated by adding 20-pmol aliquots of (pyren-1-yl)hexanoic acid to the reaction mixture in the absence of the enzyme while recording pyrene monomer fluorescence emission. Between the assays the Teflon®-coated magnetic stirring bars

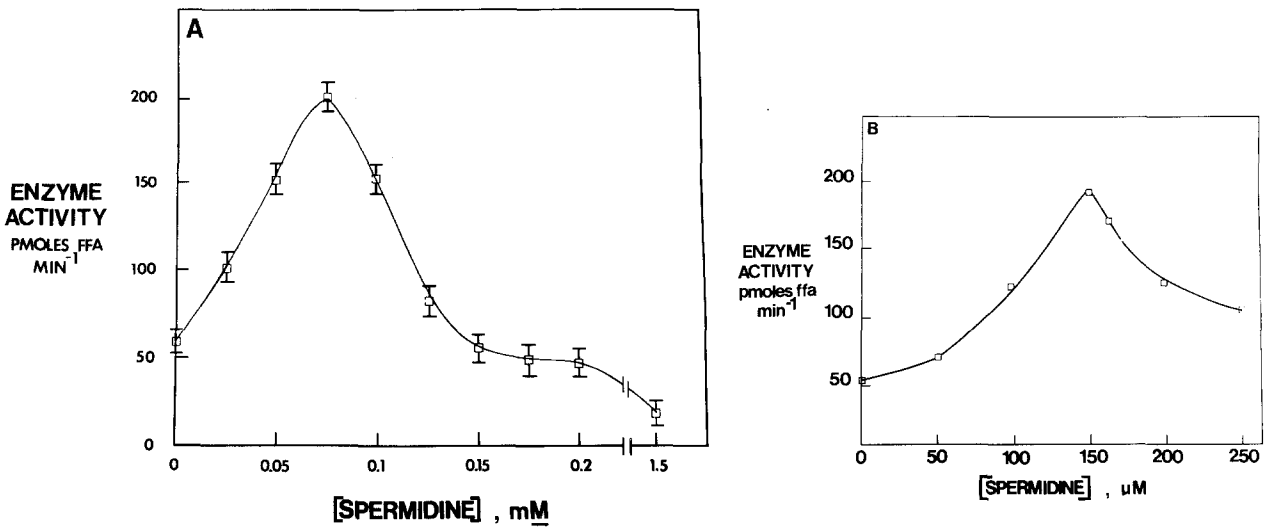

Fig. 2. Activation of PLA2 catalyzed hydrolysis of PPHPG by spermidine. (*A*) 6.2 μM and (*B*) 12.2 μM PPHPG. Conditions as in Fig. 1

were cleaned from any contaminating PLA2 by keeping them in boiling water for 5 min. Enzyme activities were expressed as pmol free fatty acid (ffa) per minute. Assays were performed in triplicate and had an average variation of 12%.

Pancreatic PLA2 contains in its active site a tightly associated calcium required in catalysis [40]. Some of the enzyme molecules are likely to be inactive due to the dissociation of calcium from the active site in the very dilute enzyme solutions used in this study. In our assay system $CaCl_2$ in the concentration range from 1 to 5 mM activated the hydrolysis of PPHPG and PPHPC by PLA2 twofold.

Specific activities of PLA2 in the absence of exogenous CaCl for PPHPG and PPHPC were 1.3 and 0.12 μmol min^{-1} mg^{-1}, respectively, and in the presence of 4 mM $CaCl_2$ 2.5 and 0.25 μmol min^{-1} mg^{-1}, respectively.

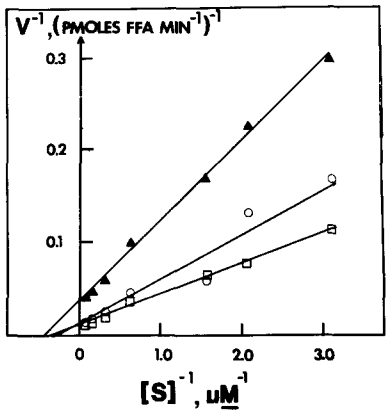

Fig. 3. Lineweaver-Burk plots of PLA2 activity *vs.* PPHPG. (▲–▲) no polyamines, (○–○) spermine/PPHPG ratio 1 : 1, (□–□) spermidine/PPHPG ratio 12 : 1. The amount of enzyme was 20 ng

Results

Effect of Polyamines on the Hydrolysis of Phosphatidylglycerol

The effects of spermine on the activity of porcine pancreatic PLA2 using PPHPG as a substrate are shown in Fig. 1. Maximally, about fourfold activation was achieved at 5.5 μM spermine which corresponds to approximate spermine/PPHPG molar ratio of 1 : 1 (Fig. 1*A*). Increasing the spermine concentration further resulted in a decline in activation and at 10 μM spermine the same PLA2 reaction rate was observed as in the absence of spermine. Nearly complete inhibition was obtained with 0.5 mM spermine (Fig. 1*A*). Using a substrate concentration of 12.2 μM the maximal activation was observed at 11.0 μM spermine again corresponding to approximate spermine/PPHPG molar ratio of 1 : 1 (Fig. 1*B*).

Similarly, spermidine activated the hydrolysis of PPHPG by porcine pancreatic PLA2 (Fig. 2). A fourfold activation was observed at 70 μM spermidine corresponding to spermidine/PPHPG molar ratio of 12 : 1 (Fig. 2*A*). When spermidine concentration was further increased the activation leveled off at 150 μM spermidine and almost complete inhibition was obtained at 1.5 mM spermidine (Fig. 2*A*). At a higher substrate concentration of 12 μM similar effects as in Fig. 2*A* were reproduced by two times higher spermidine concentration (Fig. 2*B*).

Both spermine and spermidine activated PLA2 by increasing the apparent V_{max} while increasing the apparent K_m from 3.4 to 4.9 and to 4.1 μM substrate, respectively (Fig. 3). In the presence of 4.0 mM $CaCl_2$ both polyamines produced an inhibition of the hydrolysis of PPHPG by PLA2 (Fig. 4). How-

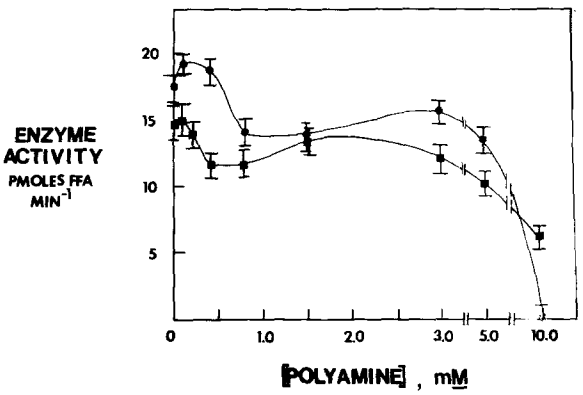

Fig. 4. Inhibition of PLA2 catalyzed hydrolysis of PPHPG by spermine (●–●) and spermidine (■–■) in the presence of 4 mM $CaCl_2$. Reaction mixture contained 6.1 μM 1-palmitoyl-2-(pyren-1-yl)hexanoyl-sn-glycero-3-phosphoglycerol in 20 mM Tris-HCl and 4 mM $CaCl_2$ buffer, pH 8.0, and the indicated polyamine concentrations. The amount of enzyme was 7 ng

ever, higher polyamine concentrations than in the absence of added calcium were needed for maximal inhibition (Fig. 4).

Regardless of the presence or absence of polyamines, addition of 0.1 mM EDTA In the reaction mixture resulted in a complete inhibition in the absence of added $CaCl_2$ (*data not shown*). Therefore, as expected, polyamines could not substitute for Ca^{2+} ions in supporting catalytic activity.

Effect of Polyamines on the Hydrolysis of Phosphatidylcholine

In the absence of added $CaCl_2$ both spermine and spermidine decreased the rate of hydrolysis of PPHPC by PLA2 (Fig. 5*A*). At concentrations of 40 and 60 μM, respectively, spermine and spermidine inhibited the reaction by 90%. However, in the presence of 4 mM $CaCl_2$ 10 μM spermine activated twofold the hydrolysis of PPHPC by PLA2 (Fig. 5*B*), whereas 10 μM spermidine did not have any significant effect under similar conditions. At higher polyamine concentrations inhibition of PLA2-catalyzed hydrolysis by both polyamines was observed (Fig. 5*B*).

Comparison of Different Diamines

Diamines 1,2-diaminopropane, 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane and 1,6-diaminohexane with different hydrocarbon spacers between the two amino groups were used to obtain more information on the structural requirements for polyamines for PLA2 activation of PPHPG hydrolysis. With the exception of 1,2-diaminopropane all the above compounds could activate PLA2. Thus, 1,3-diaminopropane (0.7 mM), 1,4-diaminobutane (2.0 mM), 1,5-diaminopentane (2.0 mM) and 1,6-diaminohexane (2.0 mM) produced three-, five-, six- and sevenfold activation, respectively (Figs. 6 and 7).

Discussion

The above results show that both spermine and spermidine can, in the absence of added calcium, activate the hydrolysis of PG and that the activation is due to an increased V_{max}. However, because of the partially two-dimensional characteristics of the PLA2 reaction, the observed V_{max} must essentially be regarded as apparent [38] and is therefore difficult to interpret.

The activating effect of polyamines on the PG-PLA2 reaction could be due to interaction of the polyamine with the enzyme protein, PG substrate or both. As polyamines in the absence of added calcium do not activate the hydrolysis of phosphatidylcholine by PLA2, it is unlikely that the activating effect is due to a direct protein-polyamine interaction. Therefore, the polyamine activation of PG-PLA2 reaction is likely to be produced at the level of the substrate which would in turn indicate binding of polyamines to phosphatidylglycerol.

For spermine and PG the complex stoichiometry appears to be 1 : 1. Yet, another complex with 2 : 1 stoichiometry of spermine to PG appears to be formed. In the latter, PG is hydrolyzed at the same rate as in the absence of spermine. Our results are supported by recent studies reporting association of polyamines with phosphatidylserine [2, 14] and also the observed stoichiometries are the same [2]. Equilibration dialysis experiments on polyamine-phospholipid complexes are currently in progress in another laboratory [23].

The effects of polyamines on the PG-PLA2 reaction are distinctly different from those on the PC-PLA2 reaction and in the latter spermine caused an inhibition. This is in agreement with the lack of binding of spermine to PC vesicles as demonstrated by Chung et al. [2] and thus indicates direct interaction of PLA2-protein with polyamines.

In the presence of 4 mM $CaCl_2$ the effects of polyamines on PLA2 reaction with PG and PC become more alike. Calcium is capable of partly reversing the inhibitory effect of high polyamine concentrations on the hydrolysis of both PPHPC and PPHPG.

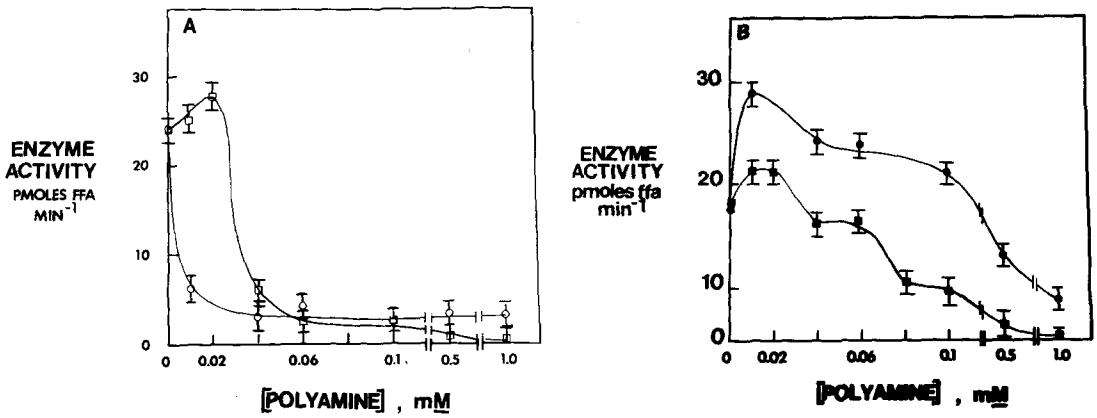

Fig. 5. Effect of spermine and spermidine on PLA2 catalyzed hydrolysis of PPHPC. (*A*) No $CaCl_2$ added. The amount of enzyme used was 200 ng. (*B*) In the presence of 4 mM $CaCl_2$. The amount of enzyme was 60 ng. Spermine ●–● and ○–○, spermidine ■–■ and □–□. Substrate was 6.0 μM 1-palmitoyl-2-(pyren-1-yl)hexanoyl-sn-glycero-3-phosphocholine in 20 mM Tris-HCl buffer, pH 8.0

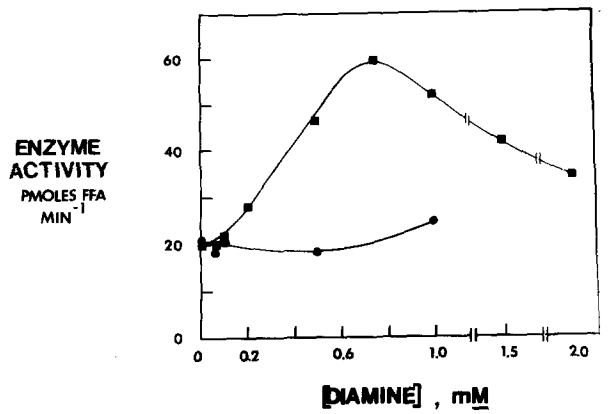

Fig. 6. Hydrolysis of PPHPG by PLA2 in the presence of 1,2-diaminopropane (●–●) or 1,3-diaminopropane (■–■). Substrate was 6.1 μM 1-palmitoyl-2-(pyren-1-yl)-hexanoyl-sn-glycero-3-phosphoglycerol in 20 mM Tris-HCl buffer, pH 8.0. Reaction was started by the addition of approximately 15 ng of porcine pancreatic PLA2

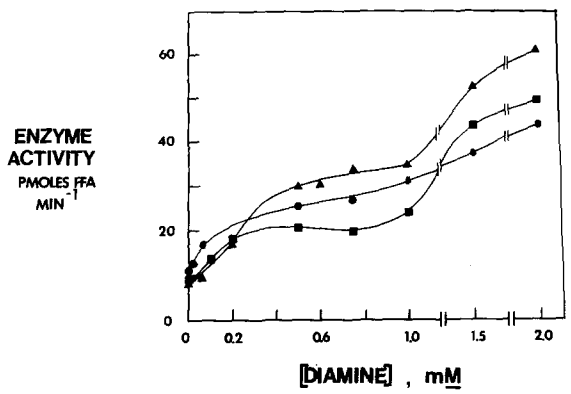

Fig. 7. Activation of PPHPG hydrolysis by PLA2 of 1,4-diaminobutane (●–●), 1,5-diaminopentane (■–■) and 1,6-diaminohexane (▲–▲). Conditions as in Fig. 6 except that the amount of enzyme was approximately 7 ng

To study the structural requirements for polyamines for the activation of PLA2 synthetic diamines were used. Two primary amino groups seemed to be required. Thus 0.7 mM 1,3-diaminopropane caused threefold activation whereas the corresponding 1,2-compound up to the concentration of 1.0 mM did not cause an activation. In the concentration range of 0 to 0.2 mM, the relative efficiencies of different diamines in PLA2 activation were 1,4-diaminobutane > 1,5-diaminopentane > 1,6-diaminohexane. Yet, these efficiencies were altered so that with 0.2 to 1.0 mM and 1.0 to 2.0 mM diamines the relative efficiencies to activate PLA2 were 1,5-diaminopentane > 1,4-diaminobutane > 1,6-diaminohexane and 1,6-diaminohexane > 1,5-diaminopentane > 1,4-diaminobutane, respectively. If the activation of PLA2 by these compounds arises due to the altered physical state of the substrate phospholipid membrane, the above effects could reflect the relative binding affinities of these diamines to PG and subsequent changes in the organization of the phospholipid lattice.

The verification of the possible biological relevance of the findings reported here warrants further studies. The pyrene-labeled phospholipids used as substrates for PLA2 differ from natural phospholipids in their packing properties [31]. Also, due to the experimental conditions the polyamine concentrations employed result in unphysiologically high polyamine/phospholipid molar ratios. However,

the present results do show that the state of membranes of acidic phospholipids can be altered by polyamines and that this alteration results in perturbation in phospholipid-protein interaction reflected in the action of PLA2. Due to the interplay *in vivo* of polyamines and calcium in the regulation of cellular processes [3, 34] and the importance of the membrane functions involved [14, 15, 21, 33] better understanding of this kind of relatively simple model systems should provide new information on the molecular mechanisms of action of polyamines.

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