Effect of Diacylglycerols on the Activity of Cobra Venom, Bee Venom, and Pig Pancreatic Phospholipases A_2

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ABSTRACT: The effects of a series of diacylglycerols (DAGs) with varying acyl chain lengths and degree of unsaturation on the activity of cobra venom, bee venom, and pig pancreatic phospholipases A_2 (PL-A_2s) were studied using two lipid substrates: dipalmitoylphosphatidylcholine (DPPC) or bovine liver phosphatidylcholine (BL-PC). The activities of the phospholipases critically depended on the chain length and degree of unsaturation of the added DAGs and on the chemical composition of the substrate. The effects of DAGs on cobra or bee venom PL-A_2s were similar, but significantly different from the pig pancreatic PL-A_2. The data, taken together with our previous NMR studies on physicochemical effects of these DAGs on lipid bilayer structure [De Boeck, H., & Zidovetzki, R. (1989) *Biochemistry 28*, 7439; (1992) *Biochemistry 31*, 623], allowed detailed correlation of the type of a bilayer perturbation induced by DAG with the activation or inhibition of the phospholipase on the same system. In general, the activation of the phospholipases correlated with the DAG-induced defects of the lipid bilayer structure. The results, however, argue against general designation of DAGs as "activators" or "inhibitors" of PL-A_2s. Thus, for example, diolein activated phospholipases with the BL-PC lipid substrate, but inhibited them with the DPPC substrate. Dihexanoylglycerol and dioctanoylglycerol inhibited pig pancreatic PL-A_2 with both lipid substrates and inhibited cobra or bee venom PL-A_2 with the DPPC substrate, but activated the latter two enzymes with the BL-PC substrate. Longer-chain DAGs (C > 12), which induce lateral phase separation of the bilayers into the regions of different fluidities, activated all PL-A_2s with both lipid substrates. The effect on pig pancreatic PL-A_2 activity was generally an order of magnitude higher than on cobra or bee venom enzymes. The effects of longer-chain DAGs and diolein on activity of pig pancreatic PL-A_2 were significant at 2.5–5 mol % of these DAGs relative to the phospholipids, which is within physiological concentrations of endogenous diacylglycerols in the biological membranes.

Phospholipase A_2 (PL-A_2),[1] which hydrolyses phospholipids at *sn*-2 chain, is a ubiquitous enzyme found in most tissues and cell types studied [see Dennis (1983), Roberts and Dennis (1989), and Jain and Berg (1989) for reviews]. Over 70 PL-A_2s, mostly of extracellular origin, have been described, and the amino acid sequences and crystal structures of many are known (Verheij et al., 1981; Dennis, 1983). Extensive studies of the properties of these enzymes have provided a wealth of information resulting in a few models accounting for the dependence of the activity of these enzymes on the physicochemical state of the lipid substrate [reviewed by Slotboom et al. (1982), Dennis (1981, 1983), and Jain and Berg (1989)]. Thus, extracellular phospholipases A_2 provide a good model system for studying the effects of the physicochemical state of lipid membranes on activities of membrane-associated enzymes.

A number of studies reported that defects in the lipid bilayer structure enhance the enzymatic activity of PL-A_2 (Jain & Cordes, 1973a,b; Wilschut et al., 1978; Upreti & Jain, 1980; Noordam et al., 1982; Jain & de Haas, 1983; Buckley, 1985; Jain & Jahagirdar, 1985; Menashe et al., 1986; Gabriel et al., 1987; Romero et al., 1987; Sen et al., 1991). Perturbations of bilayer structure by alcohols (Jain et al., 1978; Upreti et al., 1980; Kaszuba & Hunt, 1990), detergents (Jain et al., 1978), lysophospholipids, and fatty acids (Jain & Jahagirdar, 1985) or proximity to the gel to liquid crystalline phase-transition temperature (Op den Kamp et al., 1974; Wilschut et al., 1978; Noordam et al., 1982; Menashe et al., 1986) greatly activate this enzyme. Structural irregularities of the lipid bilayers are also important for the activity of intracellular PL-A_2s. Kannagi and Koizumi (1979) showed that rabbit platelet PL-A_2 preferentially hydrolyses phospholipids undergoing phase transition. Lenting et al. (1988) correlated the activity of mitochondrial phospholipase A_2 with the lipid structure determined from ^{31}P NMR experiments and found the substrate structure to be an important parameter in the activity of this intracellular PL-A_2.

Thus, in general, lipophilic molecules which induce perturbations into lipid bilayer structure may be expected to modify activity of PL-A_2. A class of lipophilic molecules that recently attracted great interest is diacylglycerols (DAGs). DAGs are endogenous second messengers, produced as a result of cell activation by a variety of stimuli [see Berridge (1987) for a review]. Several exogenously added DAGs stimulate a variety of biological responses and are studied primarily as activators of protein kinase C (Kishimoto et al., 1980; Lapetina et al., 1985; Abdel-Latif, 1986; Go et al., 1987). It was found that DAGs can directly activate both extracellular (Buckley, 1985; Kramer et al., 1987; Cunningham et al., 1989) and intracellular (Dawson et al., 1983, 1984; Kramer et al., 1987, 1988; Kolesnik & Paley, 1987; Burch, 1988) PL-A_2s.

Although a number of works addressed the question of the physicochemical effects of DAGs on the lipid membrane structure (Ohki et al., 1982; Das & Rand, 1986; Epand, 1985;

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[1] Abbreviations: PL-A_2, phospholipase A_2; DAG, diacylglycerol; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; BL-PC, PC extracted from bovine liver; NBD-PC, 1-palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine; diC_6, 1,2-*sn*-dihexanoylglycerol; diC_8, 1,2-*sn*-dioctanoylglycerol; diC_{10}, 1,2-*sn*-didecanoylglycerol; diC_{12}, 1,2-*sn*-didodecanoylglycerol; diC_{14}, 1,2-*sn*-ditetradecanoylglycerol; diC_{16}, dipalmitin; DO, diolein.

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Ortiz et al., 1988; Siegel et al., 1989; Hamilton et al., 1991), there are only few published reports where the effects of DAGs on membrane structure and on PL-A_2 activity were studied on the same system (Dawson et al., 1983, 1984; Sen et al., 1991).

In our previous NMR studies of DAG–lipid interactions, we found that different DAGs induce distinct types of perturbations of the phosphatidylcholine (PC) bilayer structure, depending on the fatty acid chain length and the degree of unsaturation of a DAG (De Boeck & Zidovetzki, 1989, 1992). In the present work, we examine the effects of the presence of these DAGs in the substrate of either dipalmitoylphosphatidylcholine (DPPC) or PC extracted from bovine liver (BL-PC) on the activity of phospholipases A_2 from three different sources: cobra venom (*Naja mocambique mocambique*), bee venom, and pig pancreas. The results demonstrate strong dependence of the activity of the phospholipases on both the chemical composition of the substrate and the length and degree of unsaturation of the added DAG molecules. Specific structural perturbations of the lipid bilayer structure induced by DAGs differentially affect PL-A_2s from different sources.

MATERIALS AND METHODS

Materials. BL-PC, DPPC, and 1-palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine (NBD-PC) were purchased from Avanti Polar Lipids (Birmingham, AL), NBD–hexanoic acid from Molecular Probes (Eugene, OR); diolein (DO), dipalmitin (diC_{16}), and phospholipases A_2 (pig pancreatic, *Naja mocambique mocambique*, and bee venom) from Sigma (St. Louis, MO). Other diacylglycerols were obtained from Serdary Research Laboratories, Inc. (London, Ontario, Canada).

Preparation of Multilamellar Vesicles. Substrates (BL-PC, DPPC) were used at a concentration of 5 mM and NBD-PC at 5 μM; and the DAG concentrations ranged from 2.5 to 35 mol %. Multilamellar vesicles were prepared by placing aliquots of the various lipid components into test tubes and by removing the chloroform and/or hexane with a stream of dry nitrogen. Then, a drop of methanol was added, and the tubes were further dried overnight under vacuum (<1 mtorr). Tris-HCl buffer (250 mM and 10 mM Ca^{2+}, pH 8.0) was then added, and the contents were put through three freeze–thaw cycles.

Phospholipase A_2 Assay. Phospholipase A_2 activity was determined according to the method of Stubbs et al. (1988) with the following modifications: the vesicles were suspended in 0.4 mL of buffer and 100 μL of phospholipase A_2 (0.5 μg of cobra venom, 0.66–0.9 μg of bee venom, 1.5 or 3.0 μg of pig pancreatic) was added to start the reaction to the total volume of 0.5 mL. The reaction times were 10 min with cobra or bee venom PL-A_2s or 20 min with pig pancreatic PL-A_2 at 37 °C (BL-PC) or 45 °C (DPPC). Kinetic experiments established that at these time points the reaction progresses linearly, and the amount of hydrolyzed lipids does not exceed 10% of the total. The reaction was stopped by the addition of 1.875 mL of chloroform–methanol (1:2, v/v), and the mixture was separated into aqueous and nonaqueous phases by the addition of 625 μL each of chloroform and water. An aliquot of the aqueous phase was removed, and the fluorescence intensity at 530 nm was determined with excitation at 470 nm in a Perkin-Elmer MPF-66 spectrofluorometer. A standard curve was obtained using known quantities of NBD–hexanoic acid. Background fluorescence was determined from the identical measurements in the absence of PL-A_2.

Table I: Initial Rates of Lipid Hydrolysis by PL-A_2s in Absence of DAGs ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)[a]

PL-A_2	substrate	initial rate
cobra venom	DPPC	50.9 ± 7.4 (23)
cobra venom	BL-PC	13.0 ± 2.7 (13)
bee venom	DPPC	30.6 ± 5.0 (17)
bee venom	BL-PC	23.6 ± 3.6 (17)
pig pancreatic	DPPC	0.17 ± 0.04 (14)
pig pancreatic	BL-PC	0.05 ± 0.01 (19)

[a] The errors are given as standard deviations; the numbers in parentheses correspond to the number of measurements.

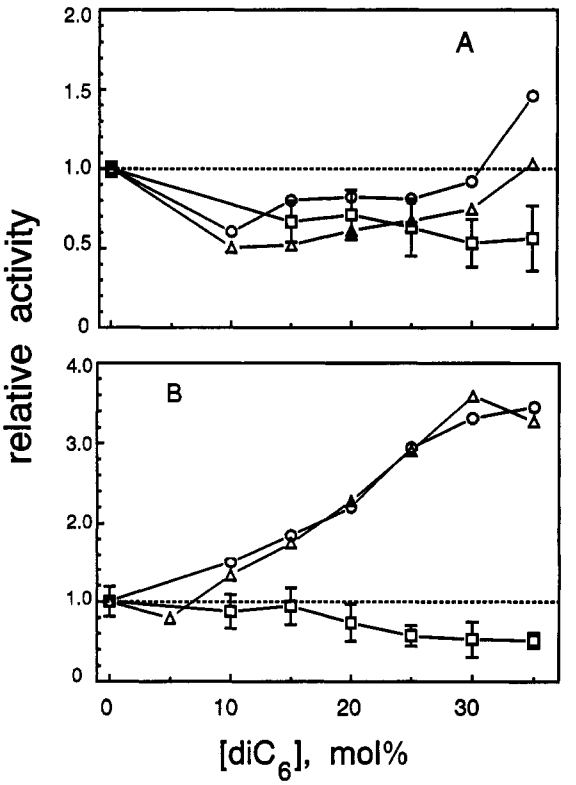

FIGURE 1: Effect of diC_6 on the activities of the phospholipases A_2. The activity of 1.0 corresponds to the absence of diC_6. The error bars are given only for the pig pancreatic PL-A_2 and omitted for the other data for clarity; the errors for other data points are of the comparable size. All assays were performed in triplicates. (△) Bee venom PL-A_2; (○) cobra venom PL-A_2; (□) pig pancreatic PL-A_2. (A) DPPC substrate; (B) BL-PC substrate.

RESULTS

Our results are presented in terms of relative activities of the PL-A_2s as the ratios of the initial rates of reaction obtained in the absence or in the presence of DAGs. The initial reaction rates of the PL-A_2s in the absence of DAGs are given in Table I.

diC_6. The dependence of the activities of the three phospholipases on the presence of different concentrations of 1,2-*sn*-dihexanoylglycerol (diC_6) is shown in Figure 1. This DAG had only a small *inhibitory* effect on the activity of pig pancreatic PL-A_2 when DPPC was used as a substrate (Figure 1A) and no significant effect with the BL-PC substrate (Figure 1B). With the DPPC substrate, diC_6 had a small but significant effect on the activity of the cobra venom or bee venom PL-A_2 (Figure 1A). The effect was biphasic: an inhibition of these two enzymes was observed up to the diC_6 concentration of 20–25 mol %, whereas further increase of diC_6 concentration resulted in decreased inhibition in the case of the bee venom PL-A_2 or even 1.5-fold activation of the cobra venom PL-A_2 by 35 mol % of diC_6. Significant activation

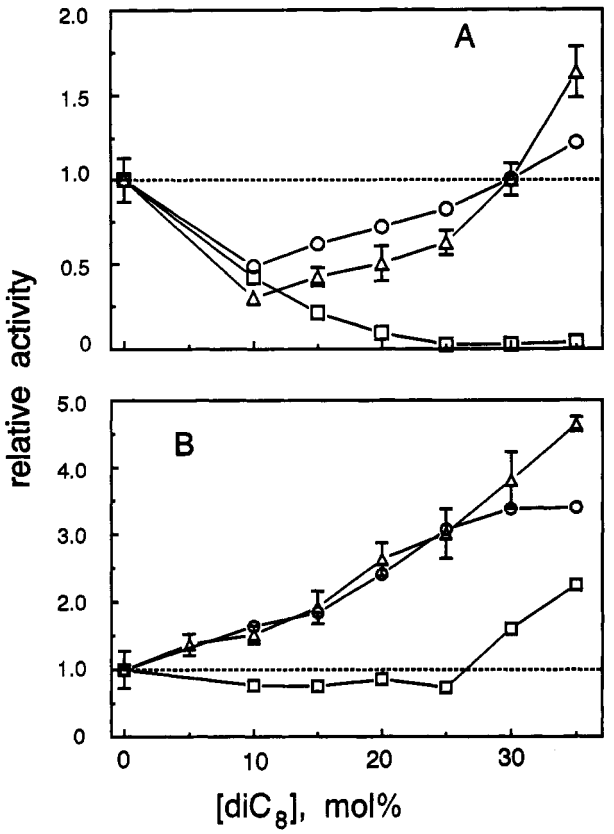

FIGURE 2: Effect of diC_8 on the activities of the PL-A_2s. (△) Bee venom PL-A_2; (○) cobra venom PL-A_2; (□) pig pancreatic PL-A_2. (A) DPPC substrate; (B) BL-PC substrate.

of both cobra and bee venom PL-A_2s was observed with BL-PC substrate: the activity increased ~3.5-fold at 30 mol % of diC_6 (Figure 1B).

diC_8. A qualitatively similar but greater effect on PL-A_2s' activity was caused by the presence of 1,2-*sn*-dioctanoylglycerol (diC_8) (Figure 2). With the DPPC substrate, the addition of diC_8 resulted in monotonously increasing inhibition of the porcine PL-A_2; the inhibition was practically complete at 20 mol % of diC_8 (Figure 2A). The effect of diC_8 on cobra or bee venom PL-A_2 was, as in the case of diC_6, biphasic: about 2-fold inhibition was caused by 10 mol % of diC_8, whereas further increase of diC_8 concentration resulted in decreased inhibition of the cobra venom PL-A_2 and even ~1.6-fold activation of the bee enzyme by 35 mol % of this DAG. With the BL-PC substrate, addition of diC_8 resulted in monotonously increased activities of cobra or bee venom PL-A_2s reaching ~4-fold activation at 35 mol % of this DAG (Figure 2B). Little effect on pig pancreatic PL-A_2 was observed up to 25 mol % of diC_8 with BL-PC substrate; further increase in diC_8 concentration activated the enzyme ~2-fold at 35 mol % of diC_8.

diC_{10}. The effects of 1,2-*sn*-didecanoylglycerol (diC_{10}) on PL-A_2 activity are shown in Figure 3. With the DPPC substrate, diC_{10}, like the shorter DAGs, had an inhibitory effect on the activity of all three PL-A_2s (Figure 3A,C); again, the degree of inhibition decreased at 35 mol % of diC_{10} in the case of the cobra or bee venom PL-A_2 (Figure 3A). No significant change of cobra or bee venom PL-A_2 activity was observed with the BL-PC lipid substrate up to 25 mol % of diC_{10}; further increase of diC_{10} concentration resulted in 1.5–2.5-fold increase of the activity of these enzymes (Figure 3B). In contrast, strong *activation* of pig pancreatic PL-A_2 was caused by the addition of diC_{10} to the BL-PC substrate; the activation reached ~25-fold with 35 mol % of diC_{10} (Figure 3D).

diC_{12}. The effects of 1,2-*sn*-didodecanoylglycerol (diC_{12}) on PL-A_2s' activity are shown in Figure 4. Only a small effect of diC_{12} on the activity of bee or cobra venom PL-A_2s was observed with the DPPC substrate up to 25 mol % of diC_{12}; the increase of diC_{12} concentration to 35 mol % resulted in ~2.2-fold activation of these enzymes (Figure 4A), while the activation was already significant at 15–20 mol % of diC_{12} with the BL-PC substrate (Figure 4B). Presence of diC_{12} resulted in a strong activation of the pig pancreatic PL-A_2 with either DPPC or BL-PC substrates. In the case of the BL-PC substrate, addition of 5 mol % of diC_{12} increased activity of the porcine PL-A_2 ~3-fold, and the activation reached ~25-fold increase at 25–35 mol % of this DAG (Figure 4D). A slightly smaller effect was observed with the DPPC substrate: the activity was enhanced 2-fold at 5 mol % of diC_{12} and reached ~18-fold of the control values at 35 mol % of diC_{12} (Figure 4C).

diC_{14}. The effects of 1,2-*sn*-ditetradecanoylglycerol (diC_{14}) on the activities of the phospholipases are summarized in Figure 5. The effects of this DAG were qualitatively similar to those induced by diC_{12}, but significantly stronger; the activation of cobra or bee venom PL-A_2s reached ~3.5-fold of the control values with DPPC or ~2-3-fold of the control values with BL-PC substrate (Figure 5A,B). Even greater effects were observed with the pig pancreatic enzyme: addition of 5 mol % of diC_{14} increased activity of this PL-A_2 ~20-fold, and the activity reached ~70-fold of the control value with 35 mol % of diC_{14} (Figure 5C). With the BL-PC substrate, addition of diC_{14} resulted in monotonous increase of the activity of the pig pancreatic PL-A_2 reaching ~20-fold activation at 25–35 mol % of this DAG (Figure 5D).

diC_{16}. Figure 6 summarizes the effect of diC_{16} on the activities of the phospholipases A_2. This DAG affected the activities of cobra and bee venom enzymes similarly to diC_{12} and diC_{14}, except that with the BL-PC substrate the effect was significant already at 5 mol % of diC_{16} and reached ~3.5-fold activation (for bee PL-A_2) at 35 mol % (Figure 6B). The strongest activation observed by us in this study was that of pig pancreatic PL-A_2 by diC_{16} with DPPC substrate: the activity was increased ~8-fold by 2.5 mol % of diC_{16} and reached ~130-fold activation at 30 mol % of diC_{16}. The activation of pig pancreatic PL-A_2 by diC_{16} was significantly lower with BL-PC substrate reaching ~35-fold of the control values at 35 mol % of diC_{16} (Figure 6D).

DO. The effect of DO on the activity of the phospholipases was critically dependent on the substrate used: DO *inhibited* all three enzymes with DPPC substrate (Figure 7A,C) while it *activated* the enzymes with BL-PC substrate (Figure 7B,D). The inhibition reached near maximum value already at 5 mol % of DO (Figure 7A,C). The presence of DO in the BL-PC substrate affected the activity of pig pancreatic PL-A_2 to a much greater degree than the cobra or bee venom enzymes: significant activation of the porcine enzyme was observed at 5 mol % of DO and reached ~32-fold of the control value at 35 mol % of DO (Figure 7D). DO had no significant effect on cobra or bee PL-A_2 with BL-PC substrate up to a concentration of 10 mol %, whereupon the enzymatic activities monotonously increased with DO concentration reaching ~3-fold activation in the case of bee venom PL-A_2 at 25–35 mol % DO (Figure 7B).

The dependence of the activities of PL-A_2s on DAG chain length are summarized in Figure 8. We found that three parameters are important in determining the resulting effect:

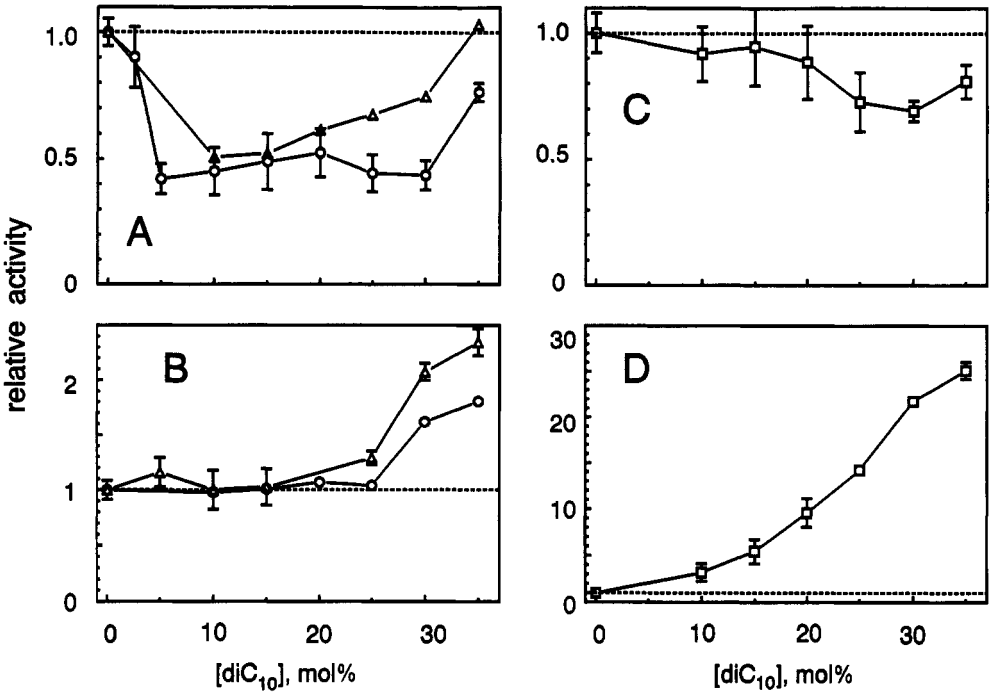

FIGURE 3: Effect of diC_{10} on the activities of the PL-A_2s. (△) Bee venom PL-A_2; (○) cobra venom PL-A_2; (□) pig pancreatic PL-A_2. (A and C) DPPC substrate; (B and D) BL-PC substrate.

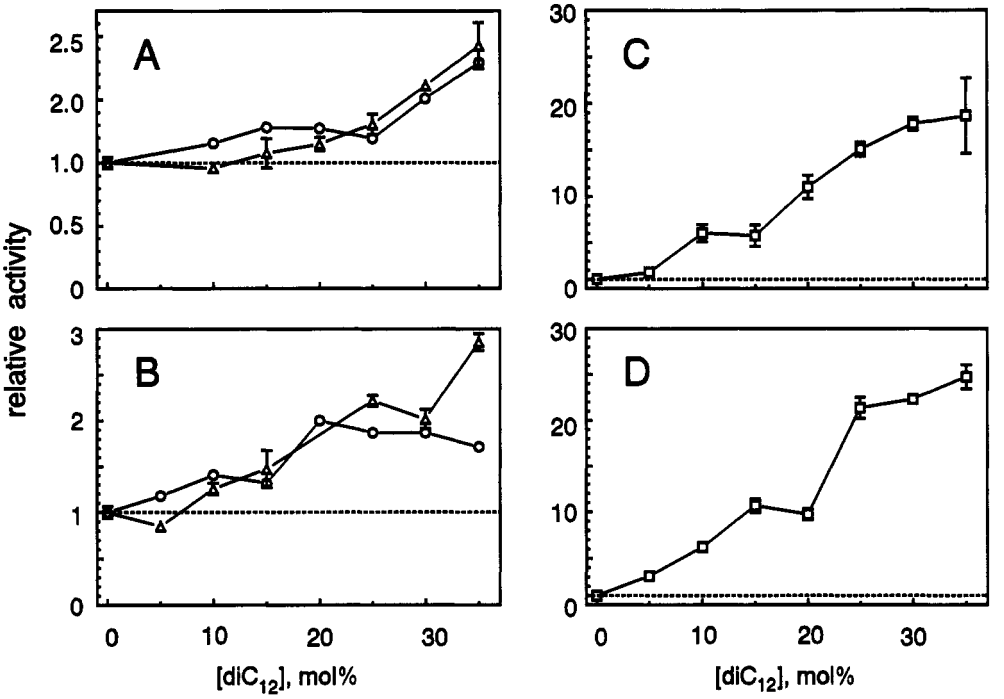

FIGURE 4: Effect of diC_{12} on the activities of the PL-A_2s. (△) Bee venom PL-A_2; (○) cobra venom PL-A_2; (□) pig pancreatic PL-A_2. (A and C) DPPC substrate; (B and D) BL-PC substrate.

(i) the length of DAG side chains; (ii) the origin of the phospholipase A_2; and (iii) the chemical composition of the substrate, i.e., DPPC or BL-PC. The effects of the DAGs on activities of cobra or bee venom PL-A_2s were essentially identical (Figure 8A,B), but these enzymes were significantly different from pig pancreatic PL-A_2 (Figure 8C,D). With the DPPC substrate, diC_6 and diC_8 inhibited all three enzymes (Figure 8A,C). Increase of the DAG side-chain length to >10 carbons resulted in the case of bee and cobra venom enzymes in the return of the activity to the control values or even a slight activation (Figure 8A). In contrast, strong activation of the porcine enzyme was observed under these conditions; 10 mol % of diC_{16} activated this enzyme ~35-fold (Figure 8C).

With the BL-PC substrate, diC_6 and diC_8 activated bee and cobra venom enzymes (Figure 8B) but had little effect on the pig pancreatic PL-A_2 (Figure 8D). diC_{10} did not affect the activity of cobra or bee venom PL-A_2s (Figure 8B) but increased the activity of pig pancreatic PL-A_2 ~3-fold (Figure 8D). The longer-chain DAGs activated all three enzymes, but the effect on pig pancreatic PL-A_2 was significantly greater (Figure 8B,D).

The effect of DAGs was also critically dependent on the nature of the phospholipid substrate: diC_6 and diC_8 inhibited

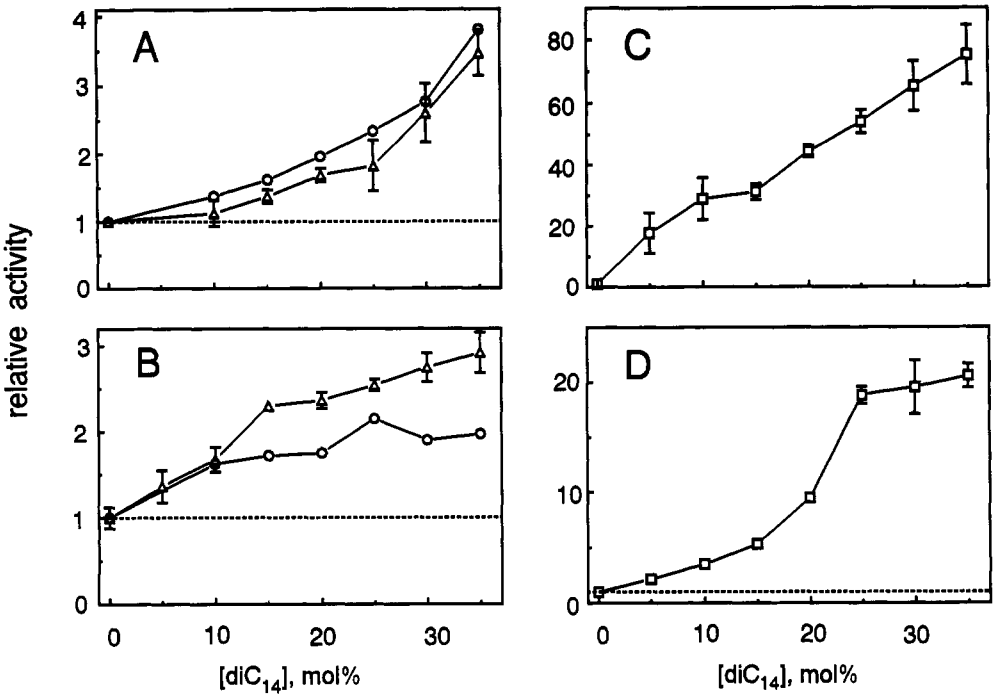

FIGURE 5: Effect of diC_{14} on the activities of the PL-A_2s. (△) Bee venom PL-A_2; (○) cobra venom PL-A_2; (□) pig pancreatic PL-A_2. (A and C) DPPC substrate; (B and D) BL-PC substrate.

cobra and bee venom PL-A_2 with DPPC substrate (Figure 8A) but *activated* the same enzymes ~1.5-fold with the BL-PC substrate (Figure 8B). Strong substrate dependence was also observed in the effects of DAGs on pig pancreatic PL-A_2; 10 mol % of diC_{10} had little effect on the activity of this enzyme with the DPPC substrate (Figure 8C), but activated it ~3-fold with the BL-PC substrate (Figure 8D). Longer-chain DAGs activated this PL-A_2 with both substrates; the effect was, however, significantly greater with the DPPC substrate (Figure 8C,D).

DISCUSSION

The present work was initiated to develop a molecular understanding of how DAGs modulate activities of membrane-associated enzymes. It extends our previous studies on physicochemical effects of DAGs on lipid bilayer structure (De Boeck & Zidovetzki, 1989, 1992) and provides a correlation of membrane effects of a series of DAGs with the DAG-induced modulation of PL-A_2 activity on the same system.

The DAGs used in the present study induce four different types of perturbations into bilayer structure (De Boeck & Zidovetzki, 1989, 1992): (i) "transverse" perturbation of the bilayer, where the order of the phospholipid side chains is increased in the region adjacent to the headgroup but decreased toward the bilayer interior (diC_6 and diC_8, with DPPC or BL-PC bilayers); (ii) lateral phase separation of the lipids into the regions of different fluidities (diC_{12}–diC_{16}, with DPPC or BL-PC bilayers); (iii) formation of nonbilayer lipid phases (DO with BL-PC membranes); and (iv) increased ordering of the lipid side chains (DO with DPPC bilayers). This information, obtained from the physicochemical studies, allows us to understand the complex effects of these DAGs on the activities of PL-A_2s observed in this report. In the following discussion we will consider how each of these bilayer-perturbing effects of DAGs affects phospholipases A_2.

Transverse Perturbation. This type of perturbation, induced by diC_6 and diC_8 (De Boeck & Zidovetzki, 1992), correlates with the inhibition of all three enzymes with the DPPC substrate (Figure 8A,C). Short-chain DAGs intercalate between the bulky PC head groups and promote tighter contact between the phospholipid side chains in the region adjacent to the head groups, which implies increased lateral surface pressure in the bilayers (De Boeck & Zidovetzki, 1992). The free volume created below the level of DAG penetration is filled by more disordered phospholipid side chains [see Zidovetzki and Lester (1992) for a model of DAG/PC bilayers]. In the case of the DPPC substrate, the net result is decreased probability for a single phospholipid molecule to dissociate from the bulk phospholipids and bind to the PL-A_2 catalytic site, leading to the observed decrease of the enzymatic activity. The diC_6- and diC_8-induced increases of the lateral surface pressure in the BL-PC bilayers are smaller than in the case of DPPC; the observed activation of the bee and cobra venom enzymes in this case (Figure 8B) correlates with the decreased order parameters in the bilayer interior. No such activation was observed for the porcine PL-A_2, indicating that the activity of this enzyme is more sensitive to the physicochemical state of the surface, rather than the interior, of the membranes. These observations can be understood in terms of the "penetrating capacity" of the phospholipases (Verger & de Haas, 1976; Gheriani-Gruszka et al., 1988). Indeed, the membrane-penetrating capacity of pig pancreatic PL-A_2 is less than half of those for cobra or bee venom enzymes (Demel et al., 1975; Zwaal et al., 1975; Van der Wiele et al., 1988) probably due to the lack of the hydrophobic region in the sequence that serves as a lipid-binding domain and is present in cobra venom PL-A_2 (Van der Wiele et al., 1988). This interpretation is further supported by the biphasic character of the effect of diC_6 and diC_8 on the activities of cobra and bee venom PL-A_2 with the DPPC substrate (Figures 1A and 2A), which correlates with the biphasic effect of these DAGs on the bilayer structure. As we have shown previously, elevation of diC_6 or diC_8 concentration above 20 mol % results in gradual decrease of the order parameters along DPPC side chains, thereby diminishing additional bilayer surface pressure (De Boeck & Zidovetzki, 1992), which allows cobra and bee venom, but not pig pancreatic, PL-A_2 to penetrate into the bilayers.

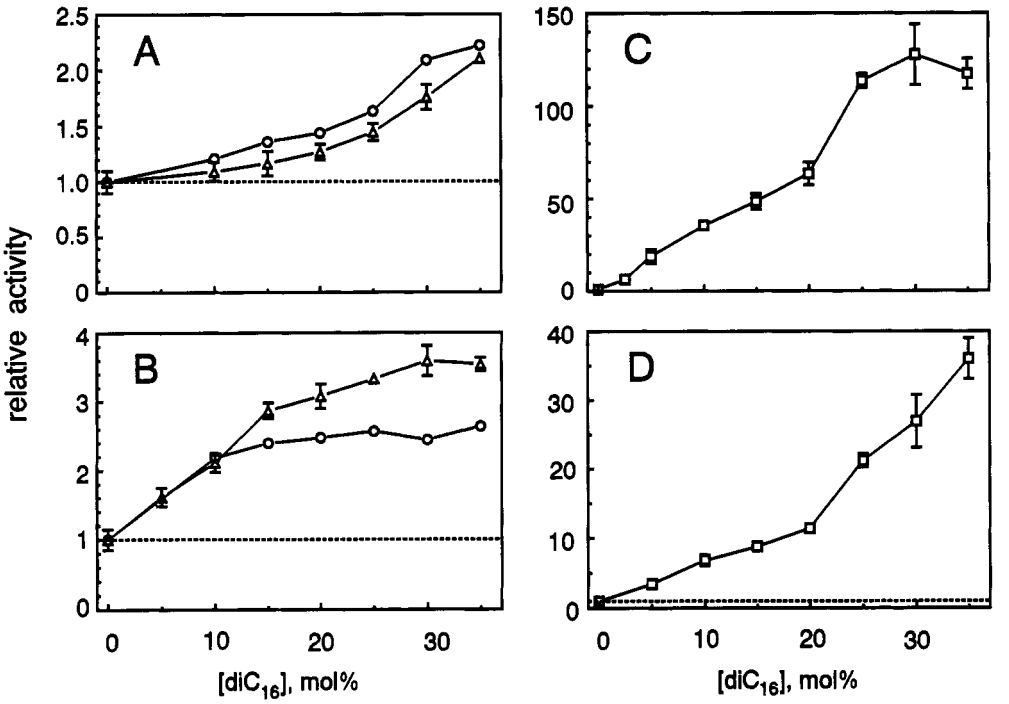

FIGURE 6: Effect of diC_{16} on the activities of the PL-A_2s. (△) Bee venom PL-A_2; (○) cobra venom PL-A_2; (□) pig pancreatic PL-A_2. (A and C) DPPC substrate; (B and D) BL-PC substrate.

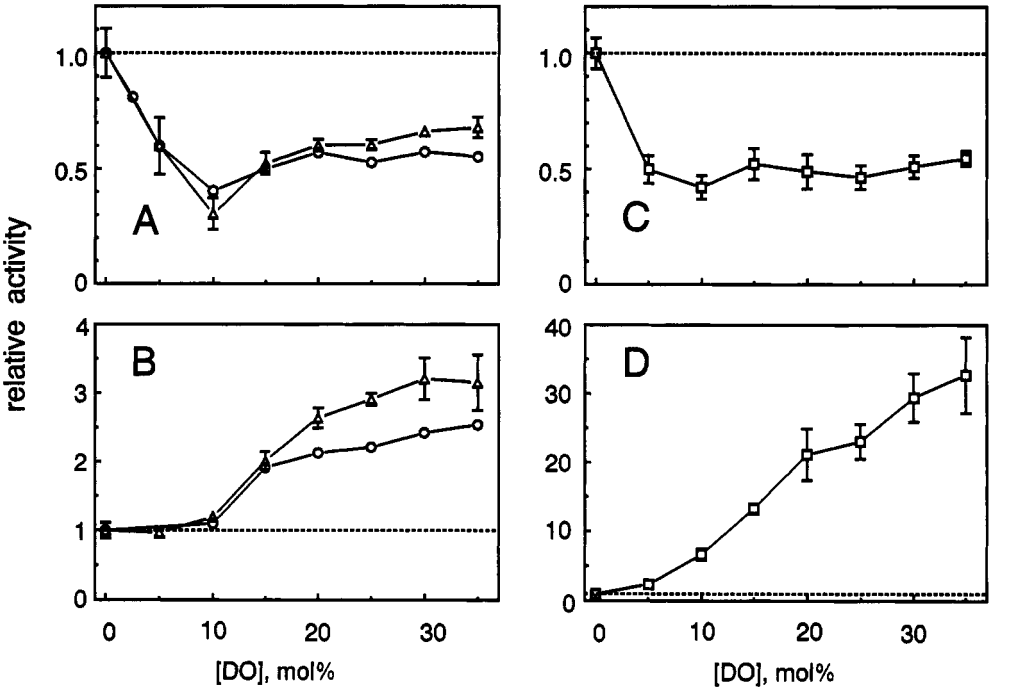

FIGURE 7: Effect of DO on the activities of the PL-A_2s. (△) Bee venom PL-A_2; (○) cobra venom PL-A_2; (□) pig pancreatic PL-A_2. (A and C) DPPC substrate; (B and D) BL-PC substrate.

Lateral Phase Separation. Longer-chain (C > 10) DAGs induce lateral phase separation of the bilayers into regions of different fluidities (De Boeck & Zidovetzki, 1989, 1992). These DAGs activate PL-A_2s with both lipid substrates (Figure 8) although the venom phospholipases with the DPPC substrate were activated only at higher DAG concentrations (Figures 4A–6A). Structural defects, associated with the coexistence of gel and liquid crystalline lipid phases, have long been implicated in the activation of PL-A_2s. Pig pancreatic and bee venom PL-A_2s efficiently hydrolyze dimyristoyl-PC or DPPC bilayers only around the phase-transition temperature, when the two phases coexist (Op den Kamp et al., 1974; Wilschut et al., 1978; Lichtenberg et al., 1986). Moreover, Jain and Jahagirdar (1985) suggested that only long-lived boundary defects (in terms of catalytic cycles of the enzyme) cause the enzyme activation. This implies that the lateral phase separation induced by the DAGs has long lifetime boundaries by this criterium. The packing defects present in the boundary region between the phases would weaken the association between the substrate molecules facilitating binding of the substrate to the catalytic site of the enzyme (Jain & Zakim, 1987). Similar interpretation of the observed activation of pig pancreatic PL-A_2 by PC–cholate mixtures was recently used by Gheriani-Gruszka et al. (1988).

Our results further demonstrate that the presence of an unsaturated side chain is not necessary for PL-A_2 activation.

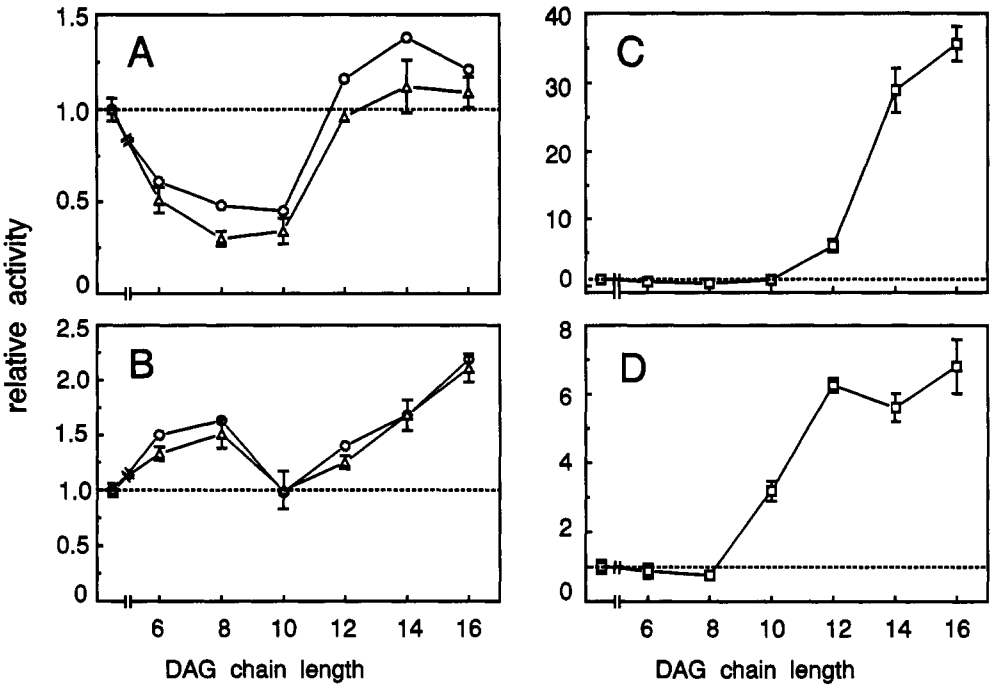

FIGURE 8: Dependence of the activities of the PL-A_2s on the DAG side-chain lengths at 10 mol % of the DAGs. (△) Bee venom PL-A_2; (○) cobra venom PL-A_2; (□) pig pancreatic PL-A_2. (A and C) DPPC substrate; (B and D) BL-PC substrate.

Such a requirement was suggested by Dawson et al. (1984) from a comparison of PL-A_2-activating capacities of diC_{10} and two DAGs with unsaturated side chains. We found that although the presence of 25 mol % of diC_{10} did enhance pig pancreatic (but not cobra or bee venom) PL-A_2 to approximately the same extent as observed by Dawson et al. (1984), the largest activation of each of these three enzymes with the BL-PC substrates was achieved not only by DO but also, to a comparable degree, by saturated diC_6, diC_8, and diC_{16} in the case of cobra venom enzymes or by diC_{10}–diC_{16} in the case of the pig pancreatic PL-A_2. Our data show that diC_{10} is probably the least "representative" or disaturated DAGs: out of six possible combinations (three PL-A_2s with two substrates each) only pig pancreatic PL-A_2 activity was significantly increased by diC_{10}, with the BL-PC substrate (Figure 8D).

Nonbilayer Lipid Phases. Nonbilayer lipid phases (hexagonal and "isotropic") are induced by DO in BL-PC membranes (De Boeck & Zidovetzki, 1989). A number of recent studies emphasized the possible role of these phases in activation of PL-A_2 and other intracellular enzymes (Hui et al., 1981; Bentz et al., 1985; Cheng et al., 1986; Epand & Bottega, 1988; Sen et al., 1991). Buckley (1985) and Sen et al. (1991) showed that several lipids which form nonbilayer structures stimulate PC hydrolysis by cobra venom, pig pancreatic, and microbial PL-A_2s. A similar correlation of the activity of PL-A_2 from rat intestinal mucosa with the presence of nonbilayer lipid phases was observed by Dawson et al. (1984). Sen et al. (1991), however, suggested that this stimulation is not due to the actual presence of a nonbilayer phase but rather is a consequence of the pretransitional packing stress and related packing defects. Our results with DO are compatible with these conclusions, because DO induces nonbilayer lipid phases with BL-PC only at the temperatures higher than those used for the PL-A_2 assays (De Boeck & Zidovetzki, 1989), which indicates increased fluctuations of the bilayer structure already at the lower temperatures, corresponding to the temperatures of the PL-A_2 activity measurements. In a recent molecular model of PC/DO bilayers we showed that intercalated into bilayers DO molecules, with their virtual absence of head groups, create "voids" between the phospholipid head groups increasing the tendency of the membranes to form nonbilayer structures (Zidovetzki & Lester, 1992). As in the above-discussed cases, the presence of these defects (or "structural fluctuations") would weaken the association between the substrate molecules promoting the catalytic process.

The association of the PL-A_2-activating effect of DO with increased bilayer fluctuations explains the apparent discrepancy of our results with those of Cunningham et al. (1988). We observed much greater activation of pig pancreatic PL-A_2 by DO (>20-fold at 25 mol %) than reported by Cunningham et al. (1989) on a similar system, where only 75% enhancement was obtained. This difference may be due to different forms of the substrates used: sonicated vesicles were used by Cunningham et al. (1989) while we used nonsonicated multilamellar lipid dispersions. Indeed, we consistently observed smaller effects when using sonicated lipid substrate. In the case of sonicated BL-PC/DO, 25 mol % of DO enhanced activity of pig pancreatic PL-A_2 only ~2-fold, as compared to >20-fold in the case of the nonsonicated substrate. The difference is probably due to the high curvature and associated increased stress of the structure of the sonicated lipids; addition of DO in this case may not induce significant additional structural defects. We chose nonsonicated lipids for this study to minimize such structural defects. We also experienced difficulties in obtaining clear suspensions of BL-PC or DPPC in the presence of ≥20 mol % of DO or diC_{16} upon sonication, suggesting possible propensity of such vesicles to fuse.

Increased Ordering of Phospholipid Side Chains. Addition of DO to DPPC results in the significant increase of order parameters of the DPPC acyl side chains and, consequently, increased lateral surface pressure of the DPPC bilayers and tighter association between the phospholipid molecules (De Boeck & Zidovetzki, 1989). This large increase of the lateral pressure in the membranes and tighter side-chain contact may prevent dissociation of the substrate molecules from the bulk lipids and correlates with the observed inhibition of the activities of all three PL-A_2s by DO with the DPPC substrate (Figure 7A,C).

Conclusions. The results obtained in this study argue against generalizations in designating amphiphilic molecules as "activators" or "inhibitors" of PL-A_2s. Thus, short-chain saturated DAGs can either activate or inhibit the same PL-A_2, depending on the nature of the substrate (Figure 8). The substrate dependence is even more prominent in the case of DO: this DAG inhibited all PL-A_2s with DPPC substrate but activated all of them with the BL-PC substrate (Figure 7). Similar observations were made by Roberts and Dennis (1989), who reported that in model PC systems, fatty acids can either activate or inhibit PL-A_2 activity depending on the source of the enzyme and the physical characteristics of the assay system.

PL-A_2 activity can be significantly affected by low (2.5–5 mol %) concentrations of DAGs, which is within physiological limits of endogenously produced DAGs (Kramer et al. 1987). Thus, for example, long-chain DAGs and DO significantly activated pig pancreatic PL-A_2 at 5 mol % membrane concentration, and, in the case of diC_{16}, ~8-fold activation was observed upon addition of only 2.5 mol % of this DAG to the DPPC substrate (Figure 6C). It was recently suggested by Ortiz et al. (1988) and Cunningham et al. (1989) that, since biomembrane lipids are under physiological conditions close to the temperature of bilayer–nonbilayer lipid phase transition (Siegel, 1986; Bentz & Ellens, 1988), the DAG-membrane system may exhibit ultrasensitive behavior [see Koshland (1987)]. Applied to the present case, this would imply that a relatively small increase in the DAG content of the membranes may trigger large changes of PL-A_2 activity. Thus, a 2–7-fold increase of DAG membrane content observed upon stimulation in some biomembranes (Wright et al., 1988; Sebaldt et al., 1990; Altin et al., 1990) might substantially increase bilayer fluctuations and significantly increase PL-A_2 activity.

The combined results of this and our previous (De Boeck & Zidovetzki, 1989, 1992) studies strongly support the notion that increased structural defects of the lipid bilayer structure, which can be associated with the transverse perturbation or order parameters of lipid side chains (induced by diC_6 and diC_8), lateral phase separation of bilayers into regions of different fluidities (induced by diC_{12}–diC_{16}), or propensity to form nonbilayer lipid phases (induced by DO) are crucial in activating PL-A_2s. Generally, the activity of pig pancreatic PL-A_2 is considerably more sensitive to the presence of DAG-induced bilayer defects as indicated by the observed significant differences in the DAG-induced changes of the activities between bee or cobra venom and pig pancreatic PL-A_2s (Figure 8). This can be a consequence of the low "penetrating capacity" of this enzyme: while cobra or bee venom PL-A_2s can efficiently hydrolyze unmodified PC substrate, the porcine enzyme's activity toward such stable bilayer substrate is extremely low (Table I), and consequently pig pancreatic PL-A_2 activity is very sensitive to and is greatly enhanced by the bilayer defect-inducing additives, such as DAGs. The results are compatible with a few models accounting for the dependence of PL-A_2 activity on the physical state of the substrate: formation of surface defects (Jain & Zakim, 1987), molecular shape of the intercalating activator (Buckley, 1985), uncoupling of phospholipid molecules from the rest of the matrix so that they are accessible for productive binding to the enzyme (Roberts et al., 1977), or increased bilayer fluctuations (Romero et al., 1987). It was pointed out previously that these models are not mutually exclusive (Barlow et al., 1988; Gheriani-Gruszka et al., 1988), for example, increased fluctuations may promote penetration of PL-A_2 into the bilayer or uncouple a lipid molecule from the membrane as proposed in the dual-phospholipid activation model. The existence of a few models, however, indicates that the exact molecular nature of these bilayer fluctuations is not yet entirely clear. We are currently studying these processes using ^2H NMR relaxation measurements.

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