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Phospholipase A2 Activity on Mixed Lipid Monolayers: Inhibition and Activation of Phospholipases A2 from Porcine Pancreas and *Crotalus adamanteus* by Anionic and Neutral Amphiphiles[1]

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The effects of anionic and neutral amphiphiles on porcine pancreatic and *Crotalus adamanteus* phospholipases A2 were studied in a monolayer system as a function of surface pressure. The insoluble amphiphile, dicetyl phosphate (DCP), inhibited the hydrolysis of didecanoylphosphatidylcholine (DDPC) by both enzymes below their normal cutoff pressures with pure DDPC. DCP, however, enhanced enzyme penetration and thus activated the pancreatic enzyme above its normal cutoff pressure. The soluble surfactants, 3,5-dibromo- and 3,5-diiodosalicyclate, acetyl salicylate, and salicylic acid, had similar effects. 1,2-Didecanoin inhibited the hydrolysis of DDPC below the normal cutoff pressures and increased the cutoff pressures for both enzymes. Zwitterionic detergents, *N*-dodecyl- and *N*-tetradecyl-*N*,*N*-dimethyl-3-aminopropanesulfonate, were found to be potent inhibitors of the pancreatic enzyme on DDPC monolayers. Relative substrate specificities for both enzymes were determined as a function of surface pressure with phosphatidylcholine, phosphatidylglycerol, and phosphatidic acid. Pancreatic phospholipase A2 was more active and penetrated to higher pressures with the anionic phospholipids, while the venom enzyme was more active with phosphatidylcholine.

Several years ago we reported on the inhibition of phospholipase A2 by cationic surface-active local anesthetics in monolayer systems (1). Inhibition by these cationic amphiphiles seemed to be a general phenomenon related to the positive charge at the lipid interface (2). In view of these results, it seemed of interest to extend these studies to include the effects of anionic and neutral amphiphiles on phospholipase A2 activity. The study of lipolytic enzyme action in monolayer systems has several advantages over bulk systems. Enzyme activity can be studied over a range of known surface pressures and the composition of the liquid interface can be more closely controlled (3, 4).

Both water-soluble and insoluble amphiphiles were included in this study. The anionic amphiphiles are dicetyl phosphate (DCP), which forms an insoluble monolayer, and salicylic acid derivatives which are soluble amphiphiles. The soluble amphiphiles have a disadvantage in that their inhibition of phospholipase activity may be related to subphase interaction with the enzyme (1) alone or in addition to surface effects at the lipid interface. The neutral amphiphiles are didecanoin (DDG), which forms insoluble monolayers, and a soluble zwitterionic detergent. In addition, substrate specificities of phospholipases A2 from porcine pancreas and *Crotalus adamanteus* with phosphatidylcholine, phosphatidylglycerol, and phosphatidic acid are reported. Although it is known that phospholipase A2 from porcine pancreas has a greater specificity for anionic phospholipids while the snake venom enzyme prefers the zwitterionic phosphatidylcholine (5), the surface pres-

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sure dependencies of these two enzymes with zwitterionic and anionic substrates have not been reported in a comparative way.

EXPERIMENTAL

Materials. The zymogen of pancreatic phospholipase A2 was isolated from porcine pancreas by the method of Nieuwenhuizen *et al.* (6). *C. adamanteus* phospholipase A2 (B form) was isolated from the venom as described by Wells (7). Stock solutions of these enzymes (1 mg/ml) were stored at 4°C. Both enzymes gave single bands after polyacrylamide gel electrophoresis. Didecanoylphosphatidylcholine (DDPC)[3] was obtained from Supelco Inc., Bellefonte, Pennsylvania, and also was synthesized as described by Jensen and Pitas (8). Didecanoylphosphatidylglycerol (DDPG) and didecanoylphosphatidic acid (DDPA) were synthesized by phospholipase D catalyzed exchange (9) from DDPC. The phospholipids were purified by HPLC through a Licroprep Si-60 (E. Merck, Darmstadt, Germany) column (1 cm id × 1 m) with chloroform–methanol solvent mixtures. The lipids were pure by TLC ($CHCl_3$:CH_3OH:NH_3:H_2O, 90:54:5.5:2). Dicetyl phosphate (DCP) was obtained from Sigma Chemical Company, St. Louis, Missouri. 1,2-Didecanoylglycerol (DDG) was prepared by pancreatic lipase hydrolysis of tridecanoin and purified by preparative TLC. Stock solutions of lipids (1 mM) were prepared in benzene (DDPC, DDPA, DDPG), benzene–methanol (24:1) (DCP) or hexane (DDG) and stored at −20°C. Zwittergent 3-12 (Z 3-12) (*N*-dodecyl-*N,N*-dimethyl-3-amino-1-propanesulfonate) and Zwittergent 3-14 (Z 3-14) (*N*-tetradecyl-*N,N*-dimethyl-3-aminopropanesulfonate) were obtained from Calbiochem-Behring Corporation, La Jolla, California. 3,5-Diiodosalicylic acid, 3,5-dibromosalicylic acid, acetyl salicylate, and salicylic acid were obtained from Eastman Kodak Company, Rochester, New York. Stock solutions of these soluble surfactants were prepared in buffer and readjusted to pH 7 if necessary. Monolayers were spread over a 0.1 M NaCl, 5 mM $CaCl_2$, 5 mM Tris-chloride buffer, pH 7, prepared from double-distilled water.

Methods. Monolayer compression isotherms were obtained by driving a mobile barrier at a rate of 1.71 cm/min (27.5 cm^2/min) across a single compartment Teflon trough (16.1 × 27.1 cm). Surface pressure was measured by the Wilhelmy method (10) using a rough-surfaced platinum plate attached to a Cahn RTL electrobalance. The temperature was maintained between 22 and 24°C.

Monolayer enzyme reactions were followed using the "zero order trough" as described by Verger and de Haas (3). The monolayers were spread over the buffer subphase (5 mM Tris, 5 mM $CaCl_2$, 0.1 M NaCl, pH 7) from 1 mM stock solutions of lipid to the desired surface pressure. Then 100 μl of enzyme solution (0.2–2 μg) was added with stirring under the monolayer in the enzyme compartment. Reactions were followed for approximately 20 min after the recorded plots became linear. Controls of pure DDPC were run between each experiment with mixed lipid monolayers and all experimental rates were expressed relative to these control rates. All runs were performed in the temperature range of 22–24°C.

RESULTS

Compression studies of mixed monolayer systems. Ideal mixing in a two-component monolayer is characterized by the equation described by Gaines (11): $A(12) = A(1)N(1) + A(2)N(2)$, where the average area per molecule, $A(12)$, is an additive function of the specific molecular areas of the two components in pure films at the same pressure, $A(1)$ and $A(2)$, and the mole fractions of the components in the mixed film, $N(1)$ and $N(2)$. Any deviation from this ideal behavior indicates nonideal mixing and component interaction. The average molecular areas for DDPC-DCP mixed monolayers as a function of mole fraction are shown in Fig. 1. Except for a slight condensing effect between 0 and 0.1 mol fraction DCP (which may or may not be significant), ideal mixing is seen at both 10 and 20 mN/m. DDPC-DDG mixed monolayers, however, show a definite condensing effect over the entire range at 5 mN/m (Fig. 1).

Kinetic studies. The kinetics of phospholipase A2 action on a lecithin monolayer are characterized by a nonlinear induction phase (presteady state) followed by a linear steady-state phase. The following kinetic expression for the steady-state rate, as developed by Verger *et al.* (4) for

[3] Abbreviations used: DDPC, 1,2-didecanoyl-*sn*-glycero-3-phosphorylcholine; DDPA, 1,2-didecanoyl-*sn*-glycero-3-phosphate; DDPG, 1,2-didecanoyl-*sn*-glycero-3-phosphorylglycerol; DCP, dicetyl phosphate; DDG, 1,2-didecanoylglycerol; Z 3-12, *N*-dodecyl-*N,N*-dimethyl-3-aminopropanesulfonate; Z 3-14, N-tetradecyl-N,N-dimethyl-3-aminopropanesulfonate; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography.

phospholipase action on a monolayer, was used for analysis of kinetic data,

$$v_m = \frac{k_{cat}E_0S}{K^*k_d/k_p},$$

where V_m is the velocity (molecules/surface/time); k_{cat}, the catalytic rate constant (time^{-1}); E_0, the total enzyme concentration (molecule/volume); S, the substrate concentration (molecules/surface); k_d, the desorption rate constant (time^{-1}); k_p, the penetration rate constant (volume/surface/time); and K^*, the interfacial Michaelis-Menton constant (molecules/surface). The reaction rate as determined with the zero-order trough is in terms of area change (of the lipid reservoir trough)/min. This value divided by the surface area (of the reaction trough) gives the rate in terms of v_m/S (min^{-1}). The rate determined in the presence of added amphiphile, v'_m/S', is expressed as a relative rate, $R = (v'_m/S')/(v_m/S)$, where v_m/S is the rate of the control (no added amphiphile) with the same total enzyme concentration. The lag time was determined by extrapolating the linear steady-state rate to zero area change.

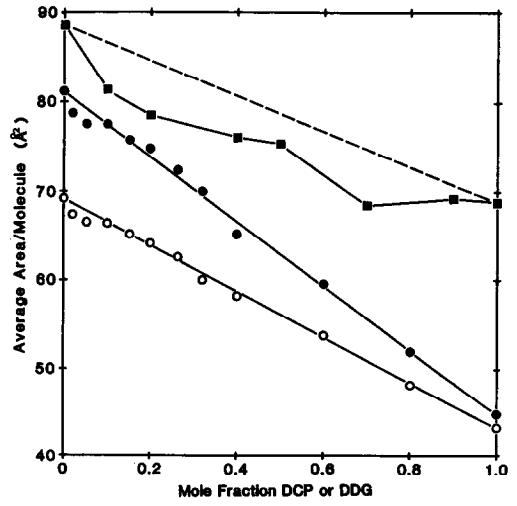

FIG. 1. Plot of average molecular area versus composition of DDPC and DCP or DDG mixed monolayers. DDPC-DDG mixed monolayers at 5 mN/m, ■; DDPC-DCP mixed monolayers at 10 mN/m, ●; and 20 mN/m, ○. All monolayers spread over 5 mM Tris buffer, pH 7, 0.1 M NaCl, 5 mM $CaCl_2$.

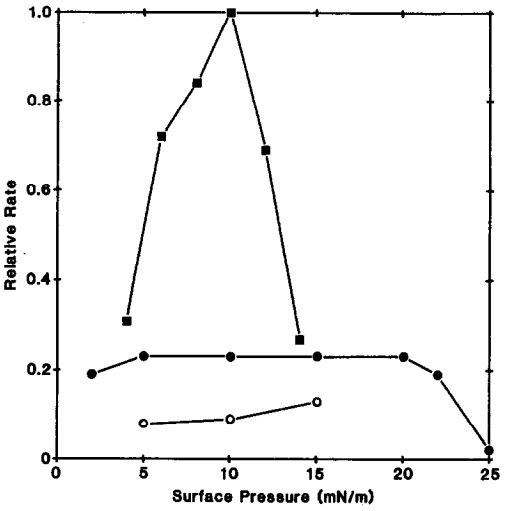

FIG. 2. Relative rate (arbitrary units) of porcine pancreatic phospholipase A2 versus surface pressure. Pure DDPC, ■; 2 mol% DCP in DDPC, ●; 5 mol% DCP in DDPC, ○.

The action of venom and pancreatic phospholipases A2 were studied as a function of surface pressure with pure DDPC and DDPC-DCP mixed monolayers. As shown in Fig. 2, a pressure optimum of about 10 mN/m and a cutoff pressure of 18 mN/m was observed for the hydrolysis of pure DDPC monolayers by porcine pancreatic phospholipase A2, in agreement with the results of Demel *et al.* (12). With DDPC-DCP mixed monolayers (2 and 5 mol% DCP), inhibition was observed below 18 mN/m while activation was observed above this pressure to a cutoff pressure of 25 mN/m. DCP thus allowed penetration of pancreatic phospholipase above its normal cutoff pressure with pure DDPC. With 2 mol% DCP the lag time was the same between 5 and 10 mN/m but less at 15 mN/m than that observed with pure DDCP. The lag time increased sharply between 20 and 25 mN/m, however, as the cutoff pressure was approached. With the venom phospholipase A2 (Fig. 3) only inhibition by 10 mol% DCP was seen and the cutoff pressure was not increased above the normal cutoff pressure of 24 mN/m observed with pure DDPC (12). The effects of DCP on both enzymes are summarized in Table I.

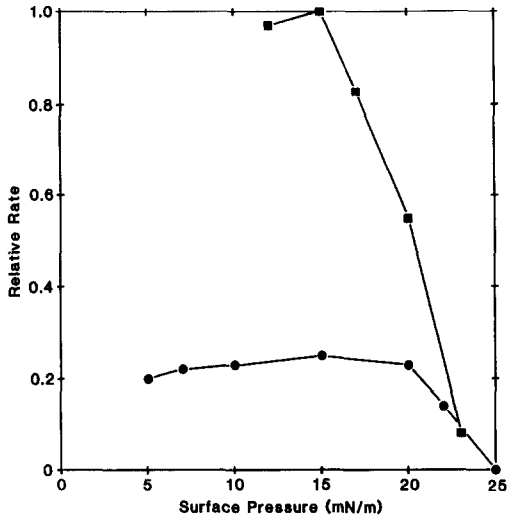

FIG. 3. Relative rate (arbitrary units) of *Crotalus adamanteus* phospholipase A2 versus surface pressure. Pure DDPC, ■; 10 mol% DPC in DDPC, ●.

Mixed DDPC-DDG monolayers showed inhibition with both enzymes below the normal cutoff pressures observed with pure DDPC; however, DDG was observed to increase the cutoff pressures and thus activate both enzymes above their normal cutoff pressures (Table I).

The soluble anionic surfactants, 3,5-diiodosalicylate, 3,5-dibromosalicylate, acetyl salicylate, and salicylic acid, all inhibited pancreatic phospholipase A2 activity with DDPC monolayers at different subphase concentrations (Table II). Dibromosalicylate (0.1 mM) inhibited both pancreatic and venom phospholipases below the normal cutoff pressures, but, like DCP, was only observed to increase the normal cutoff pressure (to 30 mN/m) for the pancreatic enzyme (Table I). Dibromosalicylate and acetyl salicylate inhibited pancreatic phospholipase activity with DDPC monolayers to about the same extent and were only slightly less inhibitory with the same enzyme and DDPG monolayers at 25 mN/m.

The zwitterionic detergents, Z 3-12 and Z 3-14, were found to be very potent inhibitors of pancreatic phospholipase A2 activity on DDPC monolayers (Table II). Interestingly, the zwittergent with the shorter hydrocarbon chain (less hydrophobic), Z 3-12, is the most potent inhibitor. The concentrations where the zwittergents inhibit are well below their CMC values (13). They do appear to penetrate into DDPC monolayers as evidenced by a decreased amount of lipid required to spread a monolayer when they are present in the subphase, and an increase in surface pressure when added under a spread

TABLE I

ACTIVITIES OF PHOSPHOLIPASE A2 WITH MIXED MONOLAYERS

	Pancreatic PLA2		*C. adamanteus* PLA2	
Monolayer[a]	Relative rate (10 mN/m)	Cutoff (mN/m)	Relative rate (15 mN/m)	Cutoff (mN/m)
DDPC	1.0	18	1.0	24
+2% DCP	0.34	25	0.32	—
+5% DCP	0.14	—	0.37	—
+10% DCP	—	—	0.26	24
+20% DCP	—	—	0.20	—
+9.5% DDG	0.75	—	0.83	—
+18.5% DDG	0.28	—	0.18	—
+38.0% DDG	0.20	26	0.17	36
+0.1 mM DBS[b]	0.68	30	0.32	24

[a] All monolayers spread over 5 mM Tris buffer, pH 7, 5 mM $CaCl_2$, 0.1 M NaCl. All rates relative to controls with pure DDPC.

[b] 3,5-Dibromosalicylate in subphase.

TABLE II

INHIBITION OF PANCREATIC PHOSPHOLIPASE A2 BY SOLUBLE SURFACTANTS

Surfactant	Monolayer substrate (concentration giving 50% inhibition, mM)[a] DDPC (10 mN/m)	DDPG (25 mN/m)
Z 3-12	0.0015	—
Z 3-14	0.0075	—
3,5-Diiodosalicylate	0.025	—
3,5-Dibromosalicylate	0.12	0.15
Acetyl salicylate	0.11	0.18
Salicylate	18.0	—

[a] All monolayers spread over 5 mM Tris buffer, pH 7, 5 mM $CaCl_2$, 0.1 M NaCl.

monolayer. There are no quantitative data, however, on the extent of penetration.

The relative rates of the pancreatic and venom phospholipases A2 with DDPC, DDPG, and DDPA as a function of surface pressures are shown in Figs. 4 and 5. The anionic phospholipids allowed penetration and hydrolysis with both enzymes above their normal cutoff pressures with DDPC. The pancreatic enzyme showed a substantial preference for the anionic phospholipids, while the venom phospholipase preferred the zwitterionic DDPC.

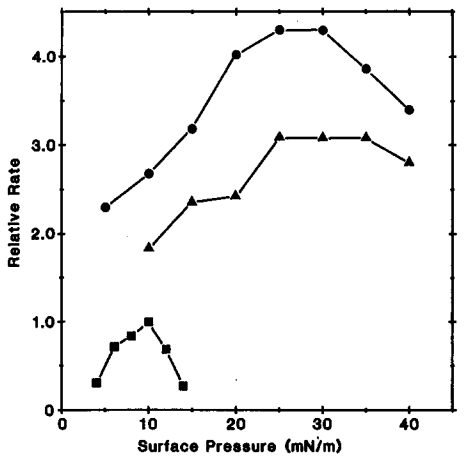

FIG. 4. Relative rate (arbitrary units) of porcine pancreatic phospholipase A2 versus surface pressure. DDPC, ■; DDPG, ●; DDPA, ▲.

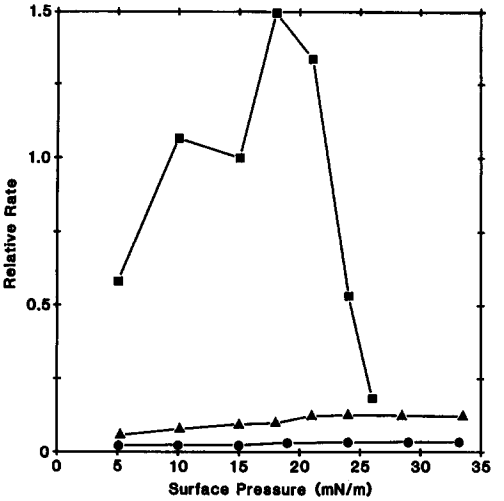

FIG. 5. Relative rate (arbitrary units) of *Crotalus adamanteus* phospholipase A2 versus surface pressure. DDPC, ■; DDPG, ▲; DDPA, ●.

DISCUSSION

Phospholipases A2 from various sources exhibit different optimal and cutoff pressures in monolayer systems (12). In a study of the effects of amphiphiles on phospholipase activity it is important to include surface pressure as a variable. In an early study of phospholipase A2 (*Naja naja*) activity on lecithin emulsions, Dawson (14) observed inhibition by DCP and other anionic amphiphiles; however, in monolayer systems he observed activation by DCP at pressures above 30 mN/m where the enzyme was not active with lecithin alone (15). Earlier, Bangham and Dawson (16) showed that DCP had a similar effect on phospholipase B (*Penicillium notatum*) activity on lecithin monolayers. DCP increased the cutoff pressure but caused inhibition below the normal cutoff pressure.

In this study, DCP inhibited both pancreatic and snake venom phospholipases A2 below their normal cutoff pressures with pure DDPC. Only in the case of the pancreatic enzyme, however, did DCP allow penetration and thus activate above the normal cutoff pressure. The cutoff pressure was shown by Verger *et al.* (17) to be a result of decreased penetration of enzyme into the interface rather than a reduction of specific activity of penetrated

enzyme. Penetration of the enzyme is a function of the structure of the enzyme penetration site and the nature of the particular lipid interface (charge, spacing, etc.). Pancreatic phospholipase A2 has been known for a long time to be more active with anionic substrates as compared to zwitterionic lecithins. Slotboom *et al.* (18) ascribed this to enhanced penetration rather than simple substrate spacing. Brockerhoff (19) proposed a hypothetical model for the active site of pancreatic phospholipase A2 which includes an electrostatic head which serves to interact with negative charges at the lipid interface. The snake venom enzyme must certainly have a different penetration site from the pancreatic enzyme. It penetrates lecithin monolayers to higher pressures and is active as a dimer (20) whereas with the pancreatic enzyme there is no evidence for dimer formation (21). Failure of DCP to increase the cutoff pressure of the venom enzyme, thus, must be due to a different penetration site. Inhibition of both enzymes below their normal cutoff pressures must be due to factors other than penetration. This could involve competitive inhibition of the penetrated enzyme or an effect of negative surface charge on catalysis at the active site. The latter would not appear to be the case for the pancreatic enzyme since it is in fact more active with anionic substrates. It would thus appear that for this enzyme, anionic nonsubstrate amphiphiles most likely inhibit by competing with substrate for binding at the active site. This hypothesis needs to be substantiated by further studies. The venom enzyme is less active with anionic substrates so that competitive inhibition would not seem to be the mechanism of its inhibition by DCP. Inhibition of the venom enzyme may well involve a different mechanism than that with the pancreatic enzyme.

Inhibition and enhancement of cutoff pressures of both enzymes by the neutral amphiphile DDG may be more an effect of substrate spacing. There is certainly interaction between DDG and DDPC molecules as evidenced by the substantial condensing effect and nonideal mixing. This may inhibit enzyme activity but enhance penetration. Demel *et al.* (12) also observed enhanced penetration of phospholipase C into lecithin monolayers in the presence of diglyceride.

Inhibition by soluble surfactants was studied only with pancreatic phospholipase A2, with the exception of 3,5-dibromosalicylate which was also studied with the snake venom enzyme. Soluble surfactants may have the same effects on phospholipase A2 as insoluble amphiphiles to the extent that they penetrate the monolayer interface. They may also, however, interact directly with the enzyme in the subphase (1). The zwitterionic detergents, Z 3-12 and Z 3-14, inhibit at very low (micromolar) subphase concentrations. Although there is evidence for monolayer penetration, the fact that the less hydrophobic homolog, Z 3-12, inhibits at a lower concentration argues for inhibition by interaction with the enzyme in the subphase in addition to a possible effect at the interface. Inhibition by the different salicylates correlates with their hydrophobicity and thus their probable penetration. Other evidence suggests that they may also inhibit by subphase effects. 3,5-Dibromosalicylate and acetyl salicylate inhibit to the same extent with DDPC monolayers at 10 mN/m and DDPG monolayers at 25 mN/m. It seems reasonable to assume that with the anionic DDPG monolayer at a higher pressure these anionic surfactants penetrate to a much smaller extent than with the DDPC monolayer. Similar inhibition with these two monolayers must thus involve subphase effects. Perhaps this involves competitive inhibition at the active site both in the subphase and at the interface.

This study shows both similarities and differences in the effects of anionic and neutral amphiphiles on pancreatic and venom phospholipases A2. Certainly surface pressure is an important parameter in determining whether an amphiphile behaves as an inhibitor or activator. Possible mechanisms of inhibition and activation are only suggested by these data. Further studies will be needed to substantiate these mechanisms. A recent study by

Verheij *et al.* (22) compares the interactions of pancreatic and venom phospholipases A2 with lipid interfaces. They suggest an additional enzyme conformational equilibrium in the interface which may prove quite different for the two sources of enzymes. This may begin to explain the differences observed in this study.

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