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Importance of the Unsaturated Fatty Acyl Group of Phospholipids in Their Stimulatory Role on Rat Adrenal Mitochondrial Steroidogenesis†

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ABSTRACT: We have investigated the relationship between chemical properties of various phospholipids and their steroidogenic activity for adrenal mitochondria prepared from dexamethasone/cycloheximide-treated quiescent rats. Phospholipids studied include those purified from bovine and rat adrenal mitochondria, obtained from commercial sources, and reduced by catalytic hydrogenation. All phospholipids were subjected to analysis of their fatty acyl groups and examined for their steroidogenic activities. From these experiments, we came to the following conclusions: (i) The degree of unsaturation in the fatty acyl moiety correlates with their steroidogenic activities regardless of head groups. Namely, polyunsaturation appears to be more important than monounsaturation with a relative insensitivity toward their head groups. (ii) Saturated phospholipids exhibit an inhibition for steroidogenic activity. (iii) Cardiolipins, which are steroidogenic, appear exceptional. Their head groups may partially participate in the activity in addition to their high content of unsaturated fatty acids. (iv) The importance of the adrenoyl (C22:4) group in phospholipids is suggested.

The rate-limiting step in adrenocorticotropic hormone (ACTH)[1]-dependent steroidogenesis is the conversion of cholesterol to pregnenolone (Stone & Hechter, 1954). An important conclusion is that ACTH stimulates this step by producing cAMP-dependent, cycloheximide-sensitive factor(s) (Garren et al., 1965). This factor seems to enhance the movement of cytosolic cholesterol to P-450$_{scc}$ in the mitochondrial inner membrane (Simpson, 1979; Kimura, 1981; Simpson & Waterman, 1983; Cheng et al., 1985). In our previous study (Igarashi & Kimura, 1984), we demonstrated that the quantities of mitochondrial PC, PE, and PI increase by ACTH administration and this increase was sensitive to cycloheximide inhibition but not to aminoglutethimide. On the other hand, CL and polyphosphoinositides were reported as activators of both mitochondrial and reconstituted steroidogenesis (Lambeth, 1981; Pember et al., 1983; Greenfield et al., 1981; Farese & Sabir, 1980; Tanaka & Strauss, 1982; Kowluru et al., 1983; Hsu et al., 1985). We have shown recently that by model experiments using cholesterol-containing liposomes and purified steroid-free P-450$_{scc}$ the unsaturated fatty acid moiety and its carbon chain length are important in the enhancement of the binding rate of cholesterol to the cytochrome (Hsu et al., 1984, 1985; Kido & Kimura, 1981; Kido et al., 1981). Although the effects of phospholipids on reconstituted liposomes may not be the same as those on mitochondria, we had a question on the role of head groups in phospholipids for their activating function.

In order to gain further insight into the action of these phospholipids, we have studied here the relationship between fatty acid compositions and steroidogenic activities of various phospholipids. We have revealed that a high content of polyunsaturated fatty acids is required for steroidogenic activities. This relationship stands for many phospholipids, except

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[1] Abbreviations: ACTH, adrenocorticotropic hormone; P-450$_{scc}$, cytochrome P-450 for cholesterol side-chain cleavage reaction; CL, cardiolipin; DPI, diphosphoinositide; lysoPC, lysophosphatidylcholine; lysoPE, lysophosphatidylethanolamine; lysoPG, lysophosphatidylglycerol; lysoPS, lysophosphatidylserine; PAF, platelet activating factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TPI, triphosphoinositide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HPLC, high-pressure liquid chromatography.

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CL. It is implicated from this study that adrenic acid (C22:4) in phospholipids plays an important role in the phospholipid-mediated activation of mitochondrial steroidogenesis.

Experimental Procedures

Animals. Male Sprague-Dawley rats of 175–200 g body weight were purchased from Charles River and kept in a temperature-controlled room at least 4 days prior to experimental use. The standard diet (Purina Lab Chow from Nobi) and water were supplied ad libitum.

In Vivo Treatments and Subcellular Particle Preparations. All the rats used in this work were preinjected intraperitoneally with 400 μg of dexamethasone dissolved in a physiological saline solution 12–14 h before sacrifice in order to suppress the stress-induced activation of steroidogenesis. Rats were injected intraperitoneally with cycloheximide (10 mg/rat), and after 20 min, rats were injected intraperitoneally with 1 IU of $ACTH^{1-24}$ or with a physiological saline solution alone. The amount of $ACTH^{1-24}$ is the submaximum effective dose under these conditions. Rats were killed by cervical dislocation 20 min after the last injection, and adrenals were immediately removed, placed in ice-cold saline solution, trimmed free of surrounding fats, decapsulated, and gently homogenized with a Teflon homogenizer in cold 10 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose (usually two adrenals per milliliter). The mitochondrial fraction was isolated from the homogenate and characterized as described previously (Igarashi & Kimura, 1984). The microsomal contamination was estimated as less than 19% by marker enzyme distribution.

Partial Hydrogenation of Phospholipids. The extraction and purification of phospholipids from bovine adrenocortical mitochondria were carried out as described previously (Igarashi & Kimura, 1984). Phospholipid solution ($CHCl_3$–CH_3OH, 2/1 v/v) was dried under N_2 gas, and the residue was dissolved in 5 mL of a methanol solution. The partial hydrogenation of phospholipid samples was carried out with a continuous flow of H_2 gas using 5 mg of palladium–carbon (10%) as a catalyst at room temperature. After 30 min, the reaction was stopped, and 10 mL of chloroform was added to dissolve hydrogenated phospholipid completely. The solution was filtrated with Whatman filter paper to remove the solid catalyst. The recovery of phospholipids was 70–80%.

Analysis of Fatty Acid Composition of Phospholipid. Preparation of fatty acid methyl esters from phospholipid was carried out essentially by the method of Morrison and Smith (1964). Briefly, the phospholipid solution (1–2 mg) was dried under N_2 gas, dissolved in 0.5 mL of 14% borofluoride–methanol solution, and boiled for 5 min at 100 °C in a water bath. After the solution was cooled, 3 mL of petroleum ether and 2 mL of H_2O were added to the tube for the extraction of fatty acid methyl esters. After low-speed centrifugation, the petroleum ether layer was dried over a small amount of sodium sulfate and was evaporated to dryness under N_2 gas. The methyl esters obtained were dissolved in 50–100 μL of hexane (HPLC grade), and an aliquot (1–10 μL) was analyzed in a Varian 6000 gas chromatograph with a flame ionization detector. The column (6 ft × 0.125 in.) was packed with Unisol 3000/Uniport C, 80/100 mesh. The initial temperature was kept at 150 °C for 10 min to remove the solvent and then programmed from 150 to 225 °C at 10 °C/min. The injection and detection temperatures were fixed at 250 °C. The flow rate of carrier gas was 15 mL/min. Standards were used for identification of the retention time of fatty acid methyl ester. Under these conditions, C_{14}–C_{24} fatty acid methyl esters were completely separated during the 40-min run. The injection of a sample was repeated at least 3 times. The columns were replaced every 150 injections to keep a fine separation. Peak area was measured and expressed as a percentage of total fatty acids.

Cholesterol Side Chain Cleavage Activity. The effect of phospholipids on the cholesterol side chain cleavage reaction was measured as follows. Adrenal mitochondria were prepared from dexamethasone- and cycloheximide-suppressed rats and incubated with or without phospholipid (dispersed in a physiological saline solution, 20 μL) at 37 °C in 200 μL of an incubation buffer containing 100 mM sucrose, 93 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), 5 mM DL-isocitrate, 1 mM NADPH, and 10 μM cyanoketone. The reaction was stopped by the addition of 2.5 mL of ice-cold CH_2Cl_2 at the time indicated, and aliquots (10–30 μL) were measured for pregnenolone by radioimmunoassay.

Purification of TPI and DPI. Some samples of TPI and DPI (Sigma, bovine brain) contained an appreciable amount (1–4%) of lysophospholipids. To remove the contaminated compounds, we carried out thin-layer chromatography using a potassium-preimpregnated silica plate and a developing solvent consisting of $CHCl_3$–CH_3COCH_3–CH_3OH–CH_3CO-OH–H_2O (40/15/13/12/8, v/v). The spot corresponding to TPI or DPI was scraped off and extracted with $CHCl_3$–CH_3OH–0.1 N HCl (1/2/0.8 v/v).

Other Analytical Methods. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Phosphorus of phospholipids was determined by the method of Ames and Dubin (1960).

Commercial Phospholipids and Chemicals. TPI (bovine brain), DPI (bovine brain), PC (egg yolk), PE (bovine brain), PS (bovine brain), PG (egg yolk), lysoPC (egg yolk), lysoPE (egg yolk), lysoPS (bovine brain), and lysoPG (egg yolk) were obtained from Sigma. PI (bovine liver), CL (bovine heart), plasmalogen (PE type, bovine brain), and PAF (1-alkyl ether 2-acetyl-PC) were purchased fro Avanti. SM (bovine brain) was from General Biochem. All standards of fatty acid methyl esters were purchased from Alltech. Borofluoride methanol (14%) was obtained from Anspec. Cyanoketone is a kind gift from Sterling-Winthrop.

Results

Effect of Partial Hydrogenation of Cardiolipin (Bovine Heart) and Bovine Adrenocortical Phospholipids on Their Steroidogenic Activities. First, we hydrogenated various phospholipids and examined the effect of hydrogenation on their steroidogenic activities. Under our experimental conditions, the hydrogenation reaction did not proceed completely. As shown in Table I, polyunsaturated fatty acids like C18:2, C20:4, and C22:4 were almost completely hydrogenated, whereas monosaturated fatty acids like C18:1 were not effectively hydrogenated. The content of total polyunsaturated fatty acids was drastically reduced from 37% to 13% in the mitochondrial phospholipids and from 74% to 16% in the CL. Using these reduced phospholipids, we examined their effects on steroidogenic activity (Figure 1). The addition of untreated CL or bovine adrenocortical mitochondrial phospholipids to adrenal mitochondria from dexamethasone/cycloheximide-treated quiescent rats caused a marked stimulation (2–3-fold) of pregnenolone formation compared with the control experiment. However, the partially hydrogenated samples did not show any stimulatory effect. These results indicate that unsaturation in fatty acyl moieties plays an important role in determining the steroidogenic activity of phospholipids.

Effect of Hydrogenation of Steroidogenic PS, PE, and CL from Bovine Adrenocortical Mitochondria on Their Activities. Among the mitochondrial phospholipids from bovine adrenal

Table I: Fatty Acid Composition of the Bovine Adrenocortical Mitochondrial Phospholipid Mixture and Cardiolipin (Bovine Heart)[a]

	bovine adrenocortical mitochondrial phospholipid		bovine heart cardiolipin	
fatty acids	untreated	hydrogenated	untreated	hydrogenated
14:0	0.3	0.2	0.6	0.3
16:0	12.9	13.3	2.5	5.0
16:1	2.3	1.5	2.3	1.7
16:2	0.9	1.2	3.4	2.0
18:0	22.3	32.9	1.6	44.9
18:1	20.2	13.4	9.1	23.7
18:2	8.2	0.3	49.3	0.5
18:3	1.5	2.4	6.4	4.3
20:0	1.8	11.1	6.5	6.0
20:1	–[b]	9.4	–	–
20:2	–	5.5	1.1	–
20:3	3.2	2.4	6.0	3.2
20:4	19.1	0.1	0.5	–
22:0	1.8	2.8	4.6	2.7
22:1	–	1.2	–	–
22:2	0.8	0.6	3.2	2.5
22:4	2.1	–	2.7	1.5
22:6	1.6	–	1.7	1.8
unsaturation[c]	58.9	38.9	85.7	41.2
polyunsaturation	37.4	12.5	74.3	15.8

[a] Results are expressed as weight percent (mean values of three determinations with error below 5% of each value). [b] Undetectable. [c] Mono plus poly.

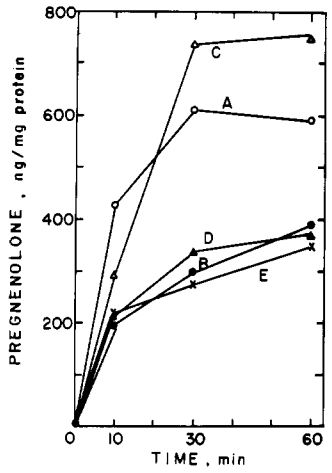

FIGURE 1: Effect of hydrogenation of cardiolipin (bovine heart) and the bovine adrenocortical mitochondrial phospholipid mixture on steroidogenic activity. Mitochondria were prepared from cycloheximide-treated rat adrenals and incubated at 37 °C in the assay medium in the presence or absence of a phospholipid dispersion (200 μM) for various incubation times. Pregnenolone was determined by radioimmunoassay. Each point displays the mean value of two assays. A, Bovine adrenocortical mitochondrial phospholipid mixture; B, hydrogenated bovine adrenocortical mitochondrial phospholipid mixture; C, cardiolipin (bovine heart); D, hydrogenated cardiolipin; E, control (no phospholipid added).

cortex, PS, PE, and CL were found to have steroidogenic activities whereas PC and PI were not steroidogenic (Table II). To see the requirement of the unsaturated fatty acyl moiety, we examined the hydrogenation effect on steroidogenic activities of respective purified phospholipids.

The result presented in Table II shows clearly that the stimulatory activity of phospholipids was completely lost upon hydrogenation. The hydrogenated PE was rather inhibitory. The fatty acid compositions of PS, PE, and CL, before and after hydrogenation, are presented in Table III, together with those of steroidogenically inactive PC, and PI from bovine

Table II: Effects of Hydrogenation of the Steroidogenic Phospholipids Phosphatidylserine, Phosphatidylethanolamine, and Cardiolipin from Bovine Adrenocortical Mitochondria on Their Steroidogenic Activity[a]

addition	concn (μM)	pregnenolone formation/20 min[b]
none		100 ± 6
PS	50	283 ± 4
	200	356 ± 14
hydrogenated PS	50	114 ± 10
	200	104 ± 6
PE	50	85 ± 22
	200	194 ± 30
hydrogenated PE	50	48 ± 12
	200	55 ± 6
CL	50	242 ± 61
	200	246 ± 57
hydrogenated CL	50	121 ± 13
	200	107 ± 6

[a] Mitochondria were prepared from cycloheximide-treated rat adrenals and incubated in the assay medium (200 μL) at 37 °C in the presence or absence of a phospholipid dispersion (50 or 200 μM) for 20 min. [b] Pregnenolone was determined by radioimmunoassay. Values display the mean ± SD of three experiments. The value for 100% was 138 ng of pregnenolone (mg of protein)$^{-1}$ (20 min)$^{-1}$.

adrenocortical mitochondria. Upon hydrogenation, polyunsaturated fatty acids were converted to more reduced forms. Untreated PE was relatively rich in the polyunsaturated fatty acids (43.8%), particularly in C20:4 (33.1%), compared with other phospholipids. An interesting point is the presence of a large amount of C22:4 (6.4%) in this phospholipid. Chang and Sweeley (1963) first reported a high content of C22:4 in canine adrenal phospholipids, especially in PE (11–13%), compared with phospholipids from other tissues which have C22:4 as a minor component (less than 1%). Accordingly, they named this fatty acid "adrenic acid". Bovine adrenocortical mitochondrial PS with a high steroidogenic activity contained the highest content of C22:4 (8.4%) among phospholipids examined, though the total content of polyunsaturated fatty acid was not so high (30%). CL (bovine adrenocortical mitochondria) had the highest content of total polyunsaturated fatty acid (84.8%), especially in C18:2 (48.1%) but much less in C20:4 and C22:4, as compared with PS and PE. In contrast, nonsteroidogenic phospholipids, PI and PC (bovine adrenocortical mitochondria), contained less polyunsaturated fatty acid (34.2% and 28.5%, respectively). They contained only small amounts of C22:4 (0.9% and 0.5%, respectively).

Comparison with the Steroidogenic Activities and Fatty Acid Composition of Commercial Phospholipids. To clarify a possible relationship between the steroidogenic activity and fatty acid composition of phospholipids, we took advantage of utilizing commercially available phospholipids. Our results are presented in Tables IV and V. CL (bovine heart) showed a high stimulatory activity (282%) for the side chain cleavage when added to mitochondria isolated from dexamethasone/cycloheximide-suppressed rat adrenals. The steroidogenic activities of TPI (bovine brain) (234%) and DPI (bovine brain) (192%) were observed only after purified samples were used. Besides these phospholipids, we discovered that plasmalogen (PE type, bovine brain) exhibited a stimulation (197%). All active phospholipids had a common feature concerning their fatty acid compositions. Namely, relatively high contents in polyunsaturated fatty acids (bovine CL, 74.3%; TPI, 46.3%; DPI, 40.6%; plasmalogen, 41.6%) were noticed. It should be stated that they also contained a large quantity of adrenic acid (C22:4) when compared with other phospholipids: bovine CL, 2.7%; TPI, 2.9%; DPI, 4.0%; plasmalogen, 7.1%.

Table III: Fatty Acid Compositions of Phosphatidylethanolamine, Phosphatidylserine, and Cardiolipin and Their Partially Hydrogenated Compounds from Bovine Adrenocortical Mitochondria[a]

fatty acids	PE	HPE	PS	HPS	CL	HCL	PC	PI
14:0	–[b]	–	–	–	0.3	0.7	–	–
16:0	5.6	5.7	5.8	10.9	6.1	13.7	24.2	4.3
16:1	4.2	0.9	1.5	2.0	1.5	3.5	2.1	0.7
16:2	1.3	1.7	3.1	5.7	1.5	4.2	1.5	2.1
18:0	31.2	31.2	34.2	30.7	5.7	32.5	19.9	42.9
18:1	10.4	7.8	19.9	11.2	21.3	18.4	25.5	11.7
18:2	3.0	0.8	2.7	2.7	48.1	5.0	8.9	1.5
18:3	–	–	2.3	–	–	–	–	1.8
20:0	–	7.0	3.8	10.8	1.9	4.9	–	1.9
20:1	–	22.4	2.3	3.9	1.0	5.0	–	1.0
20:2	–	5.1	2.7	5.0	3.4	3.2	–	2.7
20:3	–	–	2.5	3.2	2.0	3.7	2.2	1.2
20:4	33.1	0.6	6.9	–	4.4	–	15.4	25.6
22:0	–	4.6	1.7	6.3	1.0	3.7	–	1.4
22:1	–	5.8	–	0.5	–	–	–	–
22:2	–	2.9	1.4	3.9	–	1.6	–	0.5
22:4	6.4	–	8.4	–	1.6	–	0.5	0.9
24:0	–	–	0.7	1.7	–	–	–	–
unsaturation[c]	58.4	48.0	53.7	38.1	84.8	44.6	56.1	49.7
polyunsaturation	43.8	8.2	30.0	20.5	61.0	17.7	28.5	34.2

[a] Results are expressed as weight percent (mean value of three determinations with error below 5% of each value). [b] Undetectable. HPE, HPS, and HCL are hydrogenated PE, PS, and CL, respectively. [c] Mono plus poly.

Table IV: Effects of Various Commercial Phospholipids on the Cholesterol Side Chain Cleavage Reaction in Rat Adrenal Mitochondria[a]

phospholipid (source)	concn (μM)	pregnenolone formation (% of control)
none	0	100
TPI (Sigma, bovine brain)	200	234
DPI (Sigma, bovine brain)	200	192
PC (Sigma, egg yolk)	200	90
PE (Sigma, bovine brain)	200	85
PI (Avanti, bovine liver)	200	65
SM (General Biochem, bovine brain)	200	54
PS (Sigma, bovine brain)	200	98
CL (Avanti, bovine heart)	200	282
lysoPC (Sigma, egg yolk)	200	3
lysoPE (Sigma, egg yolk)	200	47
lysoPG (Sigma, egg yolk)	200	61
lysoPS (Sigma, bovine brain)	200	107
PAF (Avanti)	200	24
plasmalogen (Avanti, bovine brain)	200	197

[a] For experimental details, see the legend of Table II. Activities were expressed as a percentage of control (no phospholipid added) (mean values of at least two determinations). The concentration of phospholipid used here (200 μM) was the optimal one for the cholesterol side chain cleavage reaction. The value for 100% was 138 ng of pregnenolone (mg of protein)$^{-1}$ (20 min)$^{-1}$, respectively, for no addition of any of the phospholipids listed.

Among other phospholipids tested, lysoPC (egg yolk) and PAF (1-alkyl ether 2-acetyl-PC), which resembles lysoPC as a molecular shape, exhibited a strong inhibitory effect on the side chain cleavage activity. Other lysophospholipids like lysoPG (egg yolk) and lysoPE (egg yolk) were also inhibitory, but lysoPS (bovine brain) was neither inhibitory nor stimulatory.

The inhibitory effects of lysophospholipids on steroidogenesis in isolated mitochondrial systems have been reported by other investigators (Farese & Sabir, 1980; Tanaka & Strauss, 1982), and this phenomenon was presumed to be caused by their detergent-like effect on mitochondria since detergents such as Triton X-100 (1%) and lauryl sulfate (200 μM) exhibited the inhibitory effects on mitochondrial pregnenolone formation. Besides lysophospholipids, PI (bovine liver) and SM (bovine brain) exhibited significant inhibitory activities. The common feature of these inhibitory phospholipids was their high content in saturated fatty acids: lysoPC, 96.0%; lysoPG, 76.3%; lysoPE, 88.1%; PI (bovine liver), 69.9%; SM (bovine brain), 87.6; (Table V). On the basis of these results, we feel that the inhibitory activity of lysophospholipids is due not only to their detergent effects but also to their high content of saturated fatty acids.

Unlike PS and PE from adrenal mitochondria, commercial PS (bovine brain) and PE (bovine brain) did not exhibit any appreciable effect on mitochondrial steroidogenesis (Table IV). The fatty acid composition of commercial PS and PE was quite different from that of adrenal mitochondrial PS and PE: a low content of total polyunsaturated fatty acids (PS, 14.0%; PE, 17.3%) and only small amounts of C22:4 (below 1% for both) were characteristic (Table V).

We have already mentioned that pure polyphosphoinositides are steroidogenic. However, TPI from a bottle did not display any steroidogenic effect, and DPI from a bottle was rather inhibitory for the mitochondrial cholesterol side chain cleavage activity. A thin-layer chromatogram of these samples revealed the presence of a considerable amount (1–4%) of lysophospholipids. After the impurities were removed by thin-layer chromatography, purified TPI and DPI samples showed stimulatory activities.

Importance of Polyunsaturated Fatty Acid and Adrenic Acid (C22:4) in Adrenal Steroidogenesis. All data obtained here suggest a relationship between the steroidogenic activity and fatty acid composition of phospholipids. To demonstrate this relationship clearly, the relative steroidogenic activity was plotted against fatty acid composition. Figure 2 indicates the steroidogenic activity of a phospholipid as a function of content (percent) of total (mono plus poly) unsaturated fatty acids (A) or total polyunsaturated fatty acids (B). The correlation coefficients were 0.764 and 0.802 in (A) and (B), respectively, implying that the activity is more dependent on the content of polyunsaturated fatty acids than monounsaturated fatty acids. When a similar relationship was examined with the contents of C20:4 and C22:4 in 27 different phospholipids with various head groups, we obtained correlation coefficients of 0.185 and 0.749 for C20:4 and C22:4, respectively, indicating that the content of C22:4 in phospholipids is more important

Table V: Fatty Acid Compositions of Various Commercial Phospholipids[a]

	stimulatory phospholipids			inhibitory phospholipids				other phospholipids				
fatty acids	TPI	DPI	plasmalogen[b]	PI	SM	lysoPC[c]	lysoPG	lysoPE	lysoPS[d]	PS	PE	PC
14:0	–[c]	–	1.3	–	2.4	–	–	0.3	–	–	6.4	–
16:0	5.1	6.0	2.5	3.6	66.5	65.4	3.4	23.1	48.1	1.7	9.8	36.1
16:1	–	–	9.8	–	1.2	0.9	–	1.3	1.4	0.4	7.4	1.9
16:2	2.8	1.3	3.8	2.1	1.7	0.4	2.8	0.9	2.5	1.4	1.0	0.3
18:0	30.4	35.1	11.4	66.4	18.1	31.1	72.9	64.3	21.0	43.1	18.9	12.6
18:1	12.1	16.1	20.6	9.3	8.3	2.7	2.3	6.5	1.4	34.0	37.2	28.2
18:2	3.5	0.4	–	2.1	–	–	4.5	1.3	5.5	1.0	0.7	15.7
18:3	–	0.4	1.6	–	–	–	6.0	–	4.8	–	1.4	0.6
20:1	0.4	0.9	6.9	–	1.2	–	3.8	–	–	5.5	2.2	–
20:2	7.2	3.3	–	–	–	–	–	0.9	5.3	0.7	0.4	–
20:3	–	–	–	–	–	–	–	–	–	–	–	–
20:4	28.5	30.3	11.1	10.3	–	–	3.4	0.7	4.1	0.8	12.9	3.8
22:0	–	–	0.2	–	–	–	–	0.5	–	0.6	0.7	–
22:2	2.4	0.9	6.4	0.6	–	–	0.9	–	3.4	2.0	–	–
22:4	2.9	4.0	7.1	0.7	–	–	–	0.3	1.6	1.7	0.6	0.4
22:5	1.5	–	3.9	–	–	–	–	–	–	1.2	–	–
22:6	–	–	5.7	–	–	–	–	–	0.5	5.3	–	0.4
unsaturation	61.2	57.6	78.9	30.1	12.4	4.0	23.7	11.9	30.5	53.7	58.1	51.3
polyunsaturation	46.3	40.6	41.6	16.8	1.7	0.4	17.6	4.1	27.6	14.0	17.3	21.2

[a] Results are expressed as weight percents (mean value of three determinations with error below 5% of each value). [b] Data showed the fatty acid composition at the C_2 position (ester bonded) in plasmalogen. [c] Undetectable. The sources of phospholipids are as follows: TPI, bovine brain; DPI, bovine brain; plasmalogen, bovine brain; PI, bovine liver; SM, bovine brain; lysoPC, egg yolk; lysoPG, egg yolk; lysoPE, egg yolk; lysoPS, bovine brain; PS, bovine brain; PE, bovine brain; PC, egg yolk. [d] The lyso derivative contained a large amount of unsaturated fatty acids.

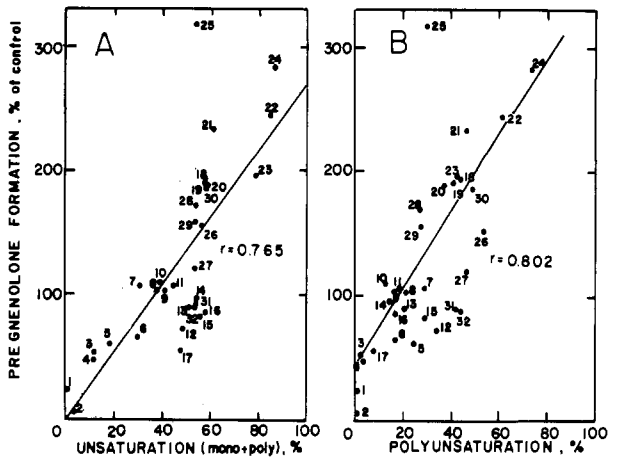

FIGURE 2: Steroidogenic activities of various phospholipids as a function of unsaturated fatty acid content (A) or polyunsaturated fatty acid content (B). The numbers indicate as follows: 1, PAF; 2, lysoPC (egg yolk); 3, SM (bovine brain); 4, lysoPE (egg yolk); 5, lysoPG (egg yolk); 6, PI (bovine liver); 7, lysoPS (bovine brain); 8, hydrogenated bovine adrenocortical mitochondrial PS; 9, hydrogenated CL (bovine heart); 10, hydrogenated bovine adrenocortical mitochondrial phospholipid mixture; 11, hydrogenated bovine adrenocortical mitochondrial CL; 12, bovine adrenocortical mitochondrial PI; 13, PC (egg yolk); 14, PS (bovine brain); 15, bovine adrenocortical mitochondrial PC; 16, PE (bovine brain); 17, hydrogenated bovine adrenocortical mitochondrial PE; 18, bovine adrenocortical mitochondrial PE; 19, DPI (bovine brain); 20, bovine adrenocortical mitochondrial phospholipid mixture; 21, TPI (bovine brain); 22, bovine adrenocortical mitochondrial CL; 23, plasmalogen (PE type, bovine brain); 24, CL (bovine heart); 25, bovine adrenocortical mitochondrial PS; 26, ACTH-stimulated rat adrenal mitochondrial phospholipids (mixture); 27, ACTH-stimulated rat adrenal microsomal phospholipids (mixture); 28, ACTH-stimulated rat adrenal mitochondrial CL; 29, ACTH-stimulated rat adrenal mitochondrial PS; 30, ACTH-stimulated rat adrenal mitochondrial PE; 31, ACTH-stimulated rat adrenal mitochondrial PC; 32, ACTH-stimulated rat adrenal mitochondrial PI; *r*, correlation coefficient.

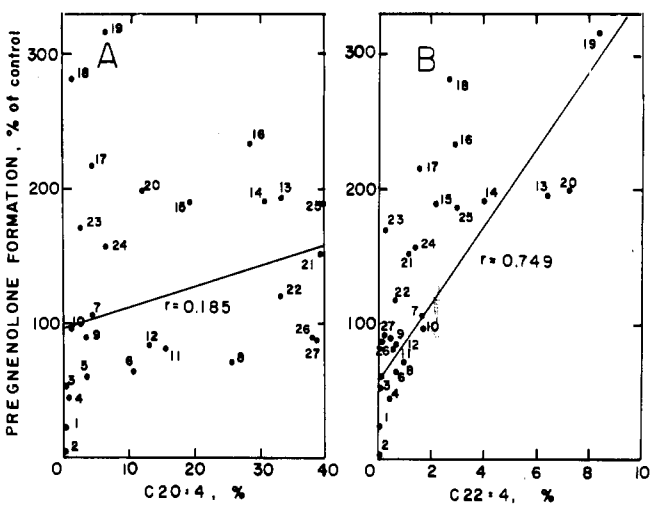

FIGURE 3: Steroidogenic activities of various phospholipids as a function of arachidonic acid (C20:4) (A) or adrenic acid (C22:4) (B) contents. The numbers indicate as follows: 1, PAF; 2, lysoPC (egg yolk); 3, SM (bovine brain); 4, lysoPE (egg yolk); 5, lysoPG (egg yolk); 6, PI (bovine liver); 7, lysoPS (bovine brain); 8, bovine adrenocortical mitochondrial PI; 9, PC (egg yolk); 10, PS (bovine brain); 11, bovine adrenocortical mitochondrial PC; 12, PE (bovine brain); 13, bovine adrenocortical mitochondrial PE; 14, DPI (bovine brain); 15, bovine adrenocortical mitochondrial phospholipid mixture; 16, TPI (bovine brain); 17, bovine adrenocortical mitochondrial CL; 18, CL (bovine heart); 19, bovine adrenocortical mitochondrial PS; 20, plasmalogen (PE type, bovine brain); 21, ACTH-stimulated rat adrenal mitochondrial phospholipid mixture; 22, ACTH-stimulated rat adrenal microsomal phospholipid mixture; 23, ACTH-stimulated rat adrenal mitochondrial CL; 24, ACTH-stimulated rat adrenal mitochondrial PS; 25, ACTH-stimulated rat adrenal mitochondrial PE; 26, ACTH-stimulated rat adrenal mitochondrial PC; 27, ACTH-stimulated rat adrenal mitochondrial PI; *r*, correlation coefficient.

than that of C20:4 in terms of steroidogenic activation (Figure 3). In Figure 3B, data points 17, 18, and 23 belong to CL's, and point 16 belongs to TPI. These points deviate from the average line, indicating that these acidic phospholipids are exceptional and their head groups may participate at least partially in the activation.

Discussion

The importance of phospholipids has been implicated for the binding of cholesterol to purified cytochrome P-450 for the cholesterol side chain cleavage reaction. In a reconstituted system, emphasis has been made on two points. First, the head group of phospholipids plays an important role in the binding of cholesterol to the cytochrome. For example, Lambeth and his group (Lambeth, 1981; Pember et al., 1983) reported CL

as an effector over polyphosphoinositides. Kowluru and his group (Kowluru et al., 1983) observed that both CL and DPI stimulated the cleavage reaction with purified cytochrome P-450. Farese and Sabir (1980) described the stimulating effects by CL, DPI, and TPI on pregnenolone biosynthesis in unstimulated adrenal mitochondria, whereas Tanaka and Strauss (1982) reported CL, but not other phospholipids, as a stimulator of activity in the luteal mitochondrial cholesterol side chain cleavage reaction. Despite somehow contradictory observations by these workers, all agree with the importance of acidic head groups of phospholipids on this hydroxylation system. Second, the role of fatty acyl groups of phospholipids has been suggested for the stimulatory effect of phospholipid. For example, Lambeth et al. (1980) reported that the rate of cholesterol binding to the purified cytochrome grossly depends on the degree of unsaturation. Using a similar system, Kido and Kimura (1981) and Hsu et al. (1985) clearly demonstrated that the binding rate increases by increasing the length of carbon chains or the number of double bonds: by increasing the C-2 unit, the rate increases by approximately 50%, and by increasing one double bond, the rate increases by about 70% in a series of PC's.

In this context, there is no doubt to believe that both head and fatty acyl groups participate in the modulation of mitochondrial steroidogenesis. Yet, some puzzles on the relative importance of both groups remain to be solved.

In this study, we have clearly demonstrated the importance of unsaturated fatty acids of phospholipids with a relative insensitivity toward their head groups. Of particular importance, we realize the fact that the higher content of C22:4 gives the higher stimulatory activity with a correlation coefficient of 0.749. Contrary to this, the content of C20:4 does not correlate with the coefficient of 0.185. PS and CL, which are not induced by ACTH in quantity (Igarashi & Kimura, 1984), are effective as activators for quiescent mitochondria (this work). Adrenocortical PS and CL are rich in unsaturated acyl chains. Namely, PS is rich in C20:4 (6.9%) and C22:4 (8.4%) and CL in C18:2 (48.1%) and C18:1 (21.3%). Their steroidogenic activities can be explained by the abundance in unsaturated fatty acids. In Figure 2, the data point 25 (bovine adrenal mitochondrial PS) deviates largely from other points. When this point was excluded, the correction coefficients became 0.831 and 0.894 instead of 0.764 and 0.802 for (A) and (B), respectively. This may be explained by the fact that bovine adrenal mitochondrial PS is very rich in C22:4, which may contribute to its activity. The data point 29 for rat PS which is less in C22:4 is fairly on the statistical average line (Figure 2). Consequently, the activity of the PS series would be understood by their contents of C22:4 fatty acids (Figure 3). CL's have an exceptional feature (Figure 3B). Namely, CL's, which are good stimulators regardless of the biological sources or fatty acid composition, are abundant in C18:2 and C18:1, while poor in C22:4 and C20:4. Thus, we believe the involvement of the acidic head group in the activity is by a different mechanism. The role of the acidic head group may be related to the stimulatory effect of cations such as Ca^{2+} group in the activity by a different mechanism (Simpson et al., 1974; Mason et al., 1978; Guerra et al., 1960).

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Registry No. Pregnenolone, 145-13-1; blood platelet activating factor, 65154-06-5; arachidonic acid, 506-32-1; adrenic acid, 2091-25-0.

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