Evidence That Nonbilayer Phase Propensity of the Membrane Is Important for the Side Chain Cleavage Activity of Cytochrome P450SCC (CYP11A1)†

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ABSTRACT: To analyze whether specific protein−lipid interactions or physical features of the membrane contribute to cytochrome P450SCC (CYP11A1) activation by lipids, dimyristoylphosphatidylcholine/cardiolipin and dimyristoylphosphatidylcholine/branched phosphatidylcholine vesicles of defined acyl chain structure were studied for their ability to stimulate the side chain cleavage activity of the enzyme. Activation was found to increase with the mole percent of nonbilayer lipids in the system and the chain lengths of both the branched and main fatty acyl chains of the activator lipid. Unsaturation provided by dioleoylphosphatidylcholine as host lipid leads to a further increase in the potency of the branched phosphatidylcholines to activate the enzyme. The observed activation can be qualitatively interpreted in terms of the effect of these lipids on the hydrophobic volume of the membrane. Using differential scanning calorimetry, we showed that the branched phosphatidylcholines perturb the bilayer membrane structure of dimyristoylphosphatidylcholine and lower the bilayer to hexagonal phase transition temperature of dielaidoylphosphatidylethanolamine, i.e., promote hexagonal phase formation. We also examined the effect of eicosane on both the cytochrome P450SCC activity and the lipid polymorphism and found that eicosane increases both the activity and the hexagonal phase propensity of the vesicle membrane. Because of these correlations, we conclude that the nonbilayer phase propensity of the membrane rather than specific binding of activator lipids to the enzyme explains best the observed activation of enzymatic activity by the lipids.

Cytochrome P450SCC (P450SCC),[1] located at the matrix side of the inner mitochondrial membrane in cells of adrenal cortex and other steroid-producing tissues, catalyzes the side chain cleavage of cholesterol to yield pregnenolone, the common precursor of all steroid hormones. The activity of P450SCC can be reconstituted in phospholipid vesicles whereby the activity was found dependent on the membrane constituents used, the method of reconstitution, and the size of the vesicles [for a review, see Lambeth (1990) and citations therein]. Activation by lipids has been discussed as being caused by specific protein−lipid interactions. For instance, cardiolipin (CL)—as a specific mitochondrial lipid to date the most potent activator lipid—binds to an effector site on the enzyme in a 1:1 stoichiometry, thereby activating the enzyme. Results suggested the headgroup of CL as a major determinant of lipid interaction with P450SCC (Lambeth, 1981); however, the role of the fatty acyl chain region, particularly its unsaturation, is contradictory and not resolved (Igarashi & Kimura, 1986). A particularly important role has been attributed to certain polyunsaturated configurations of the phospholipids, whereas the saturated phospholipids investigated so far exhibited an inhibition of enzymatic activity. Dhariwal et al. (1989) observed remarkable stimulation of P450SCC by residual octyl glucoside and certain fatty acids, and several reports showed that the vesicle size might also play a role. Taken together, although a number of studies were performed the requirements for lipid stimulation and the way the lipids exert their action remained obscure.

In a recent study, we synthesized phosphatidylcholines (PC) with fully saturated branched fatty acyl chains substituted in both positions of the main chains and found a stimulatory efficiency higher than any other phospholipid and comparable to CL (Schwarz et al., 1996). Recently such branched PC were characterized as a new class of PC forming inverted nonlamellar phases (Lewis et al., 1994; Nuhn et al., 1986). These results tempted us to propose that structural changes of the membrane induced by nonbilayer lipids may play an important role in the stimulation of P450SCC activity. Moreover, it should be considered that CL together with PE, another nonbilayer lipid, comprise more than 50% of phospholipid of the inner mitochondrial membrane. We further found that P450SCC induces specific vesicle aggregation critically dependent on CL, which also

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[1] Abbreviations: P450SCC, cytochrome P450SCC (CYP11A1); PC, phosphatidylcholine; DMPC, 1,2-dimyristoyl-PC; DOPC, dioleoyl-PC; CL(bh), bovine heart cardiolipin; CL(TA), 1,1′,2,2′-tetraacyl(16:0)-cardiolipin; CHL, cholesterol; PE, phosphatidylethanolamine; DOPE, dioleoyl-PE; DEPE, 1,2-dielaidoyl-PE; DSPC, distearoyl-PC; DSC, differential scanning calorimetry; eicosane, 20-carbon-saturated alkane; 1,2-diacyl-branched PC or PC(10,6), 1,2-di-[(2-hexyl)decanoyl]-PC; PC(12,8), 1,2-di-[(2-octyl)dodecanoyl]-PC; PC(14,10), 1,2-di-[(2-decyl)tetradecanoyl]-PC; LUVET, large unilamellar vesicles prepared by extrusion techniques.

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might be connected with the formation or predisposition of formation of nonbilayer phases (Schwarz et al., 1994, 1997). Because the branched PC with the headgroup of PC but with the fatty acyl moiety similar to CL in shape caused a significant activation comparable only with that by CL, the main suggestion was that the activation process appears to be related to an alteration in the physical properties of the membrane caused by the presence of nonbilayer lipids.

The current studies were designed to examine the importance of the state of the membrane in the activation of P450SCC. Because even small amounts of impurities as residual detergent and size effects can contribute to stimulation (Dhariwal et al., 1989) and ultrasonication often gave variable results (Bolen & Sando, 1992), all studies presented here were performed with nonsonicated vesicle preparations made using the extrusion technique. These techniques provided reproducible data and results for relatively homogeneous vesicles with regard to size. The properties of the membrane (e.g., hydrophobic volume/polar headgroup spacing, unsaturation) were systematically changed by inclusion of increasing amounts of three saturated branched PC, differing in their hydrophobic volume, two CL (highly unsaturated and fully saturated ones), and various other additives well-known as promoters of the hexagonal phase in membrane systems. We report their effects on the enzymatic activity of P450SCC in vesicle systems and correlate these data with the thermotropic phase behavior of the lipid dispersions characterized by DSC. The correlation observed between the property of the lipids to activate P450SCC and to increase the hexagonal phase propensity of the membrane strongly supports the importance of the physical property of the membrane in the activation process. This new view into the P450SCC−phospholipid interaction not only explains the results presented here but also should contribute to an understanding of virtually contradictory results of previous investigations too.

MATERIALS AND METHODS

Materials. [^{14}C]Cholesterol, [^{14}C]DOPC, and [^{3}H]DPPC were from Amersham (England). DOPC and *n*-eicosane were from Sigma (USA), and bovine heart cardiolipin, DMPC, and cholesterol were from Serva (Germany). DOPE, DEPE, and the tetraacyl-CL were from Avanti Polar Lipids (Alabaster, AL). The α-branched fatty acids for diacylphosphatidylcholine synthesis were provided by Condea-Chemie (Hamburg, Germany).

P450SCC, adrenodoxin, and adrenodoxin reductase were purified from bovine adrenocortical mitochondria to electrophoretic purity using specific affinity adsorbents (Chashchin et al., 1984; Usanov et al., 1987). They were stored until use at −80 °C in relatively highly concentrated form in 50 mM phosphate buffer containing 1 M NaCl, 0.3% sodium cholate, 1 mM EDTA, and 20%(v/v) glycerol. Before use, proteins were dialyzed into side chain cleavage assay buffer (20 mM Hepes, pH 7.3, 50 mM KCl, 0.1 mM DTT), aliquoted into small vials, and frozen at −80 °C.

The three diacylphosphatidylcholines containing branched fatty acids, pictured in Figure 1, were synthesized and purified as described by Ruerup et al. (1994). The compounds were analyzed with FAB-mass spectrometry and ^{1}H- and ^{13}C-NMR and characterized by NMR and Langmuir−Blodgett monolayer investigations. NMR revealed a different headgroup orientation as compared to PC with only two straight chains. LB analysis showed larger liftoff areas and tighter acyl chain packing at the collapse point than DMPC.

Preparation and Characterization of the Vesicle Systems. Large unilamellar vesicles were prepared by extrusion of lipid suspensions through filters of convenient size (LUVET) according to the procedure described by Mayer et al. (1986). Briefly, 12 mg of the phospholipid(s) in organic solvent was mixed with usually 6 mol % (of total lipid) cholesterol including a small amount of [^{14}C]CHL in absolute ethanol in a test tube, and solvents were evaporated under N_2 and kept under vacuum for 4 h. After complete removal of the solvent, 3 mL of standard buffer (20 mM Hepes, pH 7.3, 50 mM KCl, 0.1 mM DTT) was added and vortexed. Then the suspensions were taken through five freeze/thaw cycles and extruded 10 times through two stacked polycarbonate filters having a pore size of 100 nm (Nucleopore) using a thermobarrel extruder (Lipex Biomembranes Inc., Vancouver, BC, Canada). The extruder was thermostated at 30 °C for DMPC vesicles.

Size and homogeneity of the vesicle preparations were quantitated by light scattering using the methods of cumulants as described earlier (Schwarz et al., 1988). An average size could be determined to be about 97 nm in diameter, similar to values determined previously (Mayer et al., 1986). The parameter *Q* characterizing the variance of the size distribution is around 0.25. The particle size and size distribution as well as possible changes upon storage were checked by gel chromatography using Sephacryl S-1000. The vesicles were stable in relation to their size and size distribution within the same day when they were used after preparation.

Reconstitution of P450SCC into the preformed vesicles was done according to Seybert et al. (1979) and Lambeth et al. (1980) by incubation of the LUVET (600 μM final concentration) with P450SCC (1 μM final concentration)—or at any other desired lipid:protein ratio—except that the incubation was done for 5 min at 37 °C. Successful incorporation of P450SCC could be evidenced by gel chromatography using Sepharose 4B or Sephacryl S-1000 (Pharmacia, Sweden) (not shown).

Catalytic Activity Assay. The activity of P450SCC was determined as the side chain cleavage activity of cholesterol to produce pregnenolone according to the following assay: 0.25 μM P450SCC and 7 μM adrenodoxin in standard buffer were incubated at 37 °C for 10 min with vesicles consisting of 400 μM phospholipid with 6 mol % CHL including [^{14}C]-CHL in a total volume of 0.5 mL; 0.25 μM adrenodoxin reductase was added, and the reaction was initiated by addition of 2.5 mM NADPH (to a final concentration of 250 μM). After 5 min, the reaction was terminated by addition of 0.1 mL of 0.5 N HCl. The residual substrate and the product were extracted with 2 × 2 mL of methylene chloride, and pregnenolone was separated from unreacted cholesterol by thin-layer chromatograpy on silica gel 0.25 mm, 20 × 20 cm (Merck, Darmstadt, Germany), using a solvent mixture of *n*-hexane/petroleum ether/acetic acid (15:15:1, by volume). Analysis was done by counting the ^{14}C radioactivity of CHL and pregnenolone using a Linear Analyzer LB284 (Berthold, Germany). If not stated otherwise, each analysis was done 3 times to ensure reproducibility within a standard error of less than 10%.

Differential Scanning Calorimetry (DSC). Samples used in the DSC measurements were prepared by drying the lipid

mixture as described above and hydrating it with buffer (20 mM Hepes, pH 7.3, 50 mM KCl, 0.1 mM DTT) followed by intensive vortexing of the lipid dispersions. Total lipid concentration was 4 mg/mL for the DEPE and 2 mg/mL for the DMPC samples. DSC measurements were performed on a MC-2 microcalorimeter (MicroCal Inc., Northhampton, MA) equipped with the DA-2 data acquisition system. The samples were allowed to stand in the instrument cell at 2 °C for 1 h to reach thermal equilibrium before starting the calorimetric run. A scan rate of 30 K/h was kept constant in all experiments. Instrument base lines obtained with buffer solution under identical conditions were subtracted. Data treatment was done using the ORIGIN software package (MicroCal).

Analytical Methods. The concentration of P450SCC was determined from reduced CO minus reduced difference spectra using a difference extinction coefficient of 91 mM^{-1} for A_{450} minus A_{490} according to Omura and Sato (1964). The concentrations of adrenodoxin reductase and adrenodoxin were determined using extinction coefficients of 10.9 mM^{-1} at 450 nm and 11 mM^{-1} at 415 nm, respectively (Hanukoglu et al., 1981). CHL and lipid were quantitated using ^{14}C-labeled CHL, ^{14}C-labeled DOPC, and ^{3}H-labeled DPPC, respectively. Absorption spectra were recorded at room temperature on a double-beam spectrophotometer UV2101 PC (Shimadzu, Japan).

RESULTS

Effect of Branched PC and CL on the Side Chain Cleavage Activity of P450SCC in DMPC-LUVET. Figure 1 shows the chemical structures of the 1,2-diacyl-*sn*-glycero-3-phosphocholines containing hexyl, octyl, and decyl chains in the 2-position of their decanoyl, dodecanoyl, and tetradecanoyl main fatty acid chains. The CL(bh) with its four 18:2 acyl chains is characteristic for the most abundant fatty acyl component of the otherwise heterogeneous mixture of mitochondrial CL from bovine heart (Igarashi & Kimura, 1986). CL from eukaryotic cells are typically more than about 90% unsaturated. The tetraacyl(16:0)-CL [CL(TA)] is a commercially available fully saturated CL.

Figure 2 shows the activity of P450SCC in vesicle preparations containing pure DMPC (as host lipid) and mixtures of DMPC with either PC(10,6), PC(12,8), PC(14, 10), or both CL, respectively. The curves demonstrate the effect of varying the membrane content of the different nonbilayer lipids. In each case, the total lipid content remained the same. To facilitate direct comparison of our own previous experimental data with those from others, results were normalized to 100% maximum activity. The absolute activities corresponding to the 100% values are summarized in Table 1. It can be seen from Figure 2 that incorporation of the nonbilayer lipids in the DMPC vesicles results in significant activation by a factor between 10 and 20. At higher content of branched PC in the vesicle preparations, the activity was lowered as clearly indicated for CL(bh). Comparing the three branched PC, we can see that the increase of activity is sensitive to the acyl chain structure. As the lengths of both the branched and main acyl chains increase, a smaller amount of the phospholipids with longer chains as compared to those with shorter ones is required for maximal activity.

To quantitatively compare the activator capability of the different phospholipids, it is convenient to calculate their $K_{1/2}$ values for half-maximum activation. Regarding the differences in the molecular weights of the branched PC, the differences in $K_{1/2}$ values are more significantly expressed on a mol/mol basis. Table 1 summarizes the values. The most potent activator is CL(bh); 0.08 mol/mol (i.e., 7.4 mol %) is required for half-maximum activation, compared to 0.65 mol/mol (39.4 mol %) of PC(10,6).

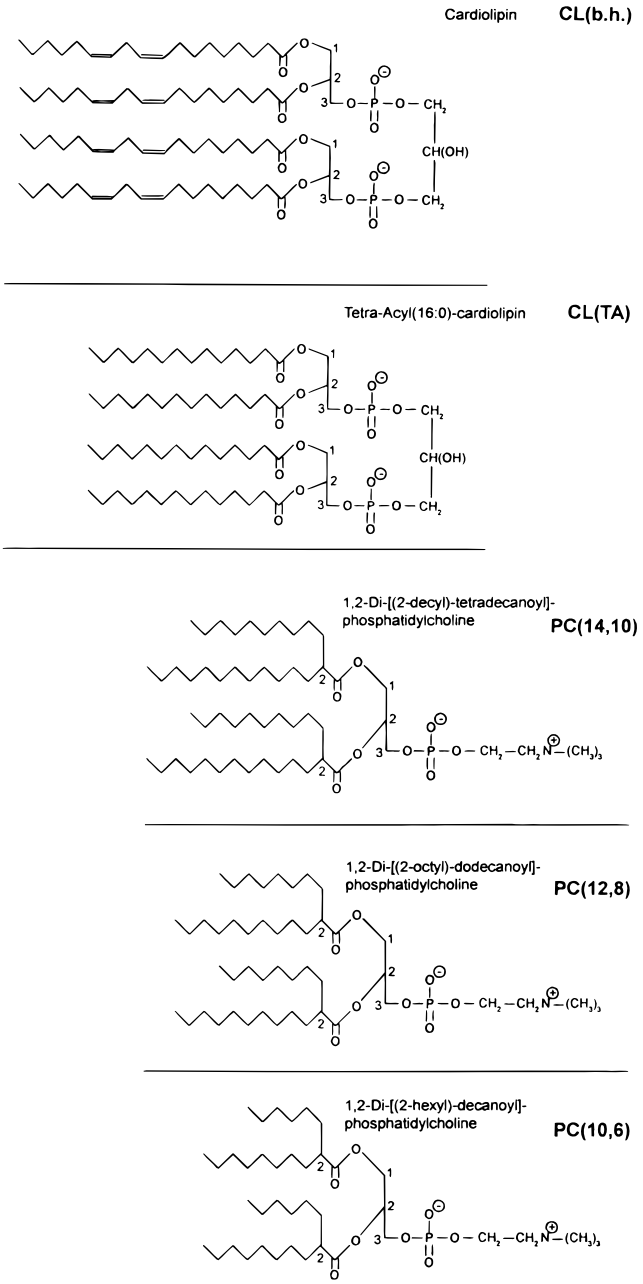

FIGURE 1: Structures of the nonbilayer lipids used for the activation of the side chain cleavage reaction by P450SCC in vesicle systems.

Effect of DEPE, DOPE, and Eicosane on the Activity of P450SCC in DMPC-LUVET. To further analyze the effect of nonbilayer lipids and their influence on membrane properties, we undertook measurements using other nonbilayer lipids, namely, PE. PE also belong to the main components of the inner mitochondrial membrane. Together with CL, it accounts for more than about 50% of the total phospholipid of the inner mitochondrial membrane. We compared the influence of DOPE and DEPE on the activity of P450SCC in vesicles consisting of DMPC/DOPE and DMPC/DEPE, respectively. The only structural difference

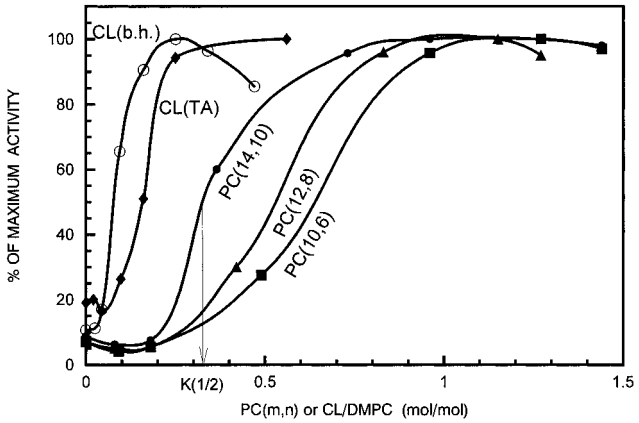

FIGURE 2: Effect of CL and branched PC on the activity of P450SCC in DMPC vesicles. (■) PC(10,6); (▲) PC(12,8); (●) PC-(14,10); (♦) CL(TA); (○) CL(bh). For each data point, vesicles were prepared consisting of a mixture of PC(*m*,*n*) or CL and DMPC containing a molar ratio of 0.06 CHL per total lipid. In each case, the total lipid content remained the same. Data were normalized to 100% maximum activity. For vesicle preparation and reconstitution of P450SCC, see the description under Materials and Methods. $K_{1/2}$ values represent the amount required for half-maximum stimulation of the activity. Values were determined as shown and expressed as mol/mol ratios of activator lipid to DMPC (host lipid). Each analysis was done 3 times to ensure reproducibility within a standard error of less than 10%.

Table 1: Half-Maximum Activation of P450SCC by Branched 1,2-Diacyl PC and CL and Maximal Activities in Cholesterol-Containing DMPC- LUVET[a]

activator lipid[d]	$K_{1/2}$ (mol/mol)[c]	no. of C atoms[e]	activity[f] [nmol of pregnenolone min^{-1} (nmol of P450SCC)$^{-1}$]
PC(10,6)	0.65	34	5.8
PC(12,8)	0.54	42	6.0
PC(14,10)	0.33	50	6.5
CL(bh)	0.08	68	4.6
CL(TA)	0.16	60	2.5
PC(14,10) in DOPC	0.077		5.3
PC(14,10) + eicosane[b]	0.18		3.5
DOPE	1.10		3.9[g]
DOPE + eicosane[b]	0.45		3.8
DEPE	>2.5		0.4[g]

[a] Mole fraction of CHL: 0.06. [b] Mole fraction of eicosane: 0.20. [c] $K_{1/2}$ values could be determined within a standard error of about 0.01. [d] Lipid host was in each case DMPC apart from one (DOPC). [e] The total number of C atoms was calculated simply by consideration of the activator lipid C atoms in their chains under inclusion of the one oxygen nearest to the glycerol backbone of the lipid molecule. [f] Absolute activities corresponding to 100% activation as shown in Figures 2, 3, 4, and 5. [g] Data represent activities measured at a molar PE:DMPC ratio of 2.4.

between DOPE and DEPE was the configuration of the double bond on the side chain. This causes DEPE to have a much higher bilayer−hexagonal phase transition temperature (around 65 °C) as compared with DOPE (around 10 °C). Figure 3 shows that the activity of P450SCC is significantly enhanced as the content of DOPE in the vesicles increases, whereas DEPE has almost no activation potential. These results demonstrating the correlation between the activation and the ability of PE to form the hexagonal phase were supported further by experiments using well-known hexagonal phase promoters as additives (Epand, 1985; Janes, 1996). For instance, in membranes containing eicosane, the potential of DOPE to stimulate the activity is even more pronounced as can be seen from Figure 3. The inclusion of

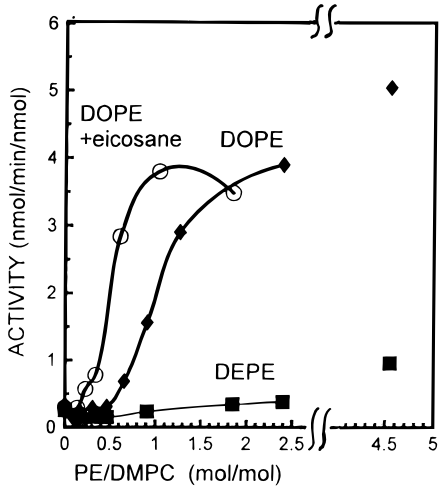

FIGURE 3: Effect of DOPE, DEPE, and eicosane on the activity of P450SCC in DMPC vesicles. (■) DEPE; (♦) DOPE; (○) DOPE + eicosane.Vesicle systems were prepared as described in the legend of Figure 1 and under Materials and Methods. In case of the curve for eicosane, for each data point vesicles were prepared containing 20 mol % eicosane. Each analysis was done 3 times to ensure reproducibility within a standard error of less than 10%.

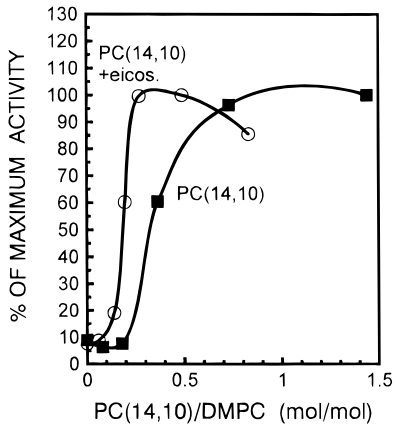

FIGURE 4: Effect of PC(14,10) on the activity of P450SCC in DMPC vesicles containing eicosane. (■) Vesicles without eicosane; (○) vesicles containing 20 mol % eicosane. For conditions, preparation, and error estimation, see the legends of Figure 2 and 3.

eicosane results in a doubling of the potential of DOPE to stimulate the side chain cleavage reaction of P450SCC. The same effect of eicosane could be evidenced in vesicle membranes containing the branched PC as can be seen from Figure 4, and the $K_{1/2}$ values listed in Table 1.

Role of Phospholipid Unsaturation on P450SCC Activity. To study the effect of unsaturation, we provided unsaturation to the membrane using two different methods: (i) by using an unsaturated host lipid (DOPC) instead of DMPC and comparing the effect of increasing amounts of the branched PC PC(14,10) in both hosts; and (ii) by using DMPC vesicles and comparing the effect of both a fully saturated CL [CL-(TA)] and the highly unsaturated CL from bovine heart [CL-(bh)] with more than 90% polyunsaturated fatty acyl chains (see Figure 1). The results shown in Figure 5 demonstrate that the introduction of unsaturation leads to a further significant increase in the potency of activation. For absolute values of the activities corresponding to 100% values of maximum activation, see Table 1. The respective half-maximum activation values, $K_{1/2}$, are listed in Table 1. From these results, it is scarcely conceivable that a specific

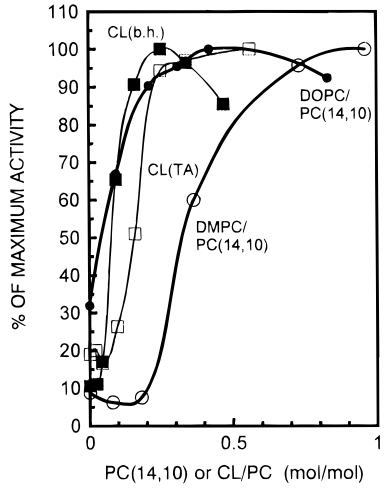

FIGURE 5: Effect of introduction of unsaturation on the activity of P450SCC. (○) DMPC/PC(14,10); (●) DOPC/PC(14,10); (□) DMPC/CL(TA); (■) DMPC/CL(bh). For experimental conditions and parameters, see the legend of Figure 2.

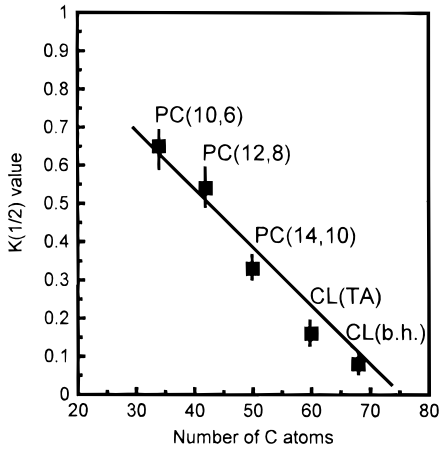

FIGURE 6: Effect of the increase of the hydrophobic volume of activator lipids on the activity of P450SCC in DMPC vesicles. Half-maximum activation values, $K_{1/2}$, characterizing the activating potency of the lipids were plotted versus the total number of C atoms in the chains of the activator lipids. This number was calculated as described in the legend of the Table 1. The straight line represents a linear regression; it gave a correlation coefficient of 0.992.

interaction between P450SCC and part of the lipid molecules, either headgroup or fatty acyl chain, is decisive for the activation. More likely, another property of the membrane dependent on unsaturation of the phospholipids determines the activation.

What property of the membrane is important for the process of activation of P450SCC? Comparing the lipid structures in Figure 1, it is evident that the molecular volume of the part of the lipid molecule including the fatty acyl moiety is apparently increasing going from PC(10,6) at the bottom to CL(bh) at the top of the schema. As evident from Figure 6, there does appear a correlation between the potency of activation of P450SCC and the hydrophobic volume defined by increased lengths of fatty acyl chains. The activity of the enzyme assayed in the five different lipid systems consisting of DMPC and the branched PC and two CL as additives, respectively, shows a linear dependence on the hydrophobic volume calculated on the basis of the number of CH_2 groups present in the activator lipid (for calculation and number of these groups, see legend of Table 1). We restricted the analysis to these five phospholipids because the size of their CL-like fatty acyl moiety can most directly be compared. Inclusion of the other systems and comparison of their relative hydrophobic sizes seem more difficult and uncertain. Taken together, this correlation tempted us to speculate that it might be mainly the increased hydrophobic volume—and the increased headgroup spacing as a consequence—which is the property imparted to the membrane by both fatty acyl chain branching and unsaturation of the activator lipids.

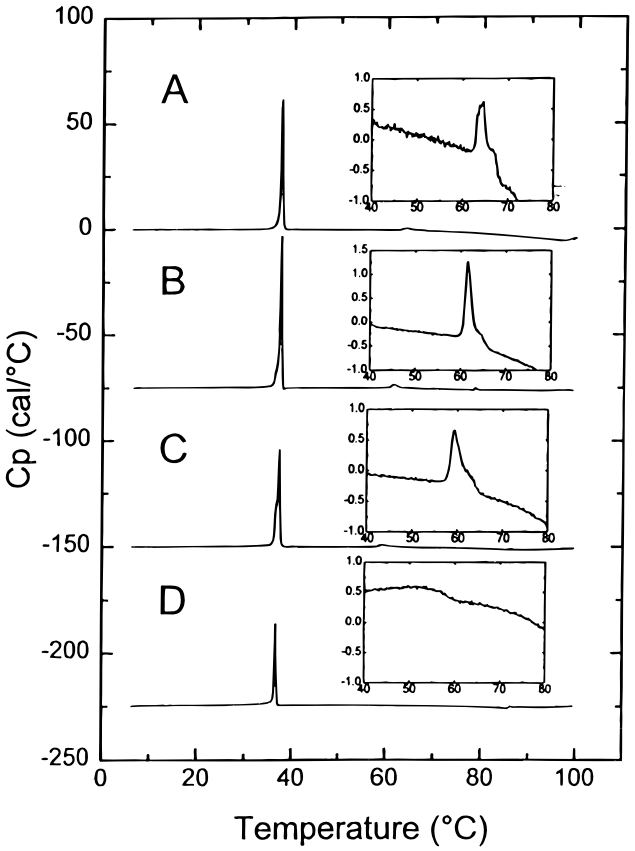

FIGURE 7: DSC curves for DEPE in the presence of increasing concentrations of PC(14,10). Molar concentration of PC(14,10): (A) 0.0, (B) 0.5, (C) 1.0, (D) 2.5 mol %. Scan rate, 30 K/h; total lipid concentration, 4 mg/mL. The inserts show parts of the scans representing an enlarged scale of the region of the bilayer to hexagonal phase transition.

Differential Scanning Calorimetry of DEPE and DMPC in the Presence of Increasing Concentrations of PC(14,10). The temperature of the bilayer to hexagonal phase transition of PE (T_H) is strongly dependent on the presence of other lipid components in the membrane (Epand, 1985; Epand et al., 1991a,b). The effect of membrane constituents on the T_H of a given lipid provides a quantitative measure of the potency of a membrane constituent to promote the nonbilayer propensity of the bilayer membrane (e.g., Janes, 1996). The effect of the branched PC on the phase transition temperature was studied by DSC. The results of the measurements are shown in Figure 7. From the figure, the two endotherms corresponding to the strong main transition (gel to liquid-crystalline phase transition around 37 °C) and the much weaker bilayer to hexagonal phase transition (around 65 °C) clearly can be seen. The enthalpy of the latter lies within 455 ± 30 cal/mol in the range reported earlier [for instance, see Epand (1985)] and is virtually unchanged below 1 mol % PC(14,10). Above that content, it is difficult to calculate

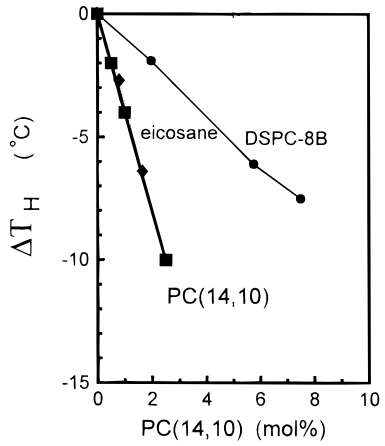

FIGURE 8: Shift of the bilayer to hexagonal phase transition temperature of DEPE in the presence of PC(14,10). ΔT_H is the change in transition temperature from 63.7 °C for DEPE. (■) DEPE + PC(14,10). For comparison: (♦) DEPE + eicosane; (●) DEPE + DSPC-8B [data taken from Epand (1985)]. For conditions and concentrations, see the legend of Figure 7.

an enthalpy due to the strong broadening of the transition. The extent to which T_H is lowered by PC(14,10) is proportional to the mole fraction of this component of the membrane. The shift in T_H (ΔT_H) of DEPE dependent on the mole fraction of PC(14,10) is shown in Figure 8. The potency to alter the T_H, for PC(14,10), is about −500 °C /mol (i.e., −4 °C/mol %). The curve demonstrates that the branched PC(14,10) is a potent bilayer destabilizer, lowering the phase transition temperature significantly. On the other hand, the gel to liquid-crystalline phase transition temperature of DEPE is 37.4 °C and is not significantly altered below 1.5 mol % PC(14,10) more than 0.2 °C. In the presence of 2.5 mol % PC(14,10), the phase transition temperature decreases by only 0.5 °C, to 36.9 °C, whereas the temperature of the bilayer to hexagonal phase transition changes by about 10 °C. The potency of the branched PC(14,10) to promote the hexagonal phase of DEPE is comparable to that of the most potent promoters reported so far (Janes, 1996). For comparison, data from Epand (1985) of other typical hexagonal phase promoters like eicosane and the branched PC di-(8-*n*-butylstearoyl)-PC (DSPC-8B) are included in the figure. The latter lipid has a pronounced stimulating effect on the protein kinase C activity (Epand et al., 1991a). PC-(14,10) has the same order of effectiveness as eicosane, but is a more potent promoter compared to the branched DSPC.

Further, the thermotropic phase behavior of bilayer membranes composed of DMPC plus various amounts of branched PC was studied. Figure 9 shows results for PC-(14,10) typical for other branched PC, too. DMPC exhibited two endotherms upon heating. The broader, lower energy transition occurring at 14.8 °C is the pretransition, which is associated with a gel-state packing rearrangement of the hydrocarbon chains. The sharper, higher energy main transition occurring at 24 °C is due to the cooperative melting of the fatty acyl chains. One can see that the presence of cholesterol completely abolishes the pretransition. At PC-(14,10) concentrations beween 2 and 42 mol %, the DSC curves consist of two components. The sharp component peak originally centered around the phase transition temperature of pure DMPC shifts significantly to lower temperatures upon addition of PC(14,10); thereby its cooperativity decreases and its enthalpy is lowered. At PC(14,10)

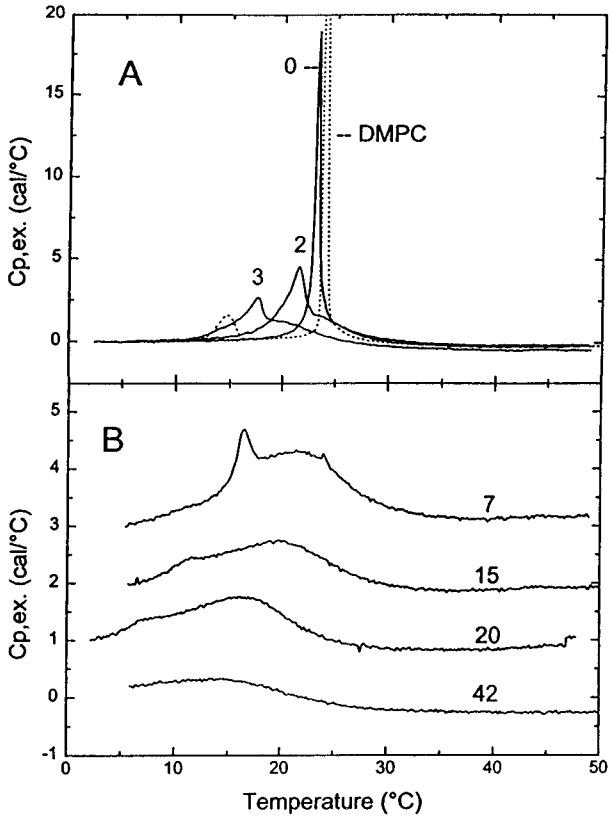

FIGURE 9: DSC curves illustrating the thermotropic phase behavior of DMPC dispersions containing increasing portions of PC(14,-10). Numbers at the curves represent fractions of PC(14,10) in mol % of total lipid. For clarity, curves with 7−42 mol % PC(14,10) are shown separetely in panel B in an enlarged scale. Their positions were shifted by about 1 cal/°C on the *y* axis. Dotted line: pure DMPC. All samples apart from "DMPC" contained 6 mol % CHL. Scan rate, 30 K/h; total lipid concentration, 2 mg/mL.

concentrations higher than about 20 mol %, it is nearly completely abolished. A second, broader peak arises at about 2 mol % PC(14,10) as a shoulder at the upper side of the main transition. It initially increased up to about 10 mol % and then broadens and decreases as the PC(14,10) content increases above 20 mol %. A similar behavior has previously been reported for several PC containing branched chain fatty acids and cholesterol or a hopanoid (Kannenberg et al., 1983) and PC−CHL, PE−CHL, and sphingomyelin−CHL mixtures [see McElhaney (1982) and citations therein]. The existence of two transitions at PC(14,10) concentrations above 2 mol % might be connected with a lateral phase separation into domains consisting of different contents of the branched PC and/or CHL.

DISCUSSION

There is now growing evidence that the lipid composition of several biological membranes appears to be regulated so that the membrane is close to, but below, the bilayer to hexagonal phase (H_{II}) transition temperature (T_H) (Lindblom et al., 1993; Rietveld et al., 1994). The data reported suggest that this physical property of the membrane plays an important role in the modulation of membrane function [e.g., see Epand (1996) and citations therein]. The general view is that nonbilayer lipids might exert some action on the membrane that might be essential for optimal functioning; e.g., the curvature stress introduced by the incorporation of nonlamellar lipids can modulate the function of membrane

proteins by mediating an optimal conformation of the protein (Gruner, 1985, 1989; Gruner et al., 1985; Hui, 1987; Hui & Sen, 1989). This view is supported by the finding that the activity of several enzymes is increased, among them protein kinase C (Goldberg et al., 1992; Zidovetsky & Lester, 1992; Senisterra & Epand, 1993; Stubbs & Slater, 1996, and citations therein), mitochondrial ubiquinol−cytochrome *c* reductase, mycoplasma Mg^{2+}-ATPase and H^+-ATPase (Yang & Hwang, 1996), lipid-metabolizing enzymes (Cornell & Arnold, 1996), and the adenine nucleotide translocator (Streicher-Scott et al., 1994). Particularly, the H_{II} phase forming propensity of branched-chain analogs of DSPC has been recently used by Epand et al. (1991a) and Epand and Lester (1990) to establish a correlation between protein kinase C activity and nonlamellar phase forming tendency. Other functions of membrane proteins like the photochemical action of rhodopsin (Brown, 1994) and the activity of ion channels (Keller et al., 1993) are promoted by lipids tending to form nonlamellar phases, too. In additon, nonbilayer lipids play an important role in membrane fusion intermediates (Chernomordik, 1996).

The results reported here using vesicle systems suggest that the hexagonal phase forming tendency of the membrane is an important factor determining the side chain cleavage activity of P450SCC, the key enzyme in mitochondrial steroidogenesis. A possible physiological role of the high content of nonbilayer lipids in the inner membrane of mitochondria where the P450SCC is localized remains to be clarified. However, it has long been known that PE and CL are able to form inverted nonbilayer phases (Cullis & DeKruijff, 1978; Rand & Sengupta, 1972) and that the content of PE+CL amounts to more than 50% of the total inner mitochondrial lipid. Our interpretation is in contrast to previous conclusions that stimulation of the activity of P450SCC by lipids is due to direct binding of lipid(s) to effector site(s) of the enzyme (Lambeth, 1981; Igarashi & Kimura, 1986; Lambeth et al., 1980; Seybert et al., 1979; Pember et al., 1983). To conduct our studies, we took advantage of a series of recently synthesized (by us) branched PC with saturated fatty acyl chains in the 2-positions of the saturated main chains, thereby approaching the structure of the hydrophobic part of CL but maintaining the headgroup of PC. These branched PC showed a high stimulatory potency, higher than any other phospholipid and comparable to CL, the most potent activator lipid to date. The first report of results was published recently (Schwarz et al., 1996).

The high propensity of nonbilayer lipids to form hexagonal phases has been explained initially on the basis of the molecular shape concept of Cullis and deKruijff (1979) and the self-assembly theory of Israelachvili (1980). According to this, nonbilayer phases are favored in the case of phospholipids whose headgroup cross-section is sufficiently reduced as compared to that of the hydrophobic part of the molecule. It was argued that only a small increase in the hydrophobic region destabilizes the lamellar phase. It might be this physical property of the membrane that is affected by both an increasing hydrophobic volume as well as unsaturation and which is important for activation of P450SCC. This conclusion is strongly supported now by systematic studies using the three branched PC and the two CL representing some kind of homologous series with increasing lengths of their fatty acyl chains. An effective acyl chain length was simply defined as the total number of carbon atoms in the chain. It is clear from Figure 6 that the potency of the nonbilayer lipids used in activation of P450SCC is increasing linearly with the increase in hydrophobic volume. Of course, this is a rather rough calculation not taking into account the different headgroups as well as the fact that unsaturation contributes to the effective hydrophobic volume. It should be emphasized that it is the effective shape of the lipid molecule that matters (Tate et al., 1991). This depends not only on chemical structure but also, e.g., on hydration, charges present, and motional dynamics of the acyl chains.

A recent study by Epand et al. (1991a) has shown that the addition of branched chain analogs of DSPC to phosphatidylserine vesicles caused activation or inhibition of protein kinase C, depending on the ability of lowering the T_H of DEPE. From the two analogs used, only the compound containing the longer branched side chain was sufficient to disrupt the hydrocarbon packing and to increase the hydrophobic volume necessary for lowering the T_H and successively to enhance activation.

The results with regard to the even higher stimulation potency by introduction of unsaturation into the membrane are in line with our interpretation. The shape theory predicts that increases in the mean acyl chain area that accompany unsaturation should promote destabilization of the bilayer, but experimental evidence is conflicting. It is known that the introduction of unsaturation into the acyl chains of PE greatly reduces T_H and promotes formation of nonbilayer phases (Tilcock & Cullis, 1987). Further, there exists some evidence that generally increasing the unsaturation of fatty acids incorporated into DEPE membranes increases the tendency of the membrane to form nonbilayer phases (Epand et al., 1991b), thereby the introduction of the first single double bond producing the largest increase in the H_{II}-forming capacity. Unsaturation could be provided to the system by either the host lipid or the activator lipid component of the membrane. As can be seen from Figure 5 and Table 1, in both cases we observed an additional increase in the capability of PC(14,10) to activate P450SCC. In the background of the discussion above, it is not surprising that the effect of PC(14,10) in DOPC vesicles is potentiated by about a factor of 4 compared to DMPC and that the introduction of nearly fully unsaturated fatty acyl chains by using CL(bh) instead of saturated CL(TA) leads to a further increase in the potency of PC(14,10) by about a factor of 2. In a recent study, Bolen and Sando (1992) came to a similar conclusion studying the effect of unsaturation on protein kinase C activity in PC/phosphatidylserine/diolein vesicles of defined acyl chain composition. Maximal activation was found to correlate with the mole percent of unsaturation in the system, whereas the degree of unsaturation and the positioning of the double bonds had little effect. A model involving headgroup spacing caused by hydrophobic volume expansion as a result of the introduction of unsaturation was discussed.

According to this discussion, an increase in the hydrophobic volume results also in an increase in the headgroup spacing which successively may promote membrane insertion of P450SCC. This may explain the sometimes reported differences in the activity and other properties of P450SCC between differently sized vesicles (Dhariwal et al., 1989; Schwarz et al., 1994). Preliminary experiments showed that the incorporation efficiency of P450SCC into vesicles with

regard to both its kinetics as well as its extent is affected by the presence of branched lipids and CL. Such experiments and investigations regarding the role of vesicle size are now under investigation.

Experimental evidence has been shown for a relationship between the tendency of the lipids to form nonbilayer phases and the increase in activity of P450SCC. However, it remains to be proved that the branched PC used by us form nonbilayer phases in pure form; such measurements (NMR and/or X-ray) are now under consideration. Nevertheless, this assumption is strongly supported by the recent reports of Lewis et al. (1994), who found that these PC containing 2-alkyl-substituted fatty acids in their main chains belong to a new class of PC forming nonbilayer phases, a possibility earlier suggested in studies of branched chain diacyl PC by Nuhn et al. (1986). From Lewis et al. (1994), it became evident that the predisposition to form nonlamellar phases increases dramatically when the length of the substituted chains is increased beyond three carbon units, i.e., butyl or more. On the other hand, we directly could show by calorimetric characterization of their influence on the T_H of DEPE that our branched PC are strong promoters of the hexagonal phase. Their potency to lower the T_H is comparable with those of most potent lipids and other membrane constituents studied so far (Janes, 1996).

Striking evidence for the decisive role of hexagonal phase forming tendency could be obtained by the comparison of the two di-18:1-acyl-PE, DOPE and DEPE, which are only different in their configuration of the double bond, cis for DOPE and trans for DEPE. This difference results in a much higher T_H of DEPE (around 65 °C) compared to DOPE (around 10 °C). This property advantageously can be used to prove by experiments the importance of the hexagonal phase forming tendency as previously done by Yang and Hwang (1996). As in the case of ubiquinol−cytochome *c* reductase, we could show that only DOPE increased the activity of P450SCC in DMPC vesicles remarkably. DEPE had almost no effect, at least in the concentration range where other membrane constituents act stimulating. These data and the finding that an additional stimulation of the potency of both DOPE and PC(14,10) can be reached by inclusion of eicosane support the conclusion that the effect of the branched PC and CL on the activity of P450SCC may be related to its ability to form nonbilayer phases. *n*-Alkanes are among the most powerful promoters of hexagonal phase formation [see Janes (1996) and citations therein].

The investigation of the thermotropic behavior of DMPC/PC(14,10) mixtures showed that the branched PC have a significant perturbing effect on the bilayer membrane of DMPC, in some relation comparable with what is known from lipid mixtures containing CHL or a bacterial hopanoid (Kannenberg et al., 1983; McElhaney, 1982). As shown in Figure 9, the shift and broadening and the almost complete abolishment of the main transition at high branched PC concentration reflect that the interaction between the hydrocarbon chains is remarkably perturbed. Moreover, the existence of a second more broad transition appears to be due to lateral phase separation in the range from 2 to 42 mol % investigated. This observation may be related to the ability of the branched PC and/or CHL to phase-separate and to form domains more or less enriched in these membrane constituents which could cause activation. Therefore, we cannot rule out the possibility that phase separation is another explanation for the activation of P450SCC in such mixed lipid systems.

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