Membrane Properties Induced by Anionic Phospholipids and Phosphatidylethanolamine Are Critical for the Membrane Binding and Catalytic Activity of Human Cytochrome P450 3A4†

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ABSTRACT: Human cytochrome P450 (CYP) 3A4, a membrane anchoring protein, is the major CYP enzyme present in both liver and small intestine. The enzyme plays a major role in the metabolism of many drugs and procarcinogens. The roles of individual phospholipids and membrane properties in the catalytic activity, membrane binding, and insertion into the membrane of CYP3A4 are poorly understood. Here we report that the catalytic activity of testosterone 6β-hydroxylation, membrane binding, and membrane insertion of CYP3A4 increase as a function of anionic phospholipid concentration in the order phosphatidic acid (PA) > phosphatidylserine (PS) in a binary system of phosphatidylcholine (PC)/anionic phospholipid and as a function of phosphatidylethanolamine (PE) content in ternary systems of PC/PE/PA or PC/PE/PS having a fixed concentration of anionic phospholipids. These results suggest that PA and PE might help the binding of CYP3A4 to the membrane and the interaction with NPR. Cytochrome b_5 (b_5) and apolipoprotein b_5 further enhanced the testosterone 6β-hydroxylation activities of CYP3A4 in all tested phospholipids vesicles with various compositions. Phospholipid-dependent changes of the CYP3A4 conformation were also revealed by altered Trp fluorescence and CD spectra. We also found that PE induced the formation of anionic phospholipid-enriched domains in ternary systems using extrinsic fluorescent probes incorporated into lipid bilayers. Taken together, it can be suggested that the chemical and physical properties of membranes induced by anionic phospholipids and PE are critical for the membrane binding and catalytic activity of CYP3A4.

The microsomal monooxygenase system oxidizes a variety of endogenous and xenobiotic compounds (*1*). This enzyme system includes cytochrome P450 (CYP[1] or P450; EC 1.14.14.1), NADPH−P450 reductase (NPR; EC 1.6.2.4), and phospholipids. CYP-dependent activities can be reconstituted under experimental conditions by mixing CYP, NPR, and phospholipids (*2*). Although the organization in phospholipid membranes of the constituent proteins of this system and their mechanism of interaction are not yet fully understood, CYP and NPR seem to be distributed randomly on the plane of membranes and interact through lateral diffusion by forming a functional complex for electron transfer (*3*). CYP is present in the membrane in large excess over the reductase, the limiting component in microsomes, with molar ratios ranging from 10:1 to 25:1 depending on treatment with inducers (*4*). Phospholipids in the immediate vicinity of CYP in liver microsomes have been reported to be highly organized as compared with those in bulk membrane (*5*), suggesting that CYP may have important interactions with the phospholipid molecules immediately surrounding the protein. It has also been proposed that the interaction of phospholipids with CYP might be necessary for maintaining an active protein conformation and the ability of CYP to interact with NPR for efficient electron transfer (*6*). We have shown previously that changes in the activity of CYPs 1A2 and 2B1 are associated with conformational changes induced by salt, phospholipids, and detergent (*7*−*9*) and that the membrane topology and activity of CYP1A2 are related to the composition of phospholipids (*10*).

In humans, CYP3A4 is generally agreed to be the most abundant CYP enzyme present in both the liver and small intestine, two major sites for oxidation of xenobiotic chemicals (*11*). The enzyme has very broad substrate specificity and catalyzes the metabolism of approximately 50% of therapeutic agents (*12*). CYP3A4 seems to have a large cytoplasmic domain anchored to the endoplasmic reticulum membrane by an amino-terminal transmembrane

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[1] Abbreviations: CYP or P450, cytochrome P450; NPR, NADPH−P450 reductase; PS, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; b_5, cytochrome b_5; apo b_5, apolipoprotein b_5; LUVs, large unilamellar vesicles; CD, circular dichroism; pyrene-PA, 1-palmitoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphate; NBD, 7-nitrobenz-2-oxa-1,3-diazole; BODIPY, 4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene; pyrene-PC, 1-palmitoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine; PyrS DHPE, *N*-(1-pyrenesulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine.

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segment (*13*). It has been reported that the anionic phospholipid PS exerted a stimulatory effect on the catalytic activity of CYP3A4 during the course of optimization of reconstituted enzyme activity (*14*, *15*).

It is well established that most biological membranes are not uniform but have a heterogeneous distribution of lipids and proteins both laterally and across the bilayer (*16*). Laterally structured lipids can be defined as lipid domains (also as lipid clustering or phase separation), which differ in lipid composition from other areas in membranes. In close relation to this lipid ordering, we have shown that the mixing properties of anionic phospholipids such as PA, PS, and PG in a PC matrix are not random but instead yield lipid domains in the liquid-crystalline phase of membranes (*17*). We also proposed that PE, another matrix phospholipid component of biological membranes, induces the lateral segregation of PS and that induced PS-enriched domains promote the membrane binding and insertion of apocytochrome *c* (*18*). In related studies, it has been shown that PE is important for the association of protein kinase C and other cytoplasmic proteins with model membranes containing PS (*19*) and that PE molecules restrict the dissipation of clustered PA phospholipids in membranes (*20*).

In the present study, we investigated the role of anionic phospholipids in a PC matrix (as a binary system) on the catalytic activity and membrane binding of human CYP3A4. The possibility that PE induces the formation of lipid domains enriched with PA (or PS) in PC/PE/(PA or PS) membranes was also tested, along with the subsequent effects of such lipid clustering on the catalytic activity, membrane binding, and insertion into the lipid bilayer of CYP3A4. We report here that the catalytic activity and membrane binding/insertion of CYP3A4 increase as a function of anionic phospholipid concentration in the order PA > PS in a binary system of PC/anionic phospholipid and as a function of PE content in ternary systems of PC/PE/PA or PC/PE/PS having a fixed concentration of anionic phospholipid. These results suggest that the chemical and physical properties of phospholipids in binary and ternary systems are critical for the interaction of CYP3A4 with membranes and NPR. The possible role of anionic phospholipids in binary systems and of PE in ternary systems in the conformational change of CYP3A4 is also discussed.

EXPERIMENTAL PROCEDURES

Chemicals. Testosterone and 6β-hydroxytestosterone were obtained from Sigma Chemical Co. (St. Louis, MO). All of the nonfluorescent phospholipids were from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. Pyrene-labeled phospholipids were purchased from Molecular Probes (Eugene, OR). The unlabeled phospholipids used here are synthetic phospholipids with acyl chains of palmitic acid at the *sn*-1 and oleic acid at the *sn*-2 position.

Protein Purification. Human liver CYP3A4 was expressed in *Escherichia coli* and purified as described (*21*). CYP3A4 was electrophoretically homogeneous and had a specific content of 14 nmol of CYP/mg of protein. Protein concentrations were estimated using a bicinchoninic acid procedure according to the manufacturer's directions (Pierce, Rockford, IL). CYP concentrations were determined by $Fe^{2+}-CO$ versus Fe^{2+} difference spectroscopy (*22*).

Recombinant rat NPR was expressed in *E. coli* and purified as described (*23*). Rabbit liver b_5 was prepared as described (*24*). Apo b_5 was prepared from rabbit b_5 as described previously (*25*).

Enzyme Activity Assays. The assay of CYP3A4 activity was done using testosterone as described elsewhere with slight modifications (*25*). The standard incubation mixture (final volume of 0.5 mL) contained CYP3A4 (0.2 μM), NPR (0.4 μM), lipid vesicles (40 μM), and testosterone (100 μM) in 100 mM potassium phosphate buffer (pH 7.4). In some cases to see the effects of b_5 and apo b_5, 0.2 μM b_5 (or apo b_5) was added to the reaction mixture. The reaction was started by adding an NADPH-generating system (0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, and 0.5 unit of glucose-6-phosphate dehydrogenase/mL), and after the sample was incubated at 30 °C for 10 min, the reaction was stopped by adding 50 μL of 1.0 N HCl containing 2.0 M NaCl and 1.5 mL of CH_2Cl_2. Organic phases were evaporated under a nitrogen stream, and product formation was determined by HPLC with a C_{18} column (4.6 × 250 mm, 5 μm). The elution was conducted with a mixture of CH_3OH/H_2O (75:25, v/v) at a flow rate of 1.0 mL/min, and detection was by UV absorbance at 240 nm.

The assay for NPR was done using cytochrome *c* as a substrate as described (*26*). The NPR-mediated reductions were measured in 100 mM potassium phosphate buffer, pH 7.4.

The rate of NADPH oxidation by the purified CYP3A4 with NPR in the presence of indicated phospholipid vesicles was measured as described (*27*). CYP3A4 (0.2 μM) was reconstituted with NPR (0.4 μM) as described for the enzyme activity assay. Reconstituted enzyme (950 μL) was preincubated for 3 min at 30 °C in the presence of indicated lipid vesicles (40 μM) and testosterone (100 μM). Reactions were initiated with the addition of 50 μL of 4.0 mM NADPH, and the decrease in A_{340} was monitored for 1 min.

Liposome Preparation. In all experiments, POPC liposomes were used as standard vesicles. To prepare vesicles containing extrinsic fluorophores such as pyrene-, BODIPY-, or NBD-labeled phospholipids, 1 mol % of pyrene-PA, 1 mol % of BODIPY-PA, or 10 mol % of NBD-PA was incorporated into liposomes. The concentrations of nonfluorescent phospholipids were determined by a phosphorus assay (*28*). The concentrations of fluorescent probes were determined spectrophotometrically at 342 nm (pyrene-PA and BODIPY-PA) and 465 nm (NBD-PA), using molar extinction coefficients of 42000 cm^{-1} for pyrene-PA, 80000 cm^{-1} for BODIPY-PA, and 22000 cm^{-1} for NBD-PA.

After the appropriate amount of lipids was mixed in chloroform, the solvent was evaporated under a stream of argon gas. The dry lipids were hydrated in buffer solution (25 mM Tris-HCl, pH 7.4, containing 100 mM NaCl and 0.5 mM Na-EDTA) by vortex mixing and subsequent brief sonication in a bath sonicator (30 s). To obtain homogeneous LUVs, the dispersion was frozen and thawed five times and extruded 25 times through two polycarbonate membranes (100 nm pore size). All LUVs used for this work were stable for at least 3 days as determined by <10% deviation in light scattering values. The concentration of liposome stock solutions was 0.5−5.0 mM, and portions of stock solutions were diluted to study the interaction of CYP3A4 and membranes.

Fluorescence Measurements. All fluorescence experiments were performed at 30 °C, maintained using a circulating water bath. Fluorescence emission spectra were recorded with a Shimazu RF-5301 PC spectrofluorometer equipped with a thermostated cuvette compartment. Emission spectra of the intrinsic fluorescence in CYP3A4 were recorded in the range of 300−450 nm with an excitation wavelength of 280 nm. The samples were incubated for 20 min before measurement. In all fluorescence experiments, 700 μL of sample solution was used in a quartz cuvette. For the measurement of the excimer (E) and the monomer (M) of pyrene-containing liposomes, the excitation wavelength was 342 nm, and the emission wavelength was 360−500 nm. The E/M ratio of pyrene-labeled PA was calculated by measuring fluorescence intensity at 375 nm (for monomer) and 480 nm (for excimer). The emission fluorescence of NBD-PA was measured at 534 nm with an excitation wavelength of 465 nm. To determine the colocalization of fluorescent probes, the excimer fluorescence intensities of pyrene-PA were measured in the presence and absence of BODIPY-PA. To prevent the excimer fluorescence quenching effect of oxygen, the buffer solution was saturated with argon gas for more than 2 h before use. In all experiments measuring membrane properties of lipids, 5 μM phospholipid in 100 mM potassium phosphate (pH 7.4) and 1 mM Na-EDTA was used, and the fluorescence intensities were monitored at 30 °C. In all fluorescence experiments, each measurement under the experimental condition was corrected for inner filter effect due to light scattering and absorption, as described elsewhere (*29*).

Binding Assays. Binding of CYP3A4 to model membranes was measured as described previously (*10*). Various concentrations of liposomes containing 1 mol % of biotinylated PE were incubated with 400 pmol of CYP3A4 for 10 min at 30 °C in 90 μL of reaction volume. Immobilized avidin (10 μL of a 7.5 mg/mL suspension) on 6% agarose beads (Pierce) was then added, and the samples were incubated with shaking. After 10 min, the liposomes were precipitated by microcentrifugation for 5 min at 14000 rpm. The supernatant was immediately assayed to determine protein concentration. The pellets were resuspended by adding 1% sodium dodecyl sulfate solution (w/v), and CYP3A4 was separated from the immobilized avidin liposome complex by centrifugation. The concentration of membrane-bound CYP3A4 was measured using the bicinchoninic acid procedure.

The energy transfer between Trp residue(s) in CYP3A4 and pyrene-PC incorporated into phospholipid bilayers was also used to measure the membrane binding and insertion into the lipid bilayer of CYP3A4. The reaction samples were incubated for 10 min at 30 °C, and then fluorescence of Trp residues at 330 nm (excitation at 280 nm) was monitored as described (*10*).

CD Spectroscopy. CD spectra in the far-ultraviolet and visible regions were monitored at room temperature with a Jasco J715 spectropolarimeter (Japan Spectroscopic, Tokyo); the optical path lengths were 0.1 and 1.0 cm, respectively. Measurements were conducted in 100 mM potassium phosphate (pH 7.4) containing 1 and 10 μM CYP3A4 for the far-ultraviolet and visible regions, respectively. The standard conditions were as follows: bandwidth, 1 nm; response, 1−2 s; step resolution, 0.1−0.2 nm. Blanks (buffer with or without phospholipid) were routinely recorded and subtracted from the original spectra. On average, data from 15 to 20 scans were accumulated.

Estimation of Binding Constants by Spectral Titrations. Because CYP3A4 is predominantly low spin (*21*), the usual binding spectral titrations (*21*) were used to determine dissociation constants (K_s) for testosterone in the presence of indicated lipid vesicles.

NADPH-Reduced, CO-Difference Spectroscopy. Reduced, CO-difference spectra were obtained using two cuvettes containing CYP3A4 (1 μM), NPR (2 μM), indicated lipid vesicles (40 μM), and testosterone (100 μM) in 100 mM potassium phosphate buffer (pH 7.4) at 30 °C. After CO bubbling into the sample cuvette and baseline correction, NADPH was added to the sample cuvette to a final concentration of 200 μM, and the difference spectra were measured after a period of 3 min. Finally, sodium hydrosulfite was added to both cuvettes, and the difference spectrum was again measured between 400 and 500 nm.

RESULTS

Effects of Phospholipids on the Catalytic Activity of CYP3A4. The effect of anionic phospholipids on the catalytic activity of CYP3A4 was examined by measuring CYP3A4-catalyzed reactions in the presence of model membranes composed of a binary mixture of PC/anionic phospholipid or PC/PE. The enzyme activity of CYP3A4 was quantified by a testosterone 6β-hydroxylation assay in the presence of NPR and NADPH (*25*). PA and PS stimulated CYP3A4 activity in a concentration-dependent manner, with PA exerting a greater effect than PS within the range examined here. As the PA and PS concentration increased to 50 mol %, CYP3A4 activity was enhanced by ∼14- and ∼6-fold, respectively, compared to activity in 100 mol % PC (Figure 1A). The enzyme activity increased remarkably when PA or PS was included at more than 20 mol % in the PC matrix, and these steep increases might be associated with stimulation of the binding and/or insertion of CYP3A4 to the membranes. When we replaced PC with PA or PS up to 50 mol %, we could not detect any aggregates of the protein and/or vesicles as assayed by light scattering. However, by further increasing the concentration of PA or PS (>60%), protein-induced aggregation of membranes and a concomitant rapid decrease of the enzyme activity were observed (results not shown).

There was no apparent change of enzymatic activity when PE was included in the PC matrix at levels even up to 50 mol % (Figure 1A). However, when replacing PC content with PE in liposomes with fixed concentrations of 10 or 20 mol % PA (or PS) (that is, ternary systems of PC/PE/PA or PC/PE/PS), CYP3A4 activity increased as a function of PE concentration. The enzyme activity was enhanced by 2.3−3.5-fold when PE was included at 40 mol % compared to the activity level in PC/PA or PC/PS alone (80:20 or 90:10 by molar ratio) (Figure 1B). PE-induced enhancement of enzyme activity in the PA-containing system was greater than that in the PS-containing system. These results suggest that PA and PE might help the binding of CYP3A4 to the membranes and the interaction with NPR in the membranes.

Effect of Phospholipid Composition on the Binding Affinity of CYP3A4 for Testosterone. The effect of vesicle composition on the substrate binding to CYP3A4 was examined. The

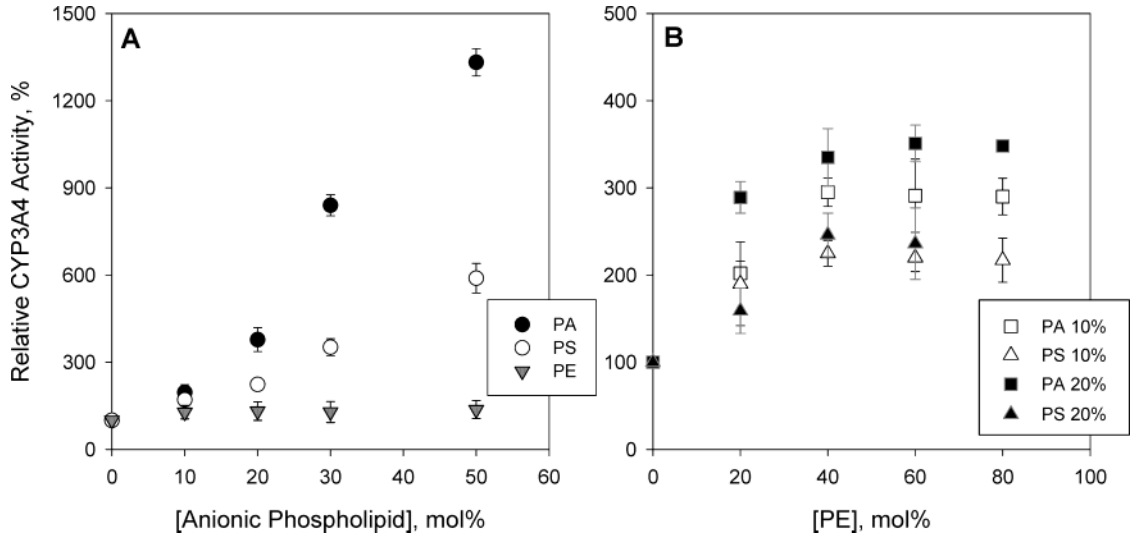

FIGURE 1: Effects of phospholipids on the catalytic activity of CYP3A4. Testosterone 6β-hydroxylation by CYP3A4 was measured in a binary system (PC/anionic phospholipid or PC/PE) with increasing concentrations of anionic phospholipid (A). In a ternary system of PC/PA/PE or PC/PS/PE (B), the concentration of PA or PS was fixed at 10 or 20 mol %, and PE content was increased up to 80 mol % at the expense of PC. The 100% activity value represents the normalized activity [0.57 nmol of product min^{-1} (nmol of CYP)$^{-1}$] for the sample containing 100% PC as a lipid component. The testosterone hydroxylation activity was measured in a reconstituted system consisting of the CYP3A4 (0.2 μM), NPR (0.4 μM), lipid vesicles (40 μM), and testosterone (100 μM) in 100 mM potassium phosphate buffer (pH 7.4). For other details, see Experimental Procedures.

Table 1: Effects of Phospholipid Composition of Vesicles on Binding Affinities of Testosterone for CYP3A4 and NADPH Oxidation by CYP3A4

vesicle composition	K_s (μM)[a]	product formation [nmol of 6β-HT[b] min^{-1} (nmol of P450)$^{-1}$][a]	NADPH oxidation [nmol of product min^{-1} (nmol of P450)$^{-1}$][a]
PC (100 mol %)	38 ± 3	0.57 ± 0.02	51 ± 2
PC/PA (50:50, mol %)	21 ± 2	7.6 ± 0.3	44 ± 1
PC/PS (50:50, mol %)	30 ± 2	3.4 ± 0.3	46 ± 1
PC/PA/PE (60:20:20, mol %)	24 ± 3	1.7 ± 0.1	54 ± 2
PC/PS/PE (60:20:20, mol %)	30 ± 3	0.91 ± 0.15	52 ± 2

[a] All estimates are means ± SD (n = 3). [b] 6β-HT = 6β-hydroxytestosterone.

addition of testosterone for CYP3A4 produced a typical low- to high-spin conversion (type I difference spectrum). A titration experiment of this type yielded dissociation constants (K_s). Various phospholipids in the PC matrix did not yield apparent changes in the binding affinity for substrate (Table 1), with K_s for all types of phospholipids vesicles ranging between 21 and 38 μM. No direct correlation between activity and binding affinities to substrate was found.

NADPH Oxidation and Coupling of CYP3A4 Reaction. To see the importance of acidic phospholipids for the interaction of NPR with CYP3A4, we examined the effect of phospholipid composition in the membranes on the NADPH oxidation catalyzed by CYP3A4 and the formation of the Fe^{2+}−substrate CO complex. When the rate of NADPH oxidation was determined in the presence of various phospholipid vesicles, there were no apparent changes in the oxidation rate compared to the 100% PC vesicles (Table 1). No correlation was found between rates of NADPH oxidation and substrate oxidation in the presence of various phospholipid vesicles. This result indicates that, in the vesicles containing PA or PS, an efficient transfer of electrons from NPR to CYP3A4 may explain the increased catalytic activities.

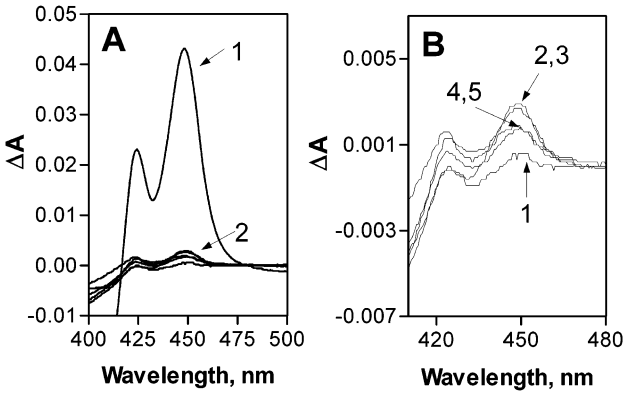

FIGURE 2: Effect of phospholipids on the CO-difference spectra of CYP3A4 reduced by NADPH and NPR. (A) CO-difference spectra of CYP3A4 reduced with sodium hydrosulfite (1) and NADPH (2) in the presence of various phospholipid vesicles. Spectrum 1 was measured in the presence of 100% PC as a lipid component. Other spectra of CYP3A4 in the presence of various phospholipid vesicles reduced by sodium hydrosulfite were very similar to spectrum 1. (B) Enlarged CO-difference spectra of CYP3A4 reduced with NADPH (indicated as 2 shown in part A): 1, 100% PC; 2, PC/PA (50:50, mol %); 3, PC/PS (50:50, mol %); 4, PC/PA/PE (60:20:20, mol %); 5, PC/PS/PE (60:20:20, mol %). CO-difference spectra of CYP3A4 were obtained using two cuvettes containing CYP3A4 (1 μM), NPR (2 μM), indicated lipid vesicles (40 μM), and testosterone (100 μM) in 100 mM potassium phosphate buffer (pH 7.4) at 30 °C.

NADPH-Dependent Heme Reduction of CYP3A4. Figure 2 shows the reduced CO-difference spectra of CYP3A4 in the presence of various phospholipid vesicles reduced by NADPH or sodium hydrosulfite. Only 1.4% of CYP3A4 was reduced by NADPH in the presence of 100% PC. When the PA or PS concentration increased to 50 mol %, 6.5−7.0% of the CYP3A4 was reduced. In the vesicles of PC/PE/PA (or PS) (60:20:20, mol %), 4.1−4.2% of the CYP3A4 was reduced. Although no direct correlation was found between catalytic activity and the extent of reduction, these results suggest that the acidic phospholipids might help the interac-

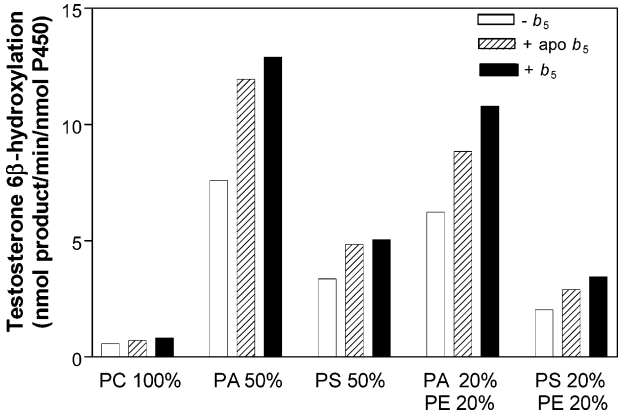

FIGURE 3: Effects of b_5 and apo b_5 on the catalytic activity of CYP3A4 in the presence of various phospholipid vesicles. The testosterone hydroxylation activity was measured in a reconstituted system consisting of the CYP3A4 (0.2 μM), NPR (0.4 μM), indicated lipid vesicles (40 μM), and testosterone (100 μM) in 100 mM potassium phosphate buffer (pH 7.4). For other details, see Experimental Procedures. All values are the means of duplicate experiments. Absolute values varied <5%.

tion of CYP3A4 with NPR in the membranes for the efficient transfer of electrons from NPR to CYP3A4.

Effects of b_5 and Apo b_5 on the Catalytic Activity of CYP3A4 in the Presence of Various Phospholipid Vesicles. It is well-known that the function of CYP3A4 is dependent on the presence of b_5 (*25*). Rates of testosterone 6β-hydroxylation by CYP3A4 increased in the presence of membranes containing acidic phospholipids (Figure 1). The effects of b_5 and apo b_5 on the catalytic activity of CYP3A4 in the presence of various phospholipid vesicles were studied (Figure 3). In the presence of b_5 or apo b_5, apparent optimal activities were observed after the addition of a 2:1 final molar ratio of NPR to CYP3A4 (results not shown). Stimulatory effects of b_5 and apo b_5 also were observed upon addition of b_5 as well as apo b_5. Catalytic activities of CYP3A4 were increased by 25−60% and 45−70% by the addition of apo b_5 and b_5, respectively (Figure 3).

Formation of PA-Enriched Domains Induced by PE. From the results described above, we expected that the increase of CYP3A4 activity by PE in PC/PE/PA or PC/PE/PS membranes is caused by PE-induced changes in membrane properties in these ternary systems. Recently, it has been shown that PE induces the formation of PS-enriched domains in PC/PE/PS membranes (*18*). To examine the effect of PE on the membrane properties of PC/PE/PA membranes, we incorporated pyrene-PA into this system. Excited-state pyrene molecules give characteristic emission fluorescence spectra for the monomer (M) and excimer (E). The E/M ratio is strictly dependent on the rate of collision of the pyrene molecules. Consequently, in lipid bilayers, measurements of pyrene-labeled lipids reflect the lateral diffusion rate of the probes (*30*) or their local concentration (*31*) or both. To determine the effect of PE on the lipid dynamics of PA, 1 mol % of pyrene-PA was incorporated into liposomes with a fixed concentration of PA (PC/PA = 90:10 or 80:20), and the E/M ratio was examined in membranes containing various concentrations of PE. The E/M ratio gradually increased as a function of PE concentration. When the concentration of PE was increased to 80 mol %, the E/M ratio was enhanced by ~56% compared to the value for PC/PA liposomes without PE (Figure 4A).

Self-quenching of the fluorescence of NBD-labeled phospholipids, which provides information on phospholipid clustering in lipid bilayers (*32*), was used to corroborate the possibility that PE molecules induce the lateral segregation of pyrene-PA. In these experiments, the quenching efficiency was enhanced as PE concentration increased (Figure 4B). At 80 mol % PE, the fluorescence of NBD-PA decreased by ~25% as compared to the value without PE. This result indicates that PE molecules induce the lateral segregation of PA in membranes.

Resonance energy transfer between pyrene-PA and BODIPY-PA was used to ascertain that domain formation of PA in PC/PE/PA mixtures is responsible for the increase in the E/M ratio of pyrene-PA and the fluorescence quenching

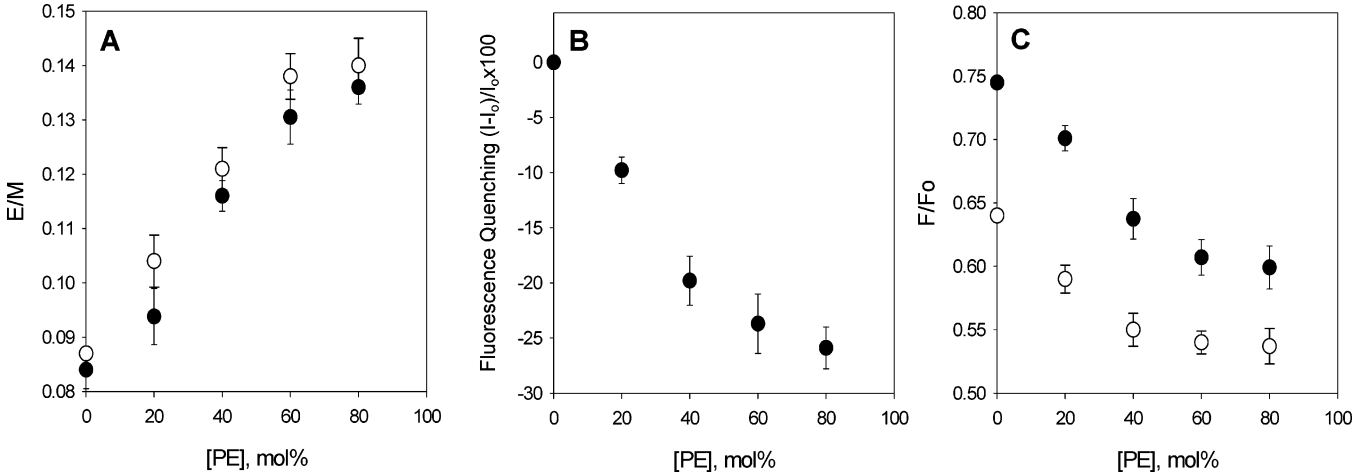

FIGURE 4: PE-dependent formation of PA-enriched domains. (A) Effect of PE concentration in membranes on the E/M ratio of pyrene-PA. The monomer (M) and excimer (E) fluorescence of pyrene-PA (1 mol %) incorporated into liposomes (PC:PA = 90:10 or 80:20) was examined by replacing PC with PE. (B) Quenching of the NBD-PA fluorescence by PE. The initial composition of vesicles (PC:PA:NBD-PA = 80:10:10) was changed by gradually replacing PC with the indicated amount of PE. I and I_0 are the fluorescence intensities at 534 nm in the absence (I_0) or in the presence (I) of PE. (C) Quenching of the excimer fluorescence of pyrene-PA by BODIPY-PA with increasing concentrations of PE. F/F_0 is the ratio of the fluorescence intensities at 480 nm for LUVs containing only 1 mol % pyrene-PA (F_0) or 1 mol % pyrene-PA and 1 mol % BODIPY-PA (F). Data points represent the mean ± SE of three independent experiments. Open and closed circles represent the liposomes containing 20 and 10 mol % of PA in the PC matrix, respectively.

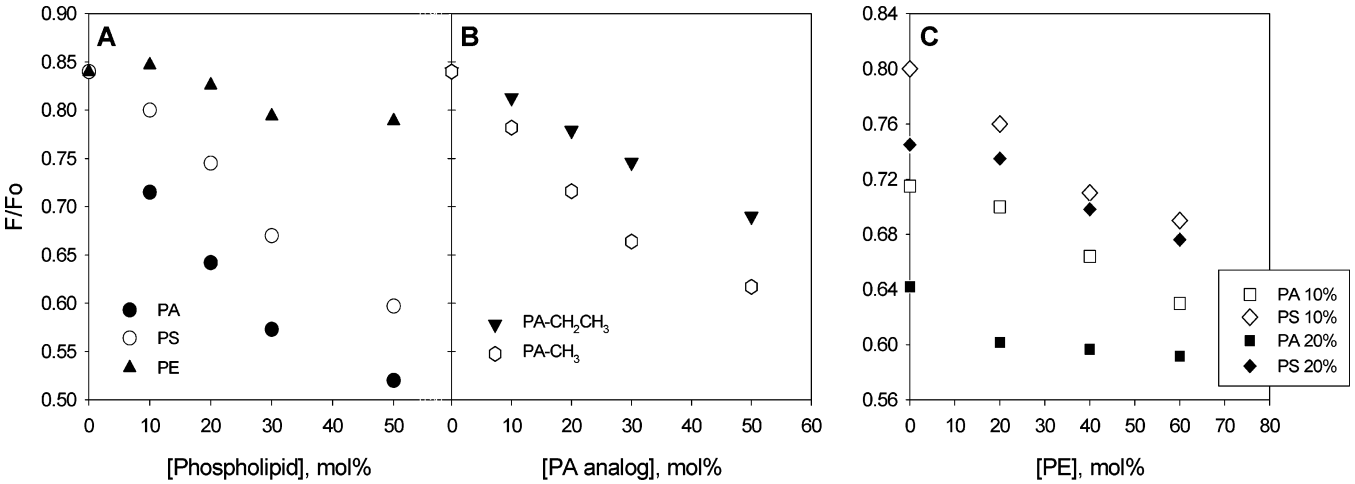

FIGURE 5: Phospholipid-dependent membrane insertion of CYP3A4. The energy transfer between Trp residues in CYP3A4 and pyrene-PC incorporated into membranes was examined in a binary system (PC/anionic phospholipid or PC/PE) by replacing PC with PA, PS, or PE (A) and with PA analogues (B) up to 50 mol %. The energy transfer was also measured in a ternary system (PC/PE/PA or PC/PE/PS) with a fixed concentration of anionic phospholipid (10 or 20 mol %) by increasing the content of PE at the expense of PC (C). F/F_0 represents the fluorescence intensity ratio at 340 nm for the sample with (F) and without (F_0) pyrene-PC incorporated in the membrane. After preparation of LUVs with or without the indicated amount of phospholipids, CYP3A4 (0.5 μM) was mixed with vesicles (100 μM) in 100 mM potassium phosphate (pH 7.4). After incubation of the sample for 10 min at 30 °C, Trp fluorescence was measured at 341 nm (excitation 295 nm).

of NBD-PA. As the dipyrromethene boron difluoride group of BODIPY shows a maximum absorption spectrum around 500 nm (*33*), it was possible to use the energy transfer between two fluorophores to estimate their colocalization as demonstrated previously (*18*, *34*, *35*). We determined the quenching efficiency (F/F_0) as a function of PE concentration, where F and F_0 are the intensities of excimer emission of pyrene-PA measured in the presence (F) and absence (F_0) of the quencher, BODIPY-PA. When the concentration of PE increased, the quenching was gradually enhanced (Figure 4C), again supporting PE-induced clustering of PA molecules.

Phospholipid-Dependent Membrane Insertion of CYP3A4. To determine whether CYP3A4 can be inserted into lipid bilayers by adding phospholipid vesicles exogenously, we measured resonance energy transfer between CYP3A4 Trp residue(s) and pyrene-PC that had been incorporated into membranes in which PC was partially replaced with anionic phospholipids such as PA and PS. In pyrene-PC, the pyrene group is located at the end of the decanoyl chain at the *sn*-2 position, and CYP3A4 has four Trp residues that are spaced throughout its sequence (*36*).

Among the phospholipids tested, PA had the most significant effect on the penetration of CYP3A4, with the lowest F/F_0 value occurring when the concentration of PA was increased at the expense of PC (Figure 5A). PS also decreased F/F_0 but was less efficient than PA. PE had no apparent effect on the penetration of CYP3A4 into membranes. To ascertain the effect of the PA headgroup on the stimulation of membrane insertion, we also measured the quenching of Trp fluorescence with the PA analogues phosphatidylmethanol and phosphatidylethanol. As expected, these analogues caused less effect on quenching than normal PA (Figure 5B). The same experiment was repeated with increasing concentrations of PE at the expense of PC in ternary systems of PC/PE/PA or PC/PE/PS with a fixed concentration of anionic phospholipid (Figure 5C). PE, which had no effect in the binary system, decreased the F/F_0 value in ternary systems containing PA or PS in a concentration-dependent manner.

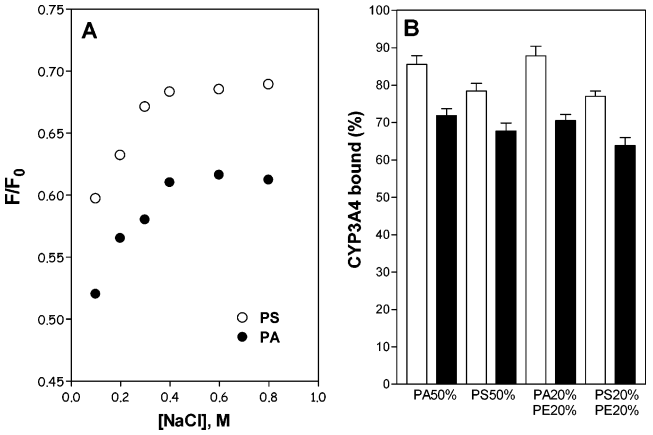

FIGURE 6: Effect of NaCl on the CYP3A4 binding to lipid bilayers.The energy transfer between Trp in CYP3A4 and pyrene-PC was measured with the same method used for Figure 3 at 50 mol % of indicated phospholipids as a function of NaCl concentration (A). The amount of membrane-bound P450 3A4 was determined at a L/P ratio of 400 after salt extraction with 1 M NaCl (B). CYP3A4 was incubated with indicated phospholipid compositions for 10 min at 30 °C, and then 1 M NaCl (final concentration) was added to the mixture. The samples were pelleted by centrifugation, and the amount of membrane-bound CYP3A4 was measured. All other conditions and procedures are the same as described for Figure 7.

This result indicates that CYP3A4 has the ability to penetrate preformed membrane bilayers depending on the type of phospholipids present in the membrane. More importantly, from these investigations, we could deduce that PE-induced membrane properties of anionic phospholipid-containing vesicles stimulate the insertion of CYP3A4 into membranes.

The energy transfer experiment was repeated with increasing concentrations of NaCl in binary systems at 50 mol % of acidic phospholipids. As a result, F/F_0 values were increased with increasing salt concentration and reached a plateau around 0.4 M NaCl, but it did not approach the value for 100% PC (Figure 6A). The result indicates that charge−charge interaction as well as other binding forces such as hydrophobic interaction between CYP3A4 and phospholipid

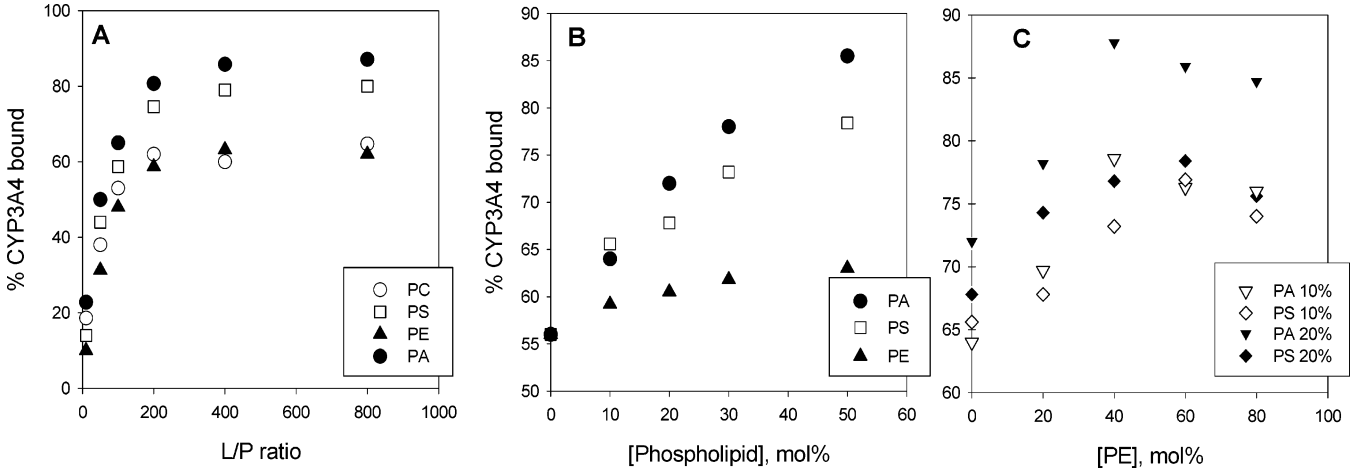

FIGURE 7: Phospholipid-dependent binding of CYP3A4 to membranes. Binding of CYP3A4 to liposomes was measured by precipitation of liposomes as described under Experimental Procedures in a binary system (PC/anionic phospholipid or PC/PE) with increasing amounts of model membranes (A) and with increasing concentrations of anionic phospholipid or PE (B). The binding experiments in ternary systems (PC/PE/PA or PC/PE/PS) with a fixed concentration of anionic phospholipid (10 or 20 mol %) were done by increasing the content of PE at the expense of PC (C). The L/P ratio in the experiments of (B) and (C) was 400.

molecules participated in the protein binding to membranes. We obtained a similar result when lower concentrations of anionic phospholipids were used instead of 50% (results not shown), which is a different observation when compared with the case of CYP1A2 (*10*). This suggests that CYP3A4 has a membrane topology more resistant to be detached from lipid bilayer than CYP1A2.

To discriminate membrane binding of CYP3A4 from insertion into bilayers, we measured the membrane-bound amount of CYP3A4 after salt extraction with 1 M NaCl. The binding was decreased for all lipid compositions, but the decrease was not so much for both binary and ternary systems (10−20% decrease of the bound protein before treatment of 1 M NaCl) (Figure 6B). The result also coincides well with the result obtained from energy transfer between Trp residue(s) and pyrene (Figure 6A). From these results, we could anticipate that membrane-inserted CYP3A4 is resistant against the salt extraction and remains in membranes by a strong association with phospholipid molecules.

To correlate the membrane insertion and the stimulation of enzyme activity of CYP3A4, the catalytic activity was measured with the salt-washed proteoliposomes. As a result, most of the activity (93−98%) in all tested phospholipid vesicles remained in the salt-washed proteoliposomes (results not shown). This result provides a direct correlation between membrane association of CYP molecules and their activity.

Effects of Phospholipids on the Binding of CYP3A4 to Membranes. To determine whether the membrane binding of CYP3A4 also depends on the phospholipid composition of the membrane, we analyzed the amounts of CYP3A4 bound to membranes with increasing concentrations of liposomes composed of PC/anionic phospholipid or PC/PE. Vesicles containing 50 mol % PA or PS increased the binding of CYP3A4 to membranes by ∼20−25%, with the efficiency in the order PA > PS, as compared to binding to vesicles of 100% PC (about 60% binding at an L/P ratio of 400) (Figure 7A). However, PE-containing liposomes had no effect on CYP3A4 binding. To obtain more information on phospholipid-dependent membrane binding of CYP3A4, further experiments were undertaken to study this effect as a function of anionic phospholipid concentration at an L/P ratio of 400 (Figure 7B). In these experiments, PA again exerted the greatest effect on membrane binding of CYP3A4, with PS exerting a substantial but lower effect. To examine the role of PE-induced membrane properties in the binding of CYP3A4 to membranes, we repeated the same experiment in ternary systems of PC/PE/PA or PC/PE/PS vesicles with a fixed concentration of PA or PS. As a result, again, increasing concentrations of PE exerted further stimulation of CYP3A4 binding to PA- or PS-containing membranes in the order PA > PS (Figure 7C). From these results, we could anticipate that the increase of CYP3A4 activity might be ascribed to the increase in membrane binding of the enzyme.

The effect of acidic phospholipids on the membrane binding of CYP3A4 was also investigated by measuring energy transfer between Trp residues in CYP3A4 and pyrene incorporated into membranes attached to the ethanolamine group of PE in PyrS DHPE. Figure 8A shows that, in a binary system, the anionic phospholipid PA or PS decreased the F/F_0 value in the order PA > PS. The decrease of F/F_0 value indicates an increase of CYP3A4 binding to the membrane, supporting the importance of anionic phospholipids and/or anionic phospholipid-induced membrane properties in the energy transfer. However, in the presence of PE instead of anionic phospholipids, the F/F_0 ratio was not apparently changed compared to the F/F_0 ratio for 100% PC (results not shown).

To examine the role of PE-induced membrane properties in the energy transfer between Trp residues in CYP3A4 and pyrene in PyrS DHPE membranes, we repeated the same experiment in a ternary system of PC/PE/PA or PC/PE/PS vesicles with a fixed concentration (10% or 20%) of PA or PS, replacing PC with various concentrations of PE. In these experiments, incorporation of PE further stimulated energy transfer in vesicles containing PA and PS (Figure 8B). The results obtained with the ternary system also coincided well with the results obtained from precipitation experiments (Figure 7). Taken together, these results demonstrate that both of the anionic phospholipids tested in our experiments (PA and PS) increased CYP3A4 binding to the lipid bilayer, but the extent of this effect was strictly dependent on the type of anionic phospholipids present.

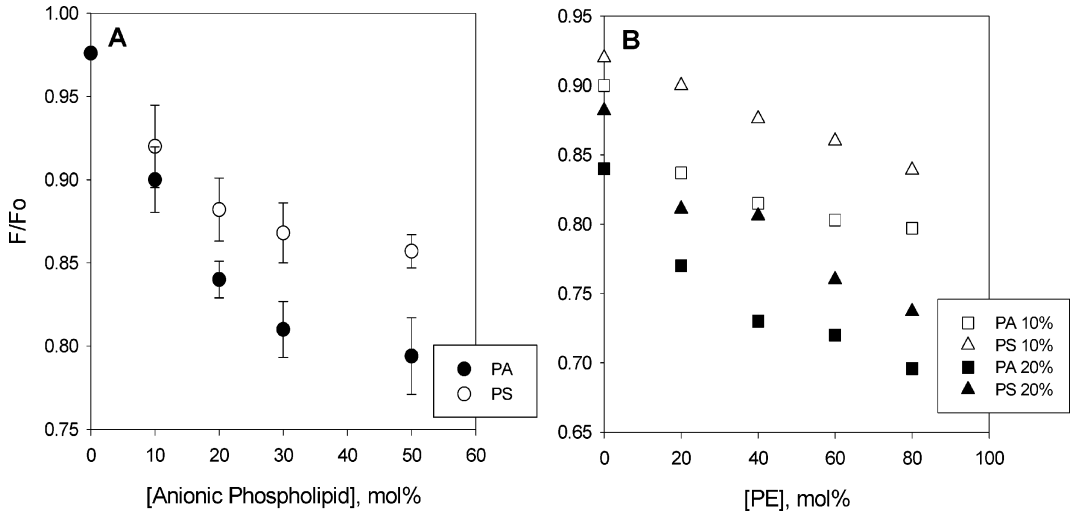

FIGURE 8: Energy transfer between Trp residues in CYP3A4 and PyrS DHPE incorporated into lipid bilayers. The effect of anionic phospholipids on the membrane binding of CYP3A4 was examined by replacing PC with the indicated phospholipid in a binary system (A). The effect of PE in a ternary system was examined by replacing PC with the indicated PE concentration with a fixed concentration of anionic phospholipid (10 or 20 mol %) (B). All other conditions and procedures for the measurement of Trp fluorescence of CYP3A4 are the same as described for Figure 5.

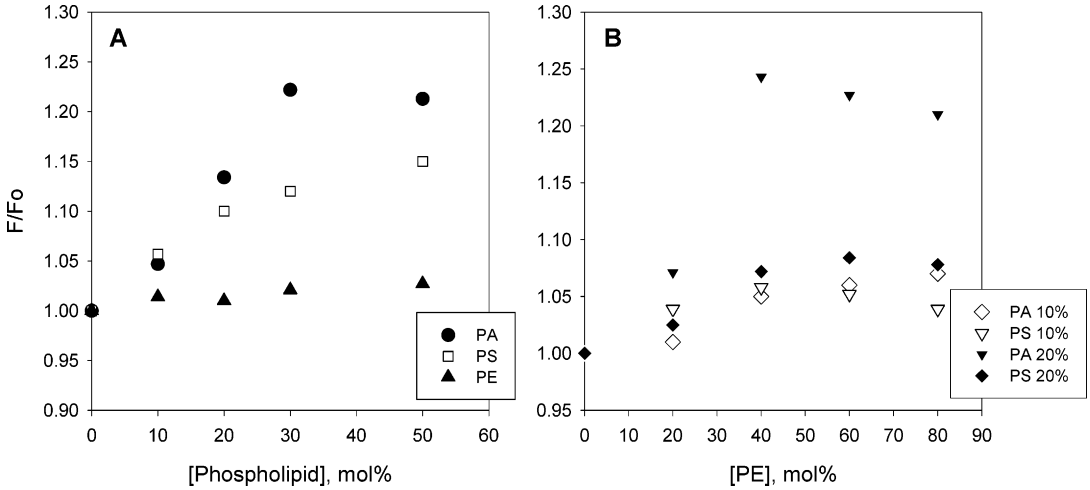

FIGURE 9: Effect of phospholipids on the Trp fluorescence spectra of CYP3A4. Spectra of 1.0 μM CYP3A4 in 100 mM potassium phosphate (pH 7.4) were recorded in the presence of the indicated phospholipid vesicles in a binary system (A) or ternary system (B) as in previous experiments.

Conformational Change upon Membrane Binding and Insertion into the Lipid Bilayer of CYP3A4. The effect of phospholipids on CYP3A4 conformation upon membrane binding and insertion into the membrane was studied by Trp fluorescence and CD spectroscopy. The fluorescence intensity generally increased with increasing concentrations of PA and PS in the PC matrix, but there were no appreciable changes in λ_{max} (Figure 9A). Although PE in the PC matrix exerted no apparent effect on intensity, PA showed the greatest effect in binary systems. Apparent PE-induced lipid domains of PA or PS (and/or PE-induced change of membrane properties) were observed in ternary systems (Figure 9B). This result indicates the possibility that model membranes and/or environmental changes such as a change in polarity near Trp residues of the protein may lead to a conformational change in CYP3A4.

In contrast to the results above, we could not detect any apparent difference in the secondary structure of CYP3A4 bound to membranes regardless of lipid composition when assayed by CD spectroscopy in the far-ultraviolet region (Figure 10A,B). The α-helical content estimated from mean residue ellipticity ($[\theta]_R$) at 222 nm was 49−53% among CYP3A4 samples with or without liposomes. However, CD spectra of the CYP3A4 in the visible region differed from one another depending on the type of phospholipids. Although CYP3A4 showed a negative Cotton effect in the Soret region regardless of the type of phospholipids, the intensity values at 420 nm for membranes containing PA and PS were greater than that for the PC membrane (Figure 10C,D). These results suggest that there are differences in the electronic structure of the heme and its vicinity. Since the CD of P450 in the visible region (negative Cotton effect at 420 nm) reflects the proximity of heme to neighboring aromatic residues (*37*), these results imply that the heme environment of CYP3A4 has been changed upon interacting with different types of phospholipids.

DISCUSSION

The present investigations established that the catalytic activity of CYP3A4 was increased by anionic phospholipids such as PA and PS in a binary system upon interaction with model membranes. Anionic phospholipids also increased the

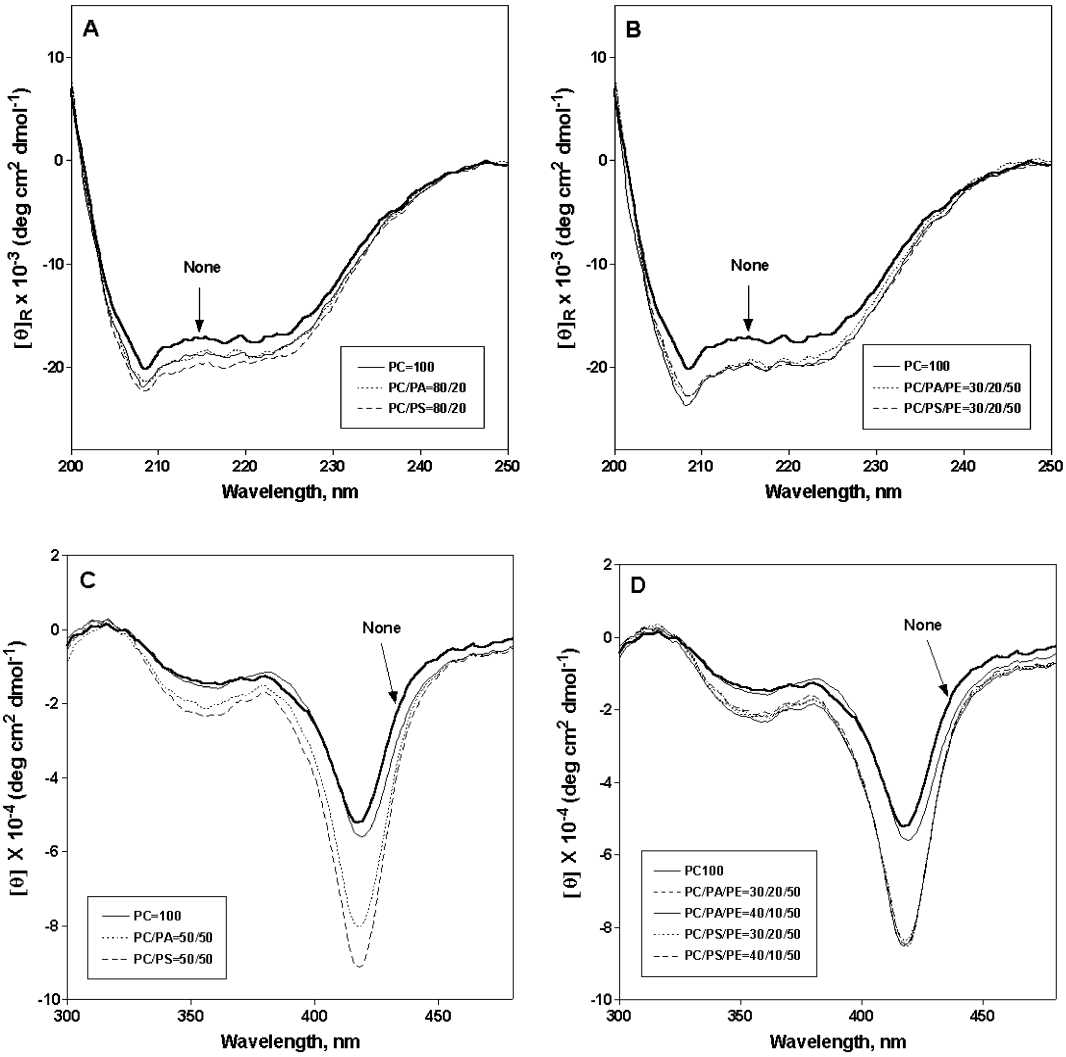

FIGURE 10: Effect of phospholipids on the CD spectra of CYP3A4. CD spectra in the far-ultraviolet (A and B) and visible (C and D) regions of 1 and 10 μM CYP3A4, respectively, in 100 mM potassium phosphate (pH 7.4) were recorded in the presence of the indicated phospholipid vesicles. Mean residue ellipticity, $[\theta]_R$, is expressed on the basis of the number of amino acids per molecule of CYP3A4. Molar ellipticity, $[\theta]$, is expressed on the basis of their molar concentration.

membrane binding and/or insertion of CYP3A4. In correlation with enzyme activity, we suggest that the increase of membrane binding/insertion of CYP3A4 to membranes is mainly responsible to acidic phospholipid-induced increase of the catalytic activity. It was deduced that the enhanced binding and concomitant stimulation of enzyme activity might be ascribed to the propensity of anionic phospholipids to form lipid domains with themselves, as suggested previously (*10*). This notion was strongly supported by the observation that PE induced PA- (or PS-) enriched domains in a ternary system of PC/PE/PA or PC/PE/PS, and this PA (or PS) clustering further stimulated the membrane binding and catalytic activity of CYP3A4. From these results, therefore, we suggest that the clustering of anionic phospholipids (and/or their tendency to form lipid domains) is important in the interaction of CYP3A4 with model membranes. It should also be emphasized that the procedure described here may serve as an adequate model for the study of problems involving the in vivo incorporation of proteins into biological membranes. This is because the role of individual lipids in the membrane insertion of proteins has not been fully understood.

We demonstrated that acidic phospholipids do not show homogeneous mixing properties with PC in a binary system and the phase separation is induced as increasing the concentration of the acidic phospholipids (*17*). Relating with our present results, we suggest that PE altered properties of acidic phospholipids to have more increased nonideal mixing propensity and the phase separation might be correlated with the stimulation of CYP3A4 activity (Figures 1B and 4). Using other techniques in addition to extrinsic fluorescent probes, it should be possible to further demonstrate that PA and PS might form self-enriched lipid domains in a concentration-dependent manner within a PC matrix and that PE molecules induce the formation of lipid domains enriched with anionic phospholipids.

PE-dependent further enhancement of the activity strongly supports the idea: In the fixed concentration of PA or PS (10%or 20%), the activity was stimulated by 2−3.5-fold as increasing concentration of PE content in membranes up to 80%. However, PE did not exert any change of CYP3A4 activity per se in the absence of acidic phospholipid. On the basis of the binding experiment, further increase of CYP activity by PE (Figure 1B) is ascribed to further augment

membrane binding of the enzyme (Figure 7C).

It seems possible that PA and PE might help the binding of CYP3A4 to membranes and the interaction with NPR in the membranes. As described, PA- and PE-induced property change of acidic phospholipids in membranes stimulated the interaction of CYP with membranes. Putting it all together, we were able to postulate the following hypothesis for the relation between membrane topology of CYP3A4 and enzyme activity: The membrane-inserted form of CYP3A4 is mainly responsible for the catalytic activity, and the PA molecule itself and/or the lipid domain enriched with PA promote(s) proper positioning of the protein in lipid bilayers and consequently its function when compared with other acidic phospholipids.

We analyzed the formation of the lipid domain by measuring fluorescence change of extrinsic membrane probes such as pyrene- and NBD-labeled phospholipid and energy transfer between pyrene and BODIPY fluorophore. But, unfortunately, we could not quantify the content of the lipid domain in the phospholipids vesicles because the fluorescence changes of extrinsic probes do not necessarily mean a linear correlation with degrees of domain formation. Furthermore, as we suggested (*17*), it seems that a certain critical concentration of acidic phospholipid is needed to represent phase separation of lipid (or clustering of lipid) in membranes. Therefore, we cannot provide a satisfactory relationship between degrees of domain formation and enzyme activity. Nonetheless, we were able to deduce that the propensity forming lipid domain stimulated the enzyme activity when considering that, at a fixed concentration of acidic phospholipids, the replacement of PC with PE (both phospholipids are not important for the activity per se) induced the increase of enzyme activity.

It has been reported that the composition of phospholipids in liver microsomes is dependent on physiological conditions (*38*, *39*). Interestingly, microsomes from rat and mouse hepatoma cells contain substantially different phospholipid composition and significantly greater amounts of PA and PS compared to normal liver microsomes (*38*). Sex differences also exist in the phospholipid composition of rat liver microsomes (*40*). Such differences in phospholipids have been suggested to be related to similar differences in catalytic activity. It is also known that dietary eritadenine, a hypocholesterolemic factor, markedly decreases the ratio of PC to PE in liver microsomes (*41*).

Proteinaceous components (e.g., SRP and SRP receptor) are well-known to be involved in protein insertion into the ER membrane (*42*). The role of individual lipids in membrane insertion of proteins has not yet been investigated, although lipids have been found to contact a nascent membrane protein, as well as a nascent secretory protein, during their insertion into the ER membrane (*43*). Our results suggest that the membrane binding and insertion process can be regulated by lipids in addition to the proteinaceous components of the translocation apparatus. The lipid-dependent insertion of proteins into membranes might be related to the structural change of proteins induced by phospholipids, as we have previously observed in the case of CYP1A2 (*8*, *10*).

At present, there is no direct information on the membrane topology of CYP3A4 because no crystal structure is yet available for the membrane-bound form, although only the N-terminal region of microsomal CYP has been proposed to penetrate the membrane (*13*). It is also not possible to determine which among the four Trp residue(s) in CYP3A4 is being inserted in this study without extensive replacement experiments. Although the exact Trp residues involved in the fluorescence studies have not been identified, our results show that the membrane binding and insertion of CYP3A4 are quite dependent upon the type of phospholipids and membrane properties.

It is known that the CD spectra in the visible region of a hemoprotein are attributable to the interaction between the heme and aromatic residue(s) in the heme pocket (*37*). Therefore, CD spectra of CYP3A4 in the visible region may reflect the steric relationship between the heme and aromatic residues in the heme pocket. The observed differences in the intensity of CD spectra (Figure 10), dependent upon the type of phospholipids, may be correlated with the differences in the interaction between the heme and nearby aromatic residue(s) of CYP3A4. It was not possible, however, to identify the aromatic residue(s) involved in the interaction because no information is available on the tertiary structure at present.

Although we could observe differences in the intrinsic fluorescence and CD spectra in the visible region of CYP3A4 upon interaction with membranes containing different types of phospholipids, CD spectra in the far-ultraviolet region of CYP3A4 were essentially unchanged (Figure 10). These results suggest the possibility that the secondary structure is not changed upon interaction with phospholipids, although the overall conformation of CYP3A4 changes depending upon the type of phospholipids, especially anionic phospholipids, in its vicinity.

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