Archives of Biochemistry and Biophysics 493 (2010) 143–150

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Functional and conformational modulation of human cytochrome P450 1B1 by anionic phospholipids

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ARTICLE INFO

Article history:
Received 25 August 2009
and in revised form 20 October 2009
Available online 24 October 2009

Keywords:
CYP1B1
Anionic phospholipids
Catalytic activity
Conformation change

ABSTRACT

We investigated the interaction of human P450 1B1 (CYP1B1) with various phospholipid bilayers using the N-terminally deleted (Δ2–4)CYP1B1 and (Δ2–26)CYP1B1 enzymes. Among anionic phospholipids, phosphatidic acid (PA) and cardiolipin specifically increased the catalytic activities, membrane binding affinities, and thermal stabilities of both CYP1B1 proteins when phosphatidylcholine matrix was gradually replaced with these anionic phospholipids. PA- or cardiolipin-dependent changes of CYP1B1 conformation were revealed by altered Trp fluorescence and CD spectra. However, both PA and cardiolipin exerted more significant effects with the (Δ2–4)CYP1B1 than the (Δ2–26)CYP1B1 implying the functional importance of N-terminal region for the interaction with the phospholipid membranes. In contrast, other anionic phospholipids such as phosphatidylserine and the neutral phospholipid phosphatidylethanolamine had no apparent effects on the catalytic activity or conformation of CYP1B1. These data suggest that the chemical and physical properties of membranes influenced by PA or cardiolipin composition are critical for the functional roles of CYP1B1.

Introduction

Cytochrome P450 (CYP or P450)[1] enzymes catalyze various types of reactions including the biotransformation of several endogenous substrates such as fatty acids, steroids, and cholesterol, as well as xenobiotics, environmental carcinogens, and mutagens [1–3]. This enzyme system includes cytochrome P450 (EC 1.14.14.1), NADPH-P450 reductase (CPR; EC 1.6.2.4), and phospholipids. P450-dependent activities can be reconstituted in vitro by mixing P450, CPR, and phospholipids [4,5]. All microsomal P450s are present in membranes, and at least some P450 enzymes are believed to interact with the specific lipid components of membranes.

Human CYP1B1 is an important isoform, capable of activating and metabolizing a wide variety of chemically diverse environmental carcinogens and mutagens [6]. Although CYP1B1 is expressed in normal tissues [7,8], it is expressed at much higher levels in tumor cells compared with the surrounding normal tissue [9,10]. The overexpression of CYP1B1 has been implicated in premalignant progression [11], and given its differential expression in tumor tissue it may be a promising drug and vaccine target for the treatment of several types of cancer [12].

CYP1B1 expression is clinically relevant in neoplastic progression, tumor metabolism, and cancer treatment. Although CYP1B1 expression has been observed in multiple cancers examined to date, particularly high expression of CYP1B1 was shown in many hormone-mediated cancers [13,14]. CYP1B1 is also implicated in the etiology of hormone-mediated tumors, as it is responsible for hormone metabolism and the formation of toxic metabolites from both endogenous and exogenous molecules [15–17]. Thus, CYP1B1 induction is an important factor for determining risk associated with hormone-mediated cancers.

Phospholipids in the immediate vicinity of P450 in microsomes have been reported to be more highly organized than those in bulk membranes [18], suggesting that P450 may have important interactions with specific phospholipid molecules surrounding the enzymes. It has also been proposed that the interaction of phospholipids with P450 might be necessary for maintaining the active conformation of the protein and the ability of P450 to interact with CPR for efficient electron transfer [19]. We have suggested previously in a review paper that particular phospholipids and lipid-induced membrane properties play an important role in the

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[1] *Abbreviations used:* CYP or P450, cytochrome P450; CPR, NADPH-P450 reductase; PA, phosphatidic acid; CL, cardiolipin; DAG, diacylglycerol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*glycero-3-phos phoethanolamine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate; pyrene-PC, 1-palmitoyl-2-(1-pyrene decanoyl)-*sn*-glycero-3-phosphocholine; LUV, large unilamellar vesicle; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; 2-OHE_2, 2-hydroxy estradiol; 4-OHE_2, 4-hydroxyestradiol; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; ER, endoplasmic reticulum.

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doi:10.1016/j.abb.2009.10.012

functional regulation of various P450 enzymes upon interaction with the membrane [20]. In the present study, we suggest a possible role for anionic phospholipids in binary systems affecting the catalytic activity, membrane binding, and conformation of the human CYP1B1 enzyme.

Materials and methods

Materials

7-Ethoxycoumarinand17β-estradiolwerepurchasedfromSigma–Aldrich (Milwaukee, WI, USA). Pyrene-labeled phospholipids were purchased from Molecular Probes (Eugene, OR). Most of the phospholipids(POPC,POPE,POPS,POPA,Cardiolipin)werefromAvantiPolarLipids (Alabaster, AL) and used without further purification. Other chemicals were of the highest grade commercially available.

Construction of the CYP1B1 expression plasmid

The recombinant human (Δ2–26)CYP1B1 plasmid was kindly provided by Prof. Fritz F. Parl (Department of Biochemistry and Pathology, Vanderbilt University Medical Center, Nashville, TN). The cDNA for human (Δ2–4)CYP1B1 (pOTB7/CYP1B1) cloning was obtained from the Center for Functional Analysis of Human Genome, KRIBB (Daejeon, Korea) and subcloned into the bacterial expression vector pCW *Ori*. The cDNA sequence was modified to include an NdeI site (5′-gggggcatatgctttctccaaatgatccatggccgctaaacccgctg) and an XbaI $(His)_5$ site (5′-gggggtctagattaatggtgatgg tgatggtgttggcaagtttcctt) at the 5′ and 3′ ends, respectively. The polymerase chain reactions were performed with Pfu DNA polymerase as described by the manufacturer (Solgent, Daejeon, Korea) using an MJ Research PTC-200 Thermal Cycler (Reno, NV). The 1632 bp PCR product was resolved on a 1% (w/v) agarose gel, purified, digested with NdeI and XbaI, and ligated into the pCW *Ori* plasmid that had been digested with the same endonucleases.

Expression of (Δ2–4) and (Δ2–26)CYP1B1

Recombinant human (Δ2–4) and (Δ2–26)CYP1B1 containing His-tags were expressed in *Escherichia coli* as described (Fig. 1A) [21,22]. Strain DH5aF'IQ yielded the highest expression levels. Transformed (Δ2–4)CYP1B1 cells were grown in terrific broth medium containing 100 μg of ampicillin/ml at 37 °C for 12 h. Then, the cells were grown at 37 °C in the same medium until the A_{600} nm was between 0.6 and 0.9. The bacterial suspension was diluted with 100 vol of TB medium containing 1.5 mM 5-(δ)-aminolevulinic acid and 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, and the cells were incubated at 28 °C for 27 h while shaking at 150 rpm. Transformed (Δ2–26)CYP1B1 cells were grown for 12 h at 37 °C in 40 ml of modified TB medium containing 100 μg of ampicillin/ml, 25 μg of kanamycin/ml, 1 mM thiamine, and 10 mM glucose. The cells were then grown at 33 °C in the same medium until the A_{600} nm was between 0.6 and 0.9. Mild induction with 8 mM lactose yielded optimal enzyme production if 1.5 mM 5-(δ)-aminolevulinic acid was added and cells were grown at 23 °C for 40 h while shaking at 150 rpm. After expression, cells were harvested by centrifugation at 5000*g* for 20 min, and the P450 content in the bacterial cell lysate was determined by Fe^{2+}–CO versus Fe^{2+} difference spectra [23]. Bacterial membranes were prepared as described elsewhere [24].

Purification of (Δ2–4) and (Δ2–26)CYP1B1

All steps were done at 4 °C. Purification of both CYP1B1 enzymes was by modification of the method of Hanna et al. [21]. The membrane fractions [320 nmol (Δ2–4) and 395 nmol (Δ2–26)CYP1B1] were solubilized in 200 ml of solubilization buffer [100 mM $NaPO_4$ (pH 8.0), 0.4 M NaCl, 40% glycerol (v/v), 10 mM β-mercaptoethanol, 10 μM aprotinin, 0.5% CHAPS (w/v), and 5 mM imidazole], and the suspension was stirred for 5 h. Centrifugation at 100,000 *g* for 90 min yielded a clear pellet, which was discarded, and a supernatant that contained most of the P450. The supernatant was applied to a pre-equilibrated Ni–NTA column (1 ml of resin per 50 nmol of enzyme). The column was washed with at least 50 column volumes of wash buffer [100 mM $NaPO_4$ (pH 8.0), 0.4 M NaCl, 40% glycerol (v/v), 10 mM β-mercaptoethanol, 0.5% CHAPS (w/v), and 10 mM imidazole]. The His-tagged protein was eluted with two column volumes of elution buffer [100 mM $NaPO_4$ (pH 8.0), 0.4 M NaCl, 40% glycerol (v/v), 10 mM β-mercaptoethanol, 0.5% CHAPS (w/v), and 400 mM imidazole], and the

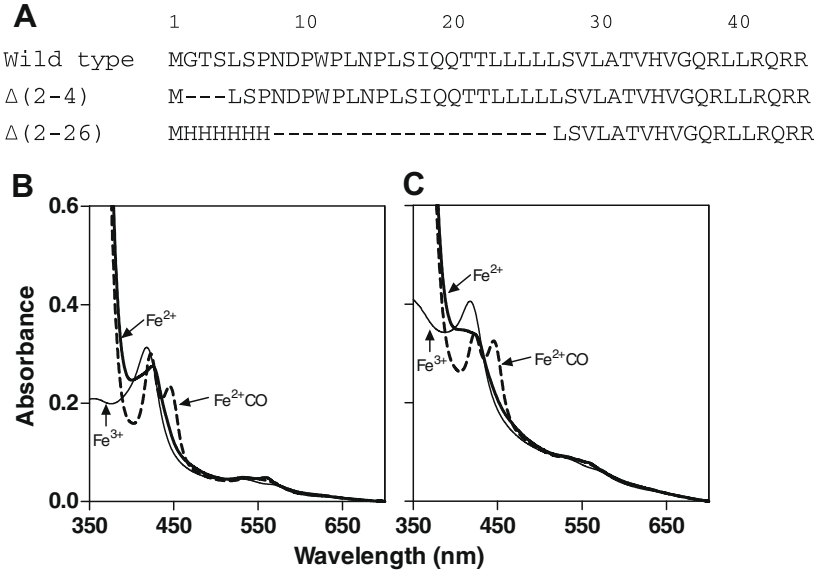

Fig. 1. Characterization of N-terminally deleted human CYP1B1. Amino acid sequence comparisons of the N-terminal sequence of wild-type and N-terminally deleted human CYP1B1 (A). Absorption spectra of (Δ2–4)CYP1B1 (B) and (Δ2–26)CYP1B1 (C) purified from *E. coli*. Spectra of ferric, sodium dithionite-reduced ferrous, and ferrous–CO complexes of CYP1B1 were recorded in 100 mM potassium phosphate buffer (pH 7.4).

eluate was dialyzed against dialysis buffer [100 mM $NaPO_4$ (pH 7.4), 0.25 M NaCl, 1 mM EDTA, 20% glycerol (v/v), and 0.1 mM dithiothreitol]. SDS–PAGE was used to assess final protein purity, and P450 concentrations were determined by Fe^{2+}–CO versus Fe^{2+} difference spectroscopy [23]. Spectroscopic properties of (Δ2–4) and (Δ2–26)CYP1B1 were confirmed by absorption spectra (Fig. 1B and C). Protein concentration was estimated using a bicinchoninic acid procedure (Pierce, Rockford, IL). Recombinant rat CPR was expressed in *E. coli* and purified as described [25,26].

CYP1B1 catalytic activity assays

7-Ethoxycoumarin and 17β-estradiol were used to compare the catalytic activities of (Δ2–4) and (Δ2–26)CYP1B1. Kinetic activities of CYP1B1 were determined using nonlinear regression analysis with GraphPad Prism software (GraphPad Software, San Diego, CA).

7-Ethoxycoumarin O-deethylation assay

The 7-Ethoxycoumarin oxidation reaction included 25 pmol CYP1B1, 50 pmol of CPR, and lipid vesicles (30 μM) in 0.50 ml of 100 mM potassium phosphate buffer (pH 7.4) along with a specified amount of the coumarin substrate. Incubations were generally performed for 20 min at 37 °C, terminated with 1 ml of CH_2Cl_2 and then centrifuged (1000*g*, 10 min). The products were extracted from the supernatant with CH_2Cl_2 (1.0 ml) followed by centrifugation at 1000*g*. The organic layers were combined, and the CH_2Cl_2 was removed under a N_2 stream. The products, 7-hydroxycoumarin and 3-hydroxy-7-ethoxycoumarin, were analyzed by HPLC using a Toso ODS-80TM octadecylsilane (C_{18}) column (4.6 × 150 mm, 5 μm) with a mobile phase of H_2O:CH_3CN (55:45, v/v) containing 10 mM $HClO_4$, a flow rate of 1.0 ml min^{-1}, and monitoring at A_{330} [27].

17β-Estradiol hydroxylation assay

The 17β-estradiol hydroxylation activities of (Δ2–4) and (Δ2–26)CYP1B1 were determined as previously described [28], with slight modifications. Briefly, purified CYP1B1 (25 pmol) was incubated with 50 pmol of CPR, lipid vesicles (30 μM), and 5 mM $MgCl_2$ in 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 0.25 ml. Ascorbic acid (1 mM) was added to the reaction mixture to protect the 17β-estradiol metabolites from oxidative degradation. After 20 min of incubation at 37 °C, reactions were terminated by extracting the metabolites twice in 1 vol of ethyl acetate. The organic layers were combined, and the ethyl acetate was removed under a N_2 stream. The reaction products, 2-OHE_2 and 4-OHE_2, were analyzed by HPLC using a Toso ODS-80TM octadecylsilane (C_{18}) column (4.6 × 150 mm, 5 μm) with a linear gradient increasing from 20% CH_3CN (v/v) in 1% CH_3CO_2H (v/v) to 30% CH_3CN (v/v) in 1% CH_3CO_2H (v/v) over 30 min with an additional 10 min elution with 30% CH_3CN (v/v) in 1% CH_3CO_2H (v/v), and the elution was monitored using A_{280} nm. The flow rate was 1.3 ml/min^{-1}. Retention time for 17β-estradiol and its metabolites were 28.4 min for 4-OHE_2, 29.2 min for 2-OHE_2, and 42.3 min for 17β-estradiol.

Liposome preparation

In all experiments, 100 mol% POPC (16:0–18:1-PC) liposomes were used as standard vesicles. After the appropriate amounts of lipids were 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), 100 mM NaCl, and 0.5 mM Na-EDTA] by vortexing and subsequent brief sonication in a bath-type sonicator (30 s). To obtain homogeneous, large unilamellar vesicles, 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 a deviation less than 10% in light scattering values. The concentration of liposome stock solutions was 1–2 mM, and portions of stock solutions were diluted to study the interaction of CYP1B1 and membranes [29].

Fluorescence measurements

All fluorescence experiments to analyze conformational changes of (Δ2–4) and (Δ2–26)CYP1B1 were performed at room temperature. Fluorescence emission spectra were recorded with a Shimadzu RF-5301 PC spectrofluorometer equipped with a thermostat-controlled cuvette compartment. After 2 μM CYP1B1 was incubated with 500 μM lipid vesicles in 100 mM potassium phosphate buffer (pH 7.4), the intrinsic fluorescence of CYP1B1 was recorded in the range of 310–450 nm with an excitation wavelength of 280 nm [29].

CD spectroscopy

CD spectra were recorded on a Jasco J715 spectropolarimeter (Japan Spectroscopic, Tokyo) at room temperature in a thermostat-controlled cuvette. Measurements were conducted in 100 mM potassium phosphate (pH 7.4) containing 2 μM (Δ2–4) and (Δ2–26)CYP1B1 with 500 μM lipid vesicles for the far-ultraviolet and visible regions. Blanks (buffer with or without phospholipid) were routinely recorded and subtracted from the original spectra. On average, data from 25 to 30 scans were accumulated [30].

Thermal stability

Inactivation of P450 was monitored by measuring the decrease of fluorescence intensity at 335 nm. The reaction mixture contained 2 μM CYP1B1 and the various phospholipids (500 μM) in 100 mM potassium phosphate buffer (pH 7.4) containing 10% glycerol (v/v). Thermal inactivation was carried out by measuring a series of fluorescence spectra from 310 to 450 nm (excitation wavelength: 280 nm) with temperatures between 20 and 70 °C in 3 °C intervals and a 90 s equilibration at each temperature [31].

Membrane binding

To examine the interaction of CYP1B1 with membranes, the energy transfer between Trp residues in CYP1B1 and pyrene-PC incorporated into membranes was measured in a binary system (PC/anionic phospholipid or PC/PE) by replacing PC with PA, PS, CL, or PE up to 40 mol%. After preparation of LUVs with or without the indicated amount of phospholipids, CYP1B1 enzymes (2 μM) were mixed with vesicles to a lipid:protein molar ratio of 250:1. After incubation of the sample for 5 min at 30 °C, Trp fluorescence was monitored at 341 nm (excitation wavelength: 295 nm). The amount of membrane-bound CYP1B1 was also quantified by HPLC using a gel-filtration column (TSK-GEL G3000SWXL, 7.8 × 300 mm). The samples were prepared by pre-incubating CYP1B1 with liposomes in the lipid hydration buffer at 30 °C for 10 min at an L:P ratio of 250:1. HPLC was performed at room temperature using a flow rate of 1 ml/min. The protein peaks were detected by absorbance measurements at 220 nm [30].

Results

Effect of anionic phospholipids on the catalytic activity of CYP1B1

In order to investigate the effect of membrane phospholipid composition on CYP1B1 catalytic activity, we measured CYP1B1-catalyzed reactions in the presence of various phospholipid

bilayers. The enzyme activity was quantified by measuring its ability to catalyze the *O*-deethylation and 3-hydroxylation of 7-ethoxycoumarin, and 4-hydroxylation of 17β-estradiol in the presence of an NADPH-generating system [27,28]. The activity of (Δ2–4)CYP1B1 increased by about 3–4-fold depending on the specific assay system compared to the 100% PC membrane as the PA concentration was increased to 50 mol% (Fig. 2A and B). CL also increased the catalytic activity to a similar degree in a lipid concentration-dependent manner. In contrast, there was no apparent increase of the enzymatic activity when the PC matrix was replaced with another anionic phospholipid, PS, or a neutral phospholipid, PE. Therefore, this result suggests that the presence of the anionic phospholipids PA and CL in the lipid bilayer specifically enhances the activity of CYP1B1. Although this effect is not general to anionic lipids, as PS did not stimulate CYP1B1 activity, it may not be limited to these two phospholipids, as other anionic phospholipids such as PIP_2 and PG were not tested in this study.

When (Δ2–26)CYP1B1 was used, PA also stimulated the enzyme, but to a significantly reduced level when compared to (Δ2–4)CYP1B1, with an approximately 1.6-fold increase in catalytic activity observed (Fig. 2C and D). Moreover, CL had little effect on the activity of (Δ2–26)CYP1B1.

The 4-hydroxylation of 17β-estradiol by CYP1B1 was also stimulated by PA and CL, and again (Δ2–4)CYP1B1 showed a more significant increase in catalytic activity than the (Δ2–26) enzyme (Fig. 3A and B). However, the activity changes were complicated depending on the type of CYP1B1 enzyme. The activity of (Δ2–4)CYP1B1 was enhanced by PA and CL in a concentration-dependent manner. However, in the case of (Δ2–26)CYP1B1, the catalytic activity decreased or remained unchanged upon replacement of PC with anionic phospholipids or PE. The 2-hydroxylation of 17β-estradiol by the CYP1B1 enzymes did not vary significantly with the phospholipid composition of the membranes (data not shown).

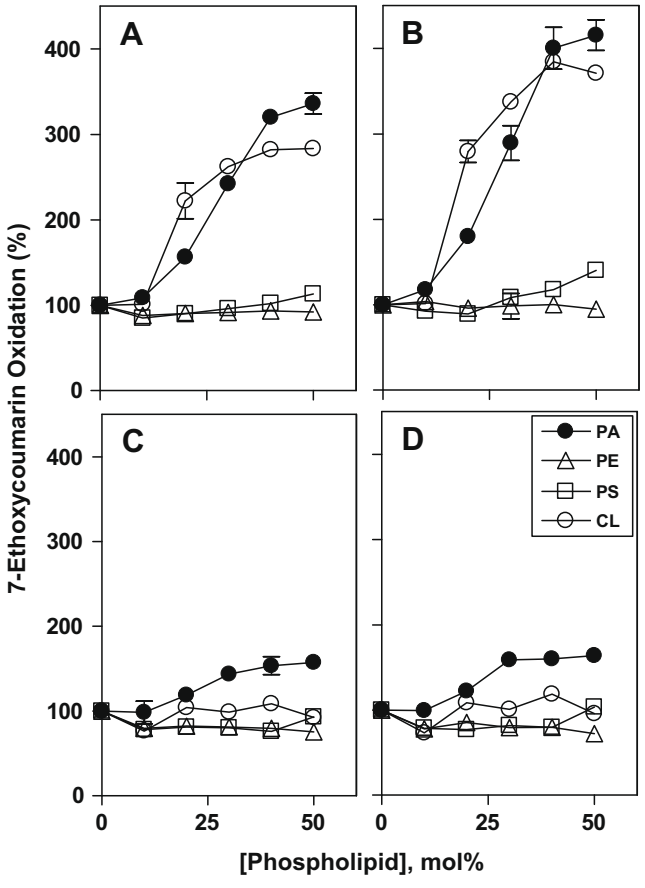

Fig. 2. Effects of phospholipids on the *O*-deethylation and 3-hydroxylation of 7-ethoxycoumarin by (Δ2–4)CYP1B1 (A and B) and (Δ2–26)CYP1B1 (C and D). *O*-Deethylation (A and C) and 3-hydroxylation (B and D) of 7-ethoxycoumarin by CYP1B1 were measured in binary systems (PC/anionic phospholipids) with increasing concentrations of the indicated phospholipids. The 100% activity value represents the normalized activity towards 7-OH coumarin and 3-OH coumarin for the samples containing 100% PC as the lipid. The *O*-deethylation and 3-hydroxylation of 7-ethoxycoumarin was measured in a reconstituted system consisting of CYP1B1 (0.025 μM), CPR (0.050 μM), lipid vesicles (30 μM), and 7-ethoxycoumarin (100 μM) in 100 mM potassium phosphate buffer (pH 7.4).

Conformational changes of CYP1B1 upon interaction with phospholipids

In order to examine the effect of phospholipids on CYP1B1 conformation upon membrane binding, structural changes of CYP1B1 were investigated using CD in the far UV region and Trp fluorescence spectroscopy. Fig. 4A and B show CD spectra of CYP1B1 in the presence of 30 mol% PA or CL in the PC matrix. The CD spectra were curve-fitted by the least squares method into reference spectra obtained from five proteins: myoglobin, lysozyme, ribonuclease A, papain, and lactate dehydrogenase [30]. Table 1 shows the calculated secondary structures of CYP1B1 enzymes derived from the curve-fitting. Interestingly, the membrane environment provided by 100% PC had no appreciable effect on the secondary structure of either CYP1B1 enzyme. In contrast, PA and CL both induced an increase in α-helix content from 40–50% to 50–60%. Notably, the amount of β-sheet structure in (Δ2–26)CYP1B1 decreased upon interaction with the membranes, but there were no significant structural changes in (Δ2–4)CYP1B1. The changes in turn structure content were small for both CYP1B1 enzymes.

The intrinsic Trp fluorescence of CYP1B1 (CYP1B1 has 7 Trp residues in its amino acid sequence) was also measured in the presence of the same types of phospholipid vesicles used in the CD experiments (Fig. 4C and D). As in the CD experiments, the PC environment had little effect on the fluorescence of the CYP1B1 enzymes. However, the emission intensity generally increased upon interaction with PA- or CL-containing vesicles compared to the 100% PC membrane without appreciable changes in λ_{max}. This result indicates that the change induced by the phospholipids

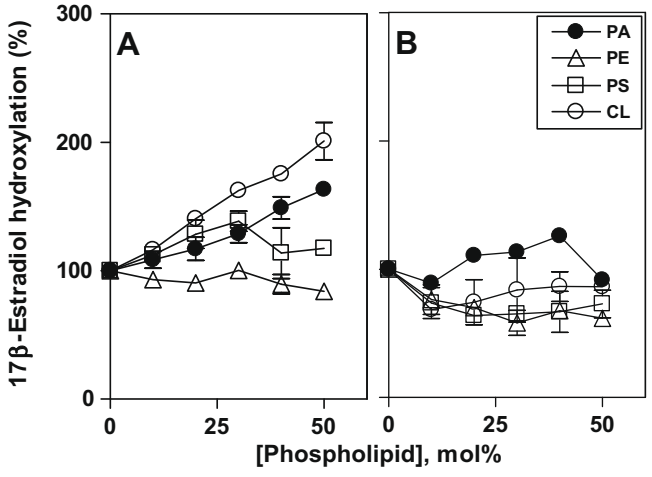

Fig. 3. Effects of phospholipids on the 4-hydroxylation of 17β-estradiol by (Δ2–4)CYP1B1 (A) and (Δ2–26)CYP1B1 (B). 4-Hydroxylation of 17β-estradiol by CYP1B1 was measured in a binary system (PC/anionic phospholipids) with increasing concentrations of the indicated phospholipids. The 100% activity value represents the normalized activity towards 4-OH 17β-estradiol for the sample containing 100% PC as the lipid component. 4-hydroxylation of 17β-estradiol was measured in a reconstituted system consisting of CYP1B1 (0.025 μM), CPR (0.050 μM), lipid vesicles (30 μM), and 17β-estradiol (100 μM) in 50 mM potassium phosphate buffer (pH 7.4).

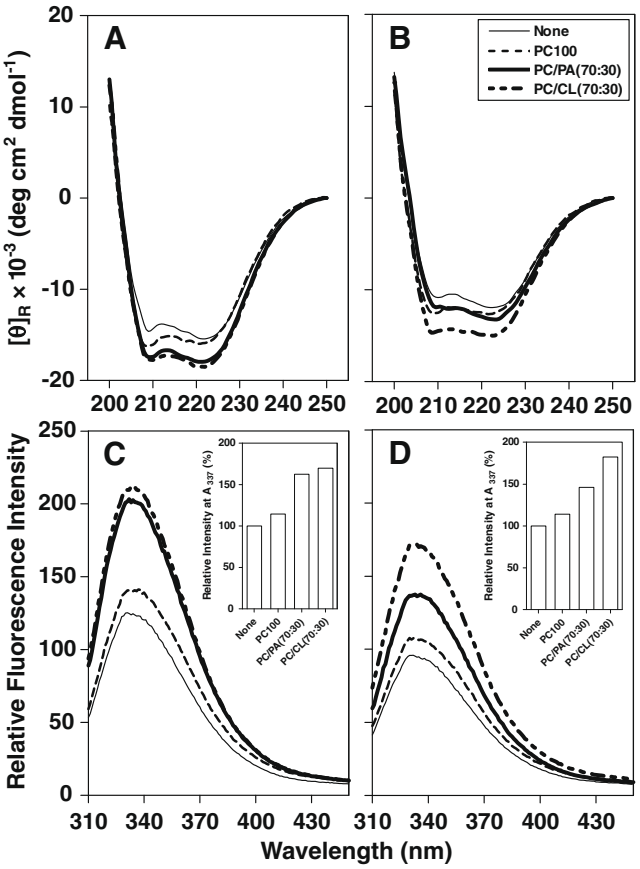

Fig. 4. Effects of phospholipids on the CD and Trp fluorescence spectra of (Δ2–4)CYP1B1 (A and C) and (Δ2–26)CYP1B1 (B and D). CD spectra of 2 μM (Δ2–4)CYP1B1 (A) and (Δ2–26)CYP1B1 (B) in 100 mM potassium phosphate (pH 7.4) were recorded in the presence of the indicated phospholipid vesicles (500 μM). Mean residue ellipticity, $[\theta]_R$, is expressed on the basis of the number of amino acids per molecule of CYP1B1. Molar ellipticity, $[\theta]$, is expressed on the basis of the molar concentration. Emission spectral changes for (Δ2–4)CYP1B1 (C) and (Δ2–26)CYP1B1 (D) were recorded in the range of 310–450 nm with an excitation wavelength of 280 nm. The inset represents the relative intensity (%) in the presence of the indicated phospholipid vesicles.

reduces the quenching of the intrinsic fluorescence in both CYP1B1 enzymes while the overall environment of the intrinsic fluorophore appears to remain unchanged. Conditions that altered intrinsic Trp fluorescence were similar to those that altered the CD spectra, with CL inducing more significant intensity changes than PA with (Δ2–26)CYP1B1, but PA and CL having nearly the same effect on the (Δ2–4)CYP1B1 enzyme.

Phospholipid-dependent thermal stability of CYP1B1

Fig. 5 shows the thermal unfolding transition profile of membrane-bound CYP1B1 measured by the intrinsic fluorescence of

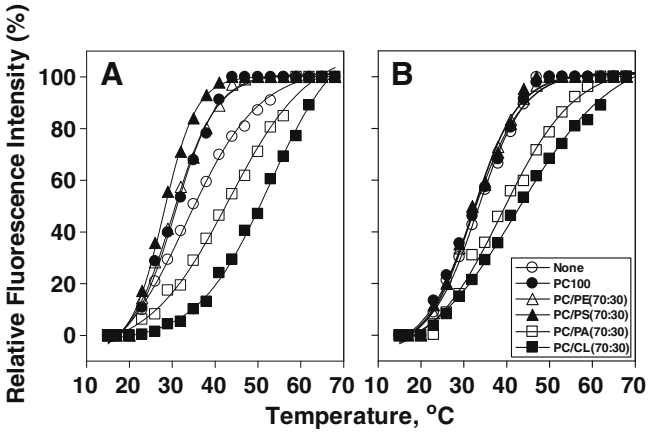

Fig. 5. Effect of phospholipids on the thermal stability of (Δ2–4)CYP1B1 (A) and (Δ2–26)CYP1B1 (B). Thermal inactivation of (Δ2–4) and (Δ2–26)CYP1B1 was conducted in the presence of the indicated phospholipid vesicles as a function of temperature. T_m values were calculated from the changes in fluorescence intensity at 335 nm. The *y*-axis is the relative change in fluorescence emission intensity with that of CYP1B1 set to zero (0) and to one (1) at the initial and final temperatures, respectively.

Trp residues in the protein. The soluble CYP1B1 enzymes had a transition temperature (T_m) of approximately 33 °C (Table 2). This indicates that both CYP1B1 enzymes have similar thermal stability in the absence of membrane components. When bound to a 100% PC membrane, the T_m of CYP1BI decreased slightly, with values of 30.6 and 32.2 °C calculated for (Δ2–4) and (Δ2–26)CYP1B1, respectively. However, the replacement of PC with PA or CL resulted in a significant increase in the T_m, rising to 38.9–52.6 °C. The PA and CL effects were more intense for the (Δ2–4)CYP1B1 construct than for the (Δ2–26) protein. This result demonstrates that PA and CL stabilize CYP1B1, possibly through induction of more compact conformation of the enzyme. The result also suggests that the increase in the thermal stability of the proteins may be responsible for the enhanced catalytic activities of CYP1B1.

Phospholipid-dependent membrane binding of CYP1B1

To investigate the binding/insertion of CYP1B1 into lipid bilayers containing anionic phospholipids, we measured resonance energy transfer between Trp residue in CYP1B1 (CYP1B1 has 7 Trp residues that are distributed throughout its sequence) and pyrene-PC that had been incorporated into membranes in which PC was partially replaced with other phospholipids. (Δ2–4)CYP1B1 has a Trp residue in N-terminal anchor region, which is expected to interact with membranes specifically. In pyrene-PC, the pyrene group is located at the end of the decanoyl chain at the *sn*-2 position.

In both CYP1B1 enzymes, PA had the most significant quenching of the Trp fluorescence with the lowest F/F_o values among the phospholipids tested (Fig. 6). Furthermore, PA induced a grad-

Table 1
Effect of phospholipids on the CD spectra of (Δ2–4)CYP1B1 and (Δ2–26)CYP1B1.

Additions	(Δ2–4)CYP1B1				(Δ2–26)CYP1B1			
	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random (%)	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random (%)
None	50 ± 1	17 ± 3	18 ± 2	15 ± 4	40 ± 3	31 ± 6	26 ± 5	3 ± 8
Phospholipids								
PC100	52 ± 1	18 ± 2	19 ± 2	12 ± 3	42 ± 2	25 ± 4	23 ± 3	9 ± 5
PC/PA(70:30)	60 ± 2	17 ± 3	18 ± 3	6 ± 4	47 ± 3	25 ± 6	23 ± 5	5 ± 8
PC/CL(70:30)	62 ± 2	15 ± 3	17 ± 3	6 ± 5	53 ± 2	19 ± 4	21 ± 3	7 ± 5

All CD spectra were recorded in 100 mM potassium phosphate (pH 7.4) in the presence of indicated phospholipid vesicles. Conditions were as described under Materials and methods. All estimates are means ± SE.

Table 2
Thermal stability of (Δ2–4) and (Δ2–26)CYP1B1 in the presence of various phospholipid vesicles.

Phospholipids	(Δ2–4)CYP1B1 T_m (°C)	(Δ2–26)CYP1B1 T_m (°C)
None	33.4 ± 0.7	33.2 ± 0.4
PC100	30.6 ± 0.4	32.2 ± 0.5
PC/PE(70:30)	30.1 ± 0.4	32.2 ± 0.3
PC/PS(70:30)	28.0 ± 0.3	32.1 ± 0.4
PC/PA(70:30)	43.2 ± 0.7	38.9 ± 0.7
PC/CL(70:30)	52.6 ± 0.7	42.7 ± 0.7

Results are representative of at least three independent determinations. Values are means ± SE.

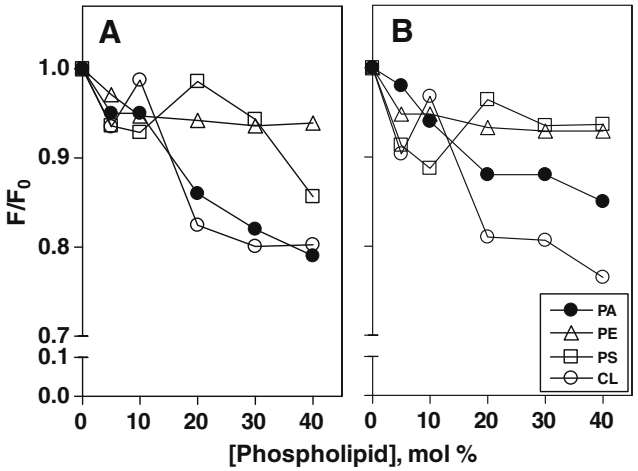

Fig. 6. Phospholipid-dependent membrane interaction of (Δ2–4)CYP1B1 (A) and (Δ2–26)CYP1B1 (B). The energy transfer between Trp residues in CYP1B1 and pyrene-PC incorporated into membranes was examined in a binary system (PC/anionic phospholipid or PC/PE) by replacing PC with PA, PS, CL, or PE up to 40 mol%. F/F_o represents the fluorescence intensity ratio at 340 nm for the sample with (F) and without (F_o) pyrene-PC incorporated in the membrane. After preparation of LUVs with or without the indicated amount of phospholipids, CYP1B1 enzymes (2 μM) were mixed with vesicles to an L:P ratio of 250:1. After incubation of the sample for 5 min at 30 °C, Trp fluorescence emission was measured at 341 nm (excitation wavelength: 295 nm).

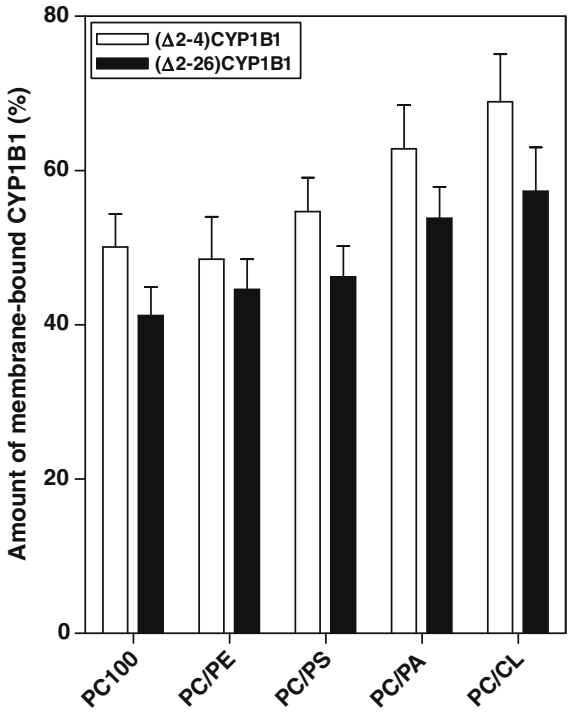

Fig. 7. Effect of phospholipids on the binding of (Δ2–4)CYP1B1 (A) and (Δ2–26)CYP1B1 (B) to lipid bilayers. Binding of the CYP1B1 enzymes to liposomes was measured by gel-filtration as described in the Materials and Methods in a binary system (PC/anionic phospholipid or PC/PE, 70:30 by mol%) at an L:P ratio of 250:1. Results are representative of at least three independent determinations. Values are means ± SE.

ual decrease in the F/F_o value with increasing concentrations, and it seems not to reach a saturation point even in the presence of 40 mol% PA. As expected, PA exhibited a stronger quenching effect on (Δ2–4)CYP1B1 than the (Δ2–26) enzyme. This result may be ascribed to a deletion of Trp residue in N-terminal anchor region of (Δ2–26) enzyme. Other phospholipids, PS and PE, also decreased F/F_o but were less efficient than PA. PS- and PE-induced quenching became saturated at 10 mol% concentration. This result indicates that CYP1B1 has the ability to bind and penetrate pre-formed membrane bilayers depending on the type of phospholipids present in the membrane.

To obtain more quantitative insight into the interaction of CYP1B1 with lipid bilayers, we determined the amount of enzyme bound to membranes with increasing concentrations of anionic phospholipids or PE in a binary system with a fixed L:P ratio of 250:1. Fig. 7 shows that approximately 60–70% of the amount of (Δ2–4)CYP1B1 was bound to membranes containing 30 mol% PA and CL. In contrast, approximately 50% of soluble (Δ2–4)CYP1B1 was bound to lipid bilayers when neutral membranes without any anionic phospholipid (100% PC or PC:PE = 70:30) were used. In addition, PS did not stimulate membrane binding. Therefore, the PA- and CL-induced increases in membrane binding of CYP1B1 might partially explain the stimulation of catalytic activity, increase in thermal stability, and increased FRET observed with labeled membranes, although the effect of PA (and CL) on the amount of membrane-bound CYP1B1 seems to be relatively small. Consistent with other results, Fig. 7 also demonstrates that (Δ2–4)CYP1B1 binds to membranes more efficiently than the (Δ2–26)CYP enzyme. This result indicates that the N-terminal region is also important for the association of the enzyme with lipid bilayers.

Discussion

In this study, we show that PA and CL specifically increased the catalytic activities, membrane binding, and thermal stability of both CYP1B1 proteins when PC matrix was gradually replaced with these anionic phospholipids. PA- or CL-dependent changes of CYP1B1 conformation were revealed. Moreover, (Δ2–4)CYP1B1 responded more strongly than the (Δ2–26)enzyme to changes in lipid composition for all of the signals investigated, demonstrating the functional importance of the N-terminal region for the interaction with the phospholipid membranes. On the contrary, other anionic phospholipids such as PS and neutral phospholipid PE had no apparent effects on catalytic activities and conformation of CYP1B1. Taken together, it can be suggested that the chemical and physical properties of membranes induced by PA or CL are critical for the functional roles of CYP1B1. CL of the mitochondrial membrane can associate with the ER membrane to affect on the structure and function of CYP1B1.

It is known that PA acts as a biosynthetic precursor for the formation of all acylglycerol lipids in cells [32]. It means that PA is rapidly converted to diacylglycerol (DAG) depending on the phospholipase D activity, short-lived in cells, and the interconversion is possible between PA and DAG. Thereby it is difficult to measure the concentration accurately. Usually, the concentrations are considered to be below

0.5 mol% in plasma and subcellular membranes. PA is also involved in diverse cellular functions such as cell growth, proliferation, reproduction, and responses to hormones [33].

Interestingly, the results for PA-mediated functional regulations of CYP1B1 parallel the previous observations that PA increased the catalytic activities of CYP1A2 and CYP3A4 with increasing the lipid concentrations in membranes [29,30]. It was also demonstrated that CYP2B1 induced clustering of the lipid PA to a greater extent than other anionic phospholipids did, and that PA increased the ability of CYP2B1 to interact with lipid monolayers [34]. Based on these observations, we anticipate that PA and/or PA-induced membrane properties play an important role in the interaction between various P450 enzymes and membranes. In addition, the requirement of anionic phospholipids for the efficient function of P450 enzymes was suggested previously [20]. At present, it is relevant that CYP1B1 interacts with PA and the biological functions can be regulated by the phospholipid. It should be also considered that the concentrations PA used in the present study seem to be higher than those found in cells. However, as we suggested previously, the mixing property of PA is not random but the phase separation occurs in the liquid–crystalline phase of membranes [35] through intermolecular hydrogen bonding [36]. In addition, PA concentration is very fluctuant as a signaling lipid depending on cellular states.

CL exists almost entirely in the mitochondrial inner membranes and is synthesized in the mitochondria matrix side. However, although the concentration is below 5 mol%, CL is also found in smooth ER [37] at which P450 enzymes exert their functions and CL-remodeling pathway was revealed in ER [38,39] suggesting a novel role for the ER in CL metabolism. It means that CL can relocate from mitochondria to ER and it was experimentally supported [40]. These results also suggest that ER plays an important role in CL biosynthesis. Moreover, CL forms raft-like microdomains [41]. Therefore, CL may be physiologically relevant to the functional regulations of CYP1B1.

On the basis of these results, it may be reasonable to perform the present investigation in CL concentration-dependent manner. It should be also notified that the mitochondrial outer membrane can associate with the ER membrane, in a structure called MAM (mitochondria-associated ER-membrane) [42]. This is important in ER-mitochondria calcium signaling and involved in the transfer of lipids between the ER and mitochondria.

The physiological significance of the results on the enzyme with the signal anchor deleted and examination of enzymes incorporated into synthetic membranes is not clear. However, (Δ2–4) CYP1B1 has truncated only three amino acids from the wild-type CYP1B1. Thus, we consider that N-terminus of this enzyme might show similar properties of wild-type enzyme. On the contrary, (Δ2–26) CYP1B1 can be represented as an N-terminal deletion mutant. Thereby, the N-terminal region and/or conformational changes induced by loss of the N-terminal region of CYP1B1 may be important for the interaction with anionic phospholipids and consequent enzymatic stimulation. Actually, the result obtained from CD showed that different conformational changes occur in the (Δ2–4) and (Δ2–26)CYP1B1 enzymes upon interaction with phospholipid membranes. The CD results also indicate that the overall conformations of both CYP1B1 enzymes change in the presence of PA- or CL-containing vesicles with different effects for the two anionic phospholipids. However, at present there does not seem to be a direct correlation between the stimulation of enzymatic activity and the alteration of secondary structure.

The signal anchor of N-terminus (Fig. 1) is the only trans-membrane helix in CYP1B1 so that the positions of the Trp (11th amino acid residue of wild-type CYP1B1) may contribute to the quenching effect upon interaction with membranes containing pyrene (Fig. 6). As expected, PA exhibited a stronger quenching effect on (Δ2–4)CYP1B1 than the (Δ2–26) enzyme. This result may be ascribed to a deletion of Trp residue in N-terminal anchor region of (Δ2–26) enzyme. We could not also exclude the possibility that other Trp residues in CYP1B1 exert their influence on the energy transfer and thus the results as well as conformational change revealed by Trp fluorescence (Fig. 4C and D) might not provide specific understanding of how the protein is interacting with the membrane. Nonetheless, the energy transfer result suggests that CYP1B1 has the ability to bind and penetrate pre-formed membrane bilayers depending on the type of phospholipids present in the membrane.

Acknowledgments

This work was supported in part by the 21C Frontier Microbial Genomics and the Application Center Program of the Ministry of Education, Science and Technology of the Republic of Korea; the Korea Science and Engineering Foundation (Grant R01-2008-000-21072-02008); and the Second Stage BK21 Project from the Ministry of Education, Science and Technology of the Republic of Korea.

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