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A Measurement Method for Cytochrome P450 3A4 (CYP3A4)-mediated Oxidation of Cholesterol in Lipid Membranes

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We have developed a measurement method for the cytochrome P450 3A4 (CYP3A4)-mediated oxidation of cholesterol in lipid membranes. This method is based on the sterol-structural selectivity for the nanopore formation of streptolysin O (SLO) in a lipid membrane. A decrease in the fraction of 4β-hydroxycholesterol in lipid membranes decreased the nanopore forming ability of SLO. The incubation of the liposomes consisting of L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), and cholesterol (0.98:0.02:0.75, mol/mol) with CYP3A4 decreased the nanopore forming ability of SLO, indicating the conversion of cholesterol to 4β-hydroxycholesterol. The change in nanopore forming ability did not occur in the presence of erythromycin, which is an inhibitor of CYP3A4.

1. Introduction

Oxysterols are oxidized forms of cholesterol, which is a component of the cellular lipid membrane in mammals, and they modulate many biomembrane processes such as membrane activities,[1] receptor function,[2,3] cell signaling,[4,5] intracellular trafficking,[6,7] and immune responses.[8,9] There are various types of oxysterol in human serum, and they are generated enzymatically[10–12] and/or through autoxidation by free radicals and reactive oxygen species (ROS).[13] Cytochrome P450 3A4 (CYP3A4), which is expressed in the liver and small intestine[14] and catalyzes a broad range of compounds,[15] increases the serum concentration of 4β-hydroxycholesterol,[16,17] because this oxysterol has a long half-life in plasma, resulting in stable plasma concentrations.[17] Furthermore, inducer compounds of CYP3A4 also lead to an increase in the amount of 4β-hydroxycholesterol.[18] These suggest that the endogenous oxysterol 4β-hydroxycholesterol may be used as a marker of CYP3A activity.[19,20]

Separation techniques such as gas chromatography–mass spectrometry (GC–MS)[21,22] and liquid chromatography tandem–mass spectrometry (LC–MS/MS) methods[23,24] were employed for the quantification of 4β-hydroxycholesterol in human serum and cells as samples. In these techniques, the oxysterol is extracted with chloroform from cellular and serum samples before the separation procedures, suggesting the difficulty in identifying the oxysterol origin, which

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may be from either cellular membranes or transporter proteins such as apolipoprotein A1, high-density lipoprotein (HDL), and low-density lipoprotein (LDL). The biological function of 4β-hydroxycholesterol has been studied[25,26] with little knowledge of where the cholesterol oxidation by CYP3A4 occurs.

Molecular dynamics (MD) simulation based on the Berger lipid force field showed that the presence of cholesterol caused the ordering and thickening of the membrane and led to the greater immersion and inclination of CYP3A4 toward the membrane,[27] suggesting that CYP3A4 catalyzes cholesterol within lipid membranes. On the other hand, there is little experimental data on cholesterol oxidation within the lipid membrane. Artificial lipid bilayer membranes, which simplify the biological membrane without containing various biomolecules except for lipids, are suitable for evaluating the enzymatic oxidation of cholesterol in cellular membranes. However, the oxidation within lipid membranes, whose thickness is about 4–5 nm, is difficult to detect directly. Previously, we described the sterol-selective recognition of streptolysin O (SLO), which forms nanopores in lipid membranes.[28] A free 3-OH group in the β-configuration is essential for the recognition. The oxidation of the ring system leads to the loss of the recognition, whereas the oxidation of the acyl chain enhances the nanopore forming ability of SLO. Furthermore, immobilized liposomes encapsulating calcein as a marker dye were successfully applied to the detection of sterol oxidation by cholesterol oxidase based on the recognition of SLO.

In this study, to demonstrate cholesterol oxidation by CYP3A4 within lipid membranes, the measurements based on the SLO recognition were performed with immobilized liposomes encapsulating calcein. The cholesterol ratio in the lipid membrane was optimized for the measurements. CYP3A4 activity was measured in the presence of glucose-6-phosphate dehydrogenase and reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is converted from oxidized NADPH ($NADP^+$) by the dehydrogenase as the source of electrons for the P450 oxidative reactions. The increase in the amount of 4β-hydroxycholesterol produced by the CYP3A4 enzymatic reaction results in the decrease in SLO nanopore forming ability, leading to the inhibition of the release of calcein from the inner solution of liposomes (Fig. 1). This calcein release can be detected by fluorescence measurements.

2. Materials and Methods

2.1 Reagents

L-α-Phosphatidylcholine (PC, purity >99%, 50 mg/mL chloroform solution) and L-α-phosphatidylethanolamine (PE, 10 mg/mL chloroform solution) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol, which was obtained from Wako Pure Chemicals (Osaka, Japan), was recrystallized three times from methanol. Streptolysin O (SLO, lyophilized powder) was purchased from Sigma Chemical (St. Louis, MO, USA). Calcein was obtained from Dojindo Laboratories (Kumamoto, Japan). Dithiothreitol (DTT) was purchased from Nacalai Tesque (Kyoto, Japan). Disuccinimidyl suberate (DSS) was obtained from Thermo Scientific (Waltham, MA, USA). All other chemicals used were of analytical reagent grade. Milli-Q water

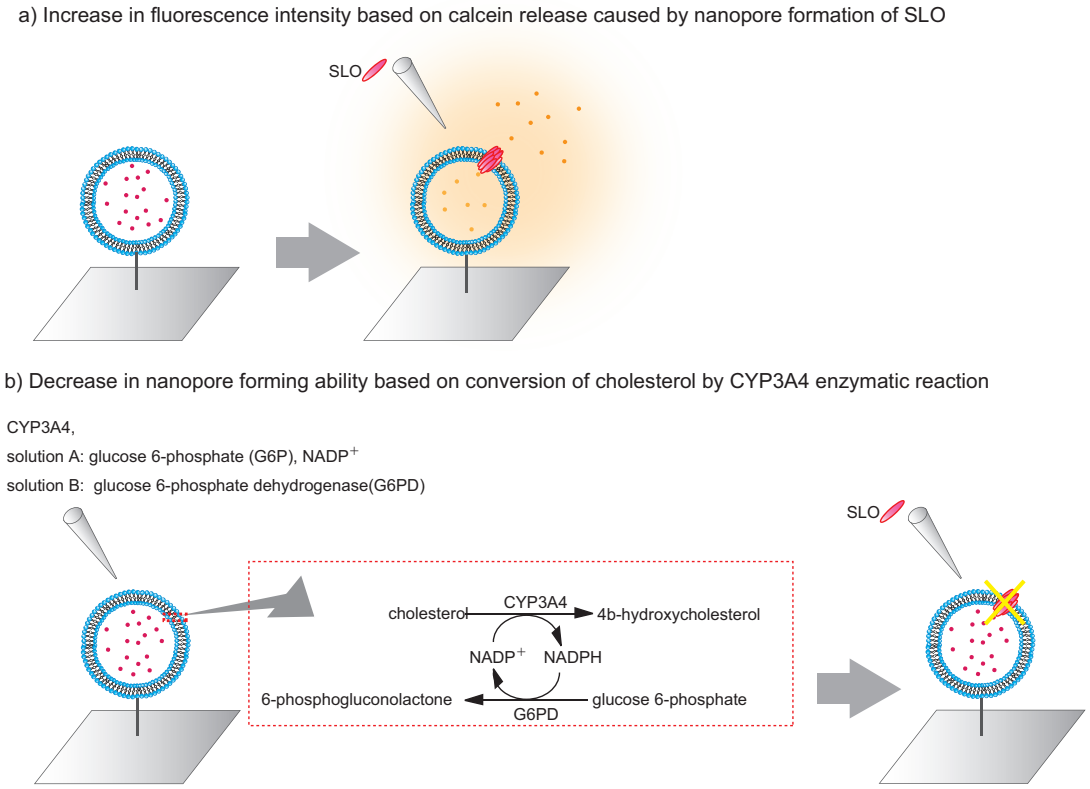

Fig. 1. (Color online) Schematic of an assay for cholesterol oxidation by CYP3A4 in lipid membrane.

(Millipore reagent water system, Bedford, MA, USA) was used throughout the experiments. CYP3A4 was obtained from Nosan Corporation (Kanagawa, Japan). NADPH regeneration system Solutions A and B were purchased from Corning Inc. (NY, USA).

2.2 Apparatus

The fluorometric intensities of liposomes in a 384-well microplate (Thermo Fisher Scientific K.K., Japan) were measured with a microplate reader (SH-9000, Corona Electric, Ibaraki, Japan). A glass electrode pH meter (model IOL30, Denki Kagaku Keiki, Tokyo, Japan) was used for pH measurements.

2.3 Immobilization of liposomes on a microplate well

A chloroform solution (120 µl) containing 4.55 mg of PC, 58 µg of PE, and 3 mg of cholesterol (0.75:0.02:1, mol/mol) was dried to a lipid film and subjected to high vacuum. The lipid film was hydrated in 1 ml of a 50 mM calcein solution containing a 50 mM phosphate buffer solution (pH 7.4, abbreviated as PBS) by vortex mixing for 10 min, followed by bath sonication for 15 min. In the latter case, excess calcein was removed from the solution by centrifugation at 12000 *g* at 4 °C for 5 min. The precipitates were washed 6–7 times with PBS containing 0.15 M NaCl. DSS was

dissolved in dimethyl sulfoxide (DMSO) at 10 mM and diluted with PBS to give a 1 mM solution. The solution hereafter is called a DSS solution. Twenty microliters of the DSS solution was added to a poly-L-lysine-coated well of a 384-well microplate. After incubation for 30 min at room temperature, the well was washed with 30 µl of PBS three times. The solution in the well was removed, and each well was incubated with 20 µl of a 100-fold-diluted liposome suspension for 1 h at room temperature. The wells were washed 7–8 times with 50 µl of PBS to remove excess calcein and unreacted liposomes. Then, 20 µl of a 1 M ethanolamine solution was added into each well and incubated for 30 min at room temperature to block the unreacted succinimidyl moiety. After removing 30 µl of the solution from each well, the wells were washed with 20 µl of PBS.

2.4 Monitoring of cholesterol oxidation in a lipid bilayer

A CYP3A4 solution was prepared immediately before its use and diluted further with PBS if necessary. SLO was dissolved in water at 0.4 mg/ml and stored at −20 °C. A 2.5 µl aliquot of the SLO solution was mixed with 2.5 µl of 10 mM DTT in PBS, and the mixture was pre-incubated for 30 min at room temperature. The mixture hereafter is called a DTT-activated SLO solution. Solutions A and B were diluted 20-fold and 100-fold with PBS, respectively. These solutions were mixed at the volume ratio of 5:1 (solution A:solution B, abbreviated as solution AB).

Each of the liposome-immobilized wells (vide supra) contained 10 µL of PBS. A 3.2 µl aliquot of a CYP3A4 solution, 1.9 µl of solution AB, and 0.88 µl of PBS were added to each well. Each well was incubated for a given time, and then 2 µl of the DTT-activated SLO solution (0.2 mg/ml) was added. After 30 min, the fluorescence intensity was measured with a microplate reader (Ex. 485 nm, Em. 510 nm). The normalized mean fluorescence intensity (ΔF_t) at time t, ascribable to calcein released from the liposomes, was obtained by subtracting the fluorescence intensity of the immobilized liposomes before adding SLO. The maximum change in normalized fluorescence intensity (ΔF_{max}), corresponding to total calcein release, was determined by the lysis of the liposomes with 0.5 µl of 12% (v/v) Triton X-100 in PBS. The calcein permeability at time t (P_t) was calculated from the ratio of $\Delta F(t)$ to ΔF_{max}.[28]

2.5 Evaluation of inhibitory effect by erythromycin

Erythromycin was dissolved in ethanol at the concentration of 1 mM. This solution was diluted with PBS at a given concentration if necessary. A 0.5 µl aliquot of the erythromycin solution was mixed with 4.5 µl of a 1 nmol/L CYP3A4 solution. A 3.2 µl aliquot of the mixture, 1.9 µl of the mixture of solutions A and B, and 0.88 µl of PBS were added to each well, followed by incubation for 150 min. As mentioned in Sect. 2.4, P_{30min}, i.e., permeability at 30 min, was calculated from the ratio of the change in fluorescence intensity at 30 min (ΔF_{30min}) to ΔF_{max} after adding a SLO solution. The permeability calculated from ΔF_{30min} in the presence of erythromycin and ΔF_{max} is defined as $P_{erythromycin}$, and the residual activity was estimated from the following equation:

$$\text{residual activity} = \frac{P_{max} - P_{erythromycin}}{P_{max} - P_{min}}, \quad (1)$$

where P_{max} is the maximum permeability obtained without CYP3A4 and P_{min} is the minimum permeability resulting from the completion of the enzymatic conversion from chol to 4β-OH chol.

3. Results and Discussion

3.1 Effect of 4β-OH chol on nanopore forming ability

CYP3A4 is known to catalyze the oxidation of cholesterol, producing 4-cholesten-3-one.[16,17] Previously, we described that the 3β-OH group of cholesterol participates commonly in SLO recognition, and the introduction of a hydroxyl group in the ring system leads to the loss of the nanopore forming ability.[28] We confirmed the nanopore forming ability using liposomes consisting of PC, PE, and 4β-OH chol (0.98:0.02:1, molar ratio). In the case of the liposomes consisting of PC and chol, the addition of SLO induced the increase in fluorescence intensity resulting from nanopore formation, and P_{30min} reached nearly 1.0, i.e., nanopores were formed in almost all liposome membranes. On the other hand, when using 4β-OH, such nanopore formation could not be observed, indicating that SLO did not recognize the 4β-OH moiety (Fig. 2).

To detect the enzymatic conversion from chol to 4β-OH chol in the lipid membrane, we investigated the effect of 4β-OH chol on the nanopore formation by SLO [Fig. 3(a)]. In the composition of lipids, i.e., PC and PE, and sterol (0.98:0.02:1, molar ratio), P_{30min} decreased in the narrow fraction range of 4β-OH chol from 50 to 75%. SLO formed nanopores in lipid membranes of all liposomes when the molar ratio of cholesterol was 0.5,[28] but nanopore formation was

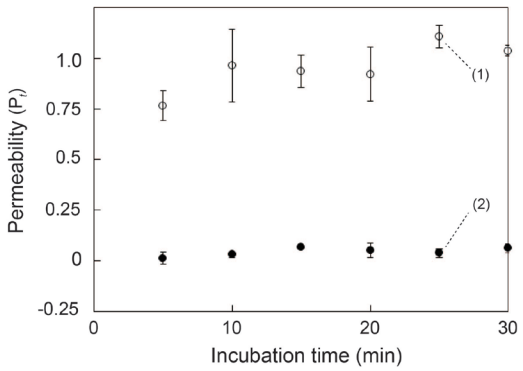

Fig. 2. Time profiles of calcein permeability (P_t) after adding 2 μl of a 0.2 mg/ml DTT-activated SLO solution to immobilized liposomes containing (1) 6 μg of PC and 3 μg of cholesterol and (2) 6 μg of PC and 3 μg of 4β-OH cholesterol. Averages of three measurements are plotted. Error bars indicate mean ± standard deviation (n = 3). Ex. 488 nm, Em. 530 nm.

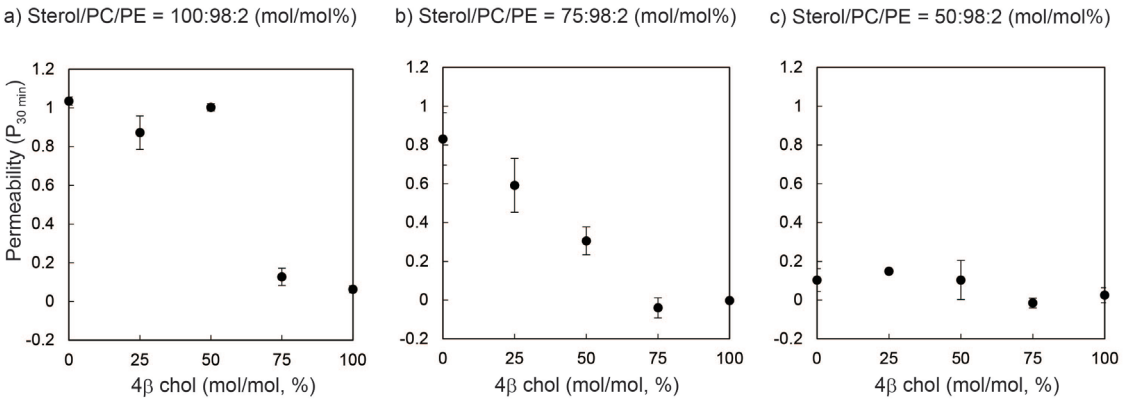

Fig. 3. Effect of 4β-OH chol on calcein permeability for immobilized liposomes. Liposomes consisting of sterol, PC, and PE at the molar ratios of (a) 100:98:2, (b) 75:98:2, and (c) 50:98:2 were immobilized on each well. Five microliters of a 0.1 mg/ml DTT-activated SLO solution was added to liposomes immobilized on a microplate. The calcein permeability at 30 min (P_{30min}) is plotted against a given molar ratio of chol and 4β-OH chol. Averages of three measurements are plotted. Error bars indicate mean ± standard deviation (n = 3). Ex. 488 nm, Em. 530 nm.

observed until the molar ratio of 25% cholesterol, which corresponds to 75% 4β-OH chol in total lipid. A tilt of 4β-OH chol affects the molecular packing and orientation of the lipid membrane, which may indirectly affect the accessibility of SLO to the 3β-OH group near the membrane surface, although SLO does not recognize 4β-OH chol. This results in nanopore formation by SLO with a lower amount of cholesterol in the lipid membrane. Furthermore, the effect of the tilt of 4β-OH chol on the packing and orientation of the lipid membrane probably induced SLO binding with chol at a rate higher than that of the diffusion of SLO. This leads to the narrow fraction range of chol, as discussed in our previous paper.[28] In Fig. 3(b), the linear relationship between P_{30min} and the fraction of 4β-OH chol was obtained in the range from 0 to 75% when using liposomes composed of lipid and sterol (1:0.75, molar ratio). The nanopore formation is diffusion-limited because of the lower ratio of chol in total lipid. The cholesterol ratio is roughly consistent with that of the cellular membrane.[29–31] On the other hand, no notable change in P_{30min} was observed for lipid sterol (1:0.5, molar ratio) owing to the insufficient amount of cholesterol [Fig. 3(c)].

3.2 Evaluation of cholesterol oxidation by CYP3A4 in a lipid bilayer

A 1 nM CYP3A4 solution was added to liposomes consisting of PC, PE, and chol (0.98:0.02:0.75, molar ratio), and the liposomes were incubated for 120 min. P_{30min} was lower than that obtained without the enzyme, indicating the conversion from chol to 4β-OH chol by the enzymatic reaction of CYP3A4 (Fig. 4). The effects of P_{30min} after the enzymatic reaction ranging from 0 to 180 min were investigated with the liposomes [Fig. 5(a)]. P_{30min} decreased with an increase in reaction time almost linearly until 180 min, and about 50% of chol was oxidized for 180 min as estimated from the relationship between P_{30min} and the fraction of 4β-OH chol [Fig. 3(b)]. At 180 min, the lower stability of liposomes may result in a longer error bar.

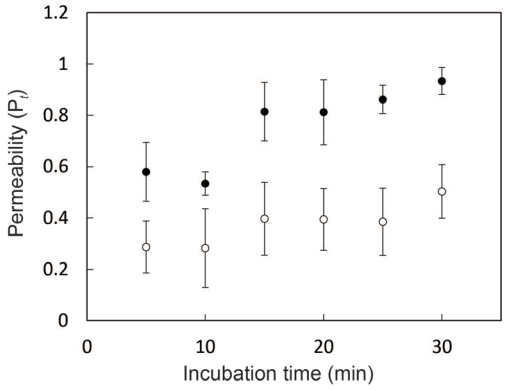

Fig. 4. Time courses of calcein permeability (P_{30min}) in the presence of CYP3A4. Liposomes consisting of chol, PC, and PE (75:98:2, mol/mol) were immobilized on each well and incubated with a 1 nM CYP3A4 solution containing solutions A and B for 120 min. The calcein permeability (P_t) was obtained by adding 5 µl of a DTT-activated SLO solution. The P_t values obtained in the presence (open circles) and absence (closed circles) of CYP3A4 are plotted against incubation time. Error bars indicate mean ± standard deviation (n = 3). Ex. 488 nm, Em. 530 nm.

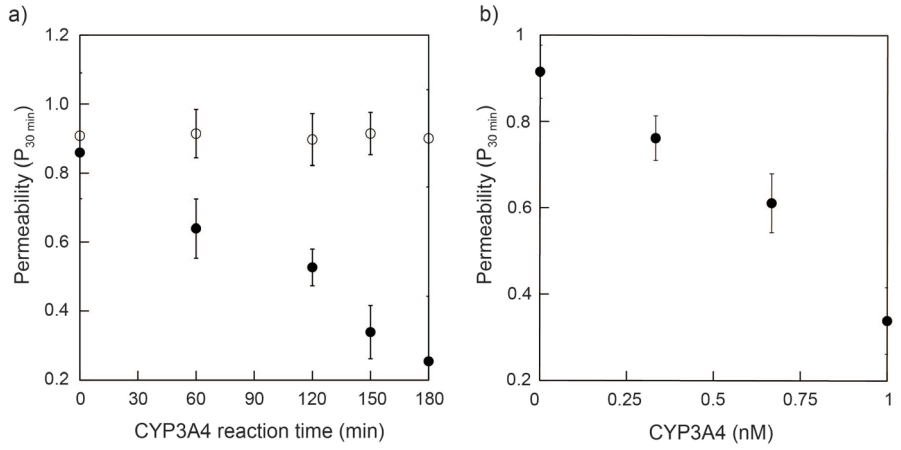

Fig. 5. CYP3A4 activity for oxidation of chol in liposome membrane. Liposomes consisting of chol, PC, and PE (75:98:2, mol/mol) were immobilized on each well. (a) Time courses of calcein permeability (P_{30min}) in the presence (closed circles) and absence (open circles) of CYP3A4. The immobilized liposomes were incubated with a 1 nM CYP3A4 solution containing solutions A and B for a given time. The calcein permeability at 30 min (P_{30min}) was obtained by adding 5 µl of a DTT-activated SLO solution. P_{30min} is plotted against CYP3A4 reaction time. (b) Effect of cholesterol oxidase concentration on calcein permeability (P_{30min}). The immobilized liposomes were incubated with a given concentration of CYP3A4 in the presence of solutions A and B. Calcein permeability (P_{30min}) after adding 5 µl of a DTT-activated SLO solution was measured. Averages of three measurements are plotted. Error bars indicate mean ± standard deviation (n = 3). Ex. 488 nm, Em. 530 nm.

On the other hand, no change in P_{30min} was observed in the case without CYP3A4. Furthermore, the fluorescence intensities obtained after 180 min of incubation of the liposomes with and without CYP3A4 were 100 ± 2.5 and 93 ± 16, respectively. These intensities were almost the same, indicating that CYP3A4 did not disturb the lipid membranes. After 150 min of enzymatic reaction of CYP3A4, the concentration dependence of P_{30min} was investigated [Fig. 5(b)]. A higher concentration of CYP3A4 led to the decrease in P_{30min} almost linearly (r^2 = 0.976). The

detection limit was 33 nM, as calculated from P_{30min} at three times the standard deviation of P_{30min} in the absence of CYP3A4, i.e., a signal-to-noise ratio of 3. These indicated that the measurements of P_{30min} allowed us to evaluate the progress of the conversion of chol to 4β-OH chol by CYP3A4.

3.3 Inhibitory effect of CYP3A4 by erythromycin

Erythromycin, which is a macrolide antibiotic, is known to inhibit CYP3A4 activity.[32] Before evaluating the CYP3A4 inhibition by erythromycin, we investigated the effect of the antibiotic and the solution of the NADPH regeneration system, i.e., $NADP^+$, glucose-6-phosphate dehydrogenase, and glucose-6-phosphate, on the stability of the liposomes [Fig. 6(a)]. An erythromycin solution was added to liposomes consisting of PC, PE, and chol (0.98:0.02:0.75, mol/mol). The fluorescence intensity of the liposomes did not change after 150 min of incubation in the presence of erythromycin, $NADP^+$, glucose-6-phosphate dehydrogenase, and glucose-6-phosphate, and the fluorescence intensity increased after the treatment of the liposomes with Triton X-100. Therefore, erythromycin and the NADPH regeneration system did not affect the stability of the liposomes significantly. In addition, erythromycin and the NAPDH regeneration system did not alter the nanopore forming ability of SLO [Fig. 6(b)]. The liposomes were incubated with a CYP3A4 solution containing erythromycin, and the fluorescence intensity was measured after adding an SLO solution. Figure 6(c) shows the relationship between the residual

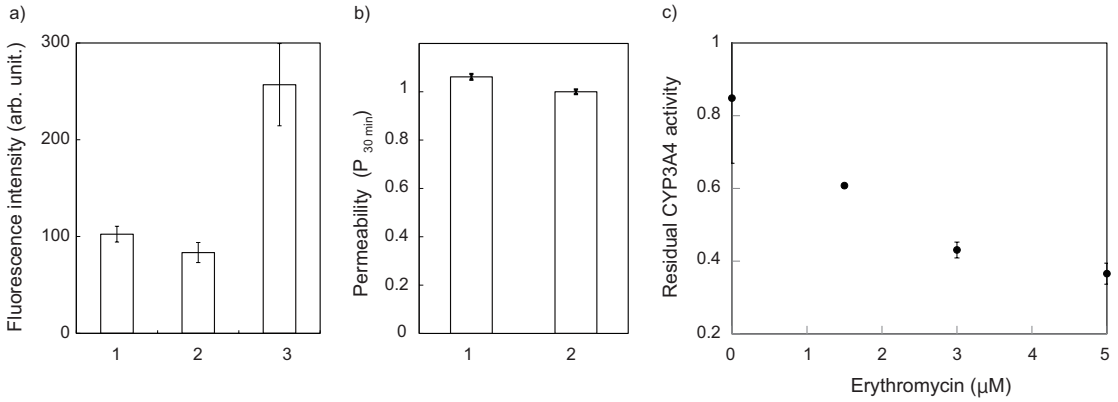

Fig. 6. Inhibition of CYP3A4 activity for oxidation of chol in liposome membrane by erythromycin. Liposomes consisting of chol, PC, and PE (75:98:2, mol/mol) were immobilized on each well. (a) Stability of liposomes encapsulating calcein. The immobilized liposomes were incubated for 150 min in the presence (2) or absence (1) of 5 μM erythromycin and a mixture of solutions A and B. (3) After the incubation of the liposomes in the presence of erythromycin and solutions A and B, the liposomes were treated with a Triton X-100 solution. (b) Effect of erythromycin on SLO nanopore forming ability. The immobilized liposomes were incubated for 120 min in the presence (2) or absence (1) of 5 μM erythromycin and a mixture of solutions A and B. After incubation for 150 min, the calcein permeability at 30 min (P_{30min}) was obtained by adding 5 μl of a DTT-activated SLO solution. (c) Inhibitory effect of erythromycin on CYP3A4 activity of chol oxidation. After a mixture of erythromycin and CYP3A4 was incubated for 30 min, solutions A and B were added into the immobilized liposomes. After incubation for 150 min, the calcein permeability at 30 min (P_{30min}) was obtained by adding 5 μl of a DTT-activated SLO solution. Averages of three measurements are plotted. Error bars indicate mean ± standard deviation (n = 3). Ex. 488 nm, Em. 530 nm.

activity of CYP3A4 and the erythromycin concentration. The residual activity decreased with increasing erythromycin concentration. A previous study on pharmacokinetic parameters showed that doses of erythromycin resulted in a plasma level on the order of μM[33] at which erythromycin may affect cholesterol metabolism in blood cell membranes, considering our data.

4. Conclusion

In this study, the CYP3A4-mediated oxidation of cholesterol in lipid membranes was measured by utilizing the SLO nanopore forming ability. Liposomes composed of PC, PE, and chol (0.98:0.02:0.75, mol/mol) were used for measurements of the CYP3A4-mediated oxidation of cholesterol because of the linear relationship between P_{30min} and 4β-OH chol fraction. The incubation of the immobilized liposome with CYP3A4 decreased P_{30min}. Furthermore, P_{30min} did not change when the liposomes were incubated with CYP3A4 in the presence of erythromycin. These indicated that the decrease in P_{30min} resulted from the conversion of chol to 4β-OH chol mediated through CYP3A4 activity. Because there was little information on cholesterol oxidation in the lipid membrane, except for MD simulation, this method can be used as a tool to understand the physiological role of cholesterol oxidation in lipid bilayers.

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