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Effect of amyloid β-peptide on the fluidity of phosphatidylcholine membranes: Uses and limitations of diphenylhexatriene fluorescence anisotropy

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ABSTRACT

There is accumulating evidence that peptide-induced perturbations in the order and dynamics of cellular membranes may play a role in the neurotoxicity of amyloid β-peptide (Aβ). Several studies have reported that Aβ decreases fluidity of membranes based on an Aβ-induced increase in the fluorescence anisotropy of diphenylhexatriene (DPH). However, the effect of Aβ on the membrane fluidity is still a subject of controversy, because other studies that employed pyrene as a fluorescent probe have shown that Aβ has the opposite effect. To reveal the reason for this discrepancy, we have examined the effect of Aβ on the fluidity of phosphatidylcholine membranes using spectroscopic methods. The fluorescence anisotropy of DPH is dramatically increased on addition of Aβ to DPH-containing phosphatidylcholine membranes. However, Aβ does not affect the Raman spectrum of the membrane, which is sensitive to the packing order of the hydrocarbon chains of lipids. We have also found that circular dichroism (CD) bands of DPH appear during incubation of DPH-containing membranes with Aβ, whereas DPH is an achiral molecule. The observed CD bands of DPH are induced by a chiral environment of Aβ but not by that of the lipids, because positive CD bands appear regardless of the D/L-chirality of phosphatidylcholine. The findings obtained from CD measurements provide evidence that DPH molecules translocate from the membrane to Aβ. The peptide-mediated extraction of DPH from the membrane may cause changes in the fluorescence anisotropy of DPH, even though Aβ does not affect the fluidity of membranes.

1. Introduction

Amyloid β-peptide (Aβ) accumulation in the brain is one of the pathological hallmarks of Alzheimer's disease [1,2]. Aβ spontaneously self-assembles to form toxic oligomers or fibrils that may be central to disease pathology [1,3]. An increasing amount of evidence supports a hypothesis that both aggregation and cytotoxic effects of Aβ are strongly associated with its ability to interact with cell membranes [4–6]. For example, Aβ is known to have affinity for negatively charged lipid bilayers which contain acidic lipids such as gangliosides [7]. Aβ can also bind to electrically neutral membranes consisting of phosphatidylcholine with the zwitterionic head group, but only when the membranes are in the gel phase [8]. Physical properties of lipid membranes such as the membrane order affect not only affinity of Aβ for membranes but also the secondary structure of Aβ. Especially, tightly packed lipid membranes serve as a platform for self-association of Aβ to form β-sheet-rich aggregates [8].

In addition to the abovementioned effects of lipid membranes on the structure and self-association of Aβ, Aβ has also been suggested to perturb the order and dynamics of membranes [5,9–19]. Changes in the membrane fluidity are proposed to affect cell membrane functions and properties including activity of membrane-associated proteins [20–23]. Although accumulating evidence suggests that the peptide-induced perturbations in membranes may play a crucial role in the neurotoxicity of Aβ, there are conflicting reports about the effects of Aβ on the fluidity of membranes [5,6]. Several studies have examined the effects of Aβ on fluidity of biological or model membranes by measuring a steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH), and concluded that Aβ rigidifies the membranes based on the increased fluorescence anisotropy of the membrane-embedded DPH [9–16]. On the other hand, the intensity ratio of the excimer/monomer fluorescence of pyrene is another sensitive index of membrane fluidity [24,25]. Studies in which pyrene was employed as a fluorescent probe have shown that Aβ increases fluidity of membranes [17–19]. At present, there is no agreement on the membrane-rigidifying properties of Aβ, in spite of its potential relevance to pathological processes.

Abbreviations: Aβ, amyloid β-peptide; DPH, 1,6-diphenyl-1,3,5-hexatriene; CD, circular dichroism; ICD, induced circular dichroism; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DPHpPC, 1-palmitoyl-2-[2-[4-(6-phenyl-trans-1,3,5-hexatrienyl)phenyl]ethyl)carbonyl)-3-*sn*-phosphatidylcholine; T_m, main phase transition temperature

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In the present study, we examine the uses and limitations of DPH fluorescence anisotropy as a method for investigating the effect of Aβ on membrane fluidity. Aβ causes a significant increase in the fluorescence anisotropy of DPH when an aggregated form of the peptide is added to DPH-containing phosphatidylcholine membranes in the liquid crystalline phase. However, Raman spectra of lipid membranes do not provide evidence that the added Aβ affects the packing order of the hydrocarbon chains of the membranes. The appearance of circular dichroism (CD) of DPH demonstrates that DPH molecules at least partly translocate from the membrane to the aggregated peptides. The extraction of DPH by Aβ may be a potential cause of the change in the fluorescence anisotropy of DPH.

2. Materials and methods

2.1. Preparation of liposomes

The L-isomer of phosphatidylcholines, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), were purchased from Avanti Polar Lipids (Alabaster, AL). D-DPPC, DL-DPPC and DPH were obtained from Sigma-Aldrich (St. Louis, MO). 1-palmitoyl-2-[2-[4-(6-phenyl-trans-1,3,5-hexatrienyl)phenyl]ethyl)carbonyl)-3-*sn*-phosphatidylcholine (DPHpPC) was purchased from Setareh Biotech (Eugene, OR). Concentrations of DPH and DPHpPC were determined in methanol by using molar extinction coefficients $\varepsilon_{350} = 91{,}000\ M^{-1}\ cm^{-1}$ for DPH and $\varepsilon_{350} = 78{,}000\ M^{-1}\ cm^{-1}$ for DPHpPC, respectively [26]. Phosphatidylcholine was dissolved in a 1:1 (v/v) chloroform/methanol mixture. For preparation of liposomes of phosphatidylcholine bilayer containing a fluorescent probe, chloroform solution of DPH or DPHpPC was added to the lipid solution. The solution was spread as a thin layer onto the wall of a round-bottom flask by removing the solvent with a rotary evaporator, and then a trace of the solvent was completely removed by drying under vacuum overnight. Sodium phosphate buffer (5 mM, pH 7.4) was then added to the flask and the lipid film was hydrated under vigorous shaking for 10 min. The suspension containing multilamellar vesicles was sonicated using an ultrasonic generator with a tip probe (Nihonseiki, US-50) until the suspension became transparent. Titanium particles from the tip probe were removed by centrifugation.

2.2. Preparation of peptides

A 42-residue human Aβ peptide, Aβ1–42 (DAEFRHDSGYEVHHQ KLVFFAEDVGSNKGAIIGLMVGGVVIA), was purchased from Wako Chemical Co. (Osaka, Japan) and used without further purification. Human Aβ1–40 peptide was synthesized on an automated peptide synthesizer (Applied Biosystems model 431A). The crude peptide was dissolved in 5 mM ammonium acetate buffer (pH 10.5) and purified by HPLC on a reversed-phase column (Asahipak ODP50-10E). The peptide was dissolved in ammonium hydroxide (pH 11), dispensed into aliquots, and lyophilized. The amount of peptide in each aliquot was determined from the UV absorption intensity of tyrosine ($\varepsilon_{275} = 1410\ M^{-1}\ cm^{-1}$).

2.3. Steady-state fluorescence anisotropy of DPH

Lyophilized Aβ peptide was dissolved at a concentration of 5 or 10 μM in phosphate buffer (5 mM, pH 7.4) that contains DPH-labeled liposomes (0.5 mM phosphatidylcholine and 10 μM DPH). A 100 μL-aliquot of the mixture was introduced into a 3 × 3 mm quartz cuvette and allowed to equilibrate at the temperature of experiment in a thermostated cuvette holder of a spectrofluorometer. Steady-state fluorescence anisotropy measurements were performed on a Jasco FR-6300 spectrofluorometer equipped with excitation and emission polarizers. Excitation and emission wavelengths were set at 360 and 454 nm, respectively. Fluorescence anisotropy (r) was calculated using the Eq. (1):

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the fluorescence intensities determined at vertical and horizontal orientations of the emission polarizer, respectively, when the excitation polarizer is set in the vertical position. The G factor, which compensates for differences in detection efficiency for vertically and horizontally polarized light, was calculated from the fluorescence intensity ratio of vertical and horizontal emissions when the excitation polarizer is set in the horizontal position (I_{HV}/I_{HH}). The fluorescence intensities were averaged over the last 10 s of total measurement time of 20 s in a time-course mode.

2.4. Raman spectroscopy

A suspension of liposomes consisting of 5 mM DPPC or that mixed with Aβ (0.1 mM) was sealed in a glass capillary tube and excited with the 488-nm line of a diode laser (Cyan-488-150, Spectra-Physics, Santa Clara, CA). The sample temperature was regulated with a constant-temperature circulator. Raman scattered light was collected with a 20× objective lens (Olympus, Japan) and dispersed on a micro-Raman spectrometer (NRS-3100, JASCO, Japan) equipped with a thermoelectrically cooled CCD detector (DU401-BV-120, Andor, UK).

2.5. CD Spectroscopy

Lyophilized Aβ peptide was dissolved at 10 μM in phosphate buffer (5 mM, pH 7.4) that contains DPH-labeled liposomes (0.5 mM phosphatidylcholine and 10 μM DPH). Induced CD (ICD) spectra of DPH were recorded on a Jasco J-820 spectropolarimeter using a quartz cell of 5-mm path length and averaged over four scans for each sample. A quartz cell of 1-mm path length was used for measurements of CD spectra of peptides. The background signal due to the quartz cell and lipid was recorded separately and subtracted from the spectra of peptide–liposome suspensions.

3. Results

3.1. Effects of Aβ on the fluorescence anisotropy of DPH

The DPH fluorescence anisotropy characterizes the rate of the probe rotational diffusion and is well correlated with the membrane fluidity when DPH is embedded in membranes [27,28]. We investigated the effect of Aβ1–42 on the steady-state fluorescence anisotropy of DPH in the liposomal bilayer of DPPC which undergoes the main phase transition at 41 °C (Fig. 1a) [29]. In the absence of Aβ, the anisotropy is as low as or below 0.1 when the membranes are in the fluid liquid crystalline phase at temperatures higher than the main phase transition temperature (T_m). The fluorescence anisotropy increases with a decrease in temperature. A steep increase in anisotropy occurs around the T_m at which the membrane undergoes the transition from the fluid liquid crystalline phase to the more rigid gel phase.

Aβ has a distinct effect on the fluorescence anisotropy of DPH when the DPPC membranes are in the liquid crystalline phase (Fig. 1a). At temperatures higher than the T_m, the DPH fluorescence anisotropy is unusually high for the liquid crystalline membrane in the presence of Aβ. At a peptide-to-lipid ratio of 1:50, Aβ raises the anisotropy to ~0.2 which is rather close to that in the gel phase membrane. A similar Aβ-induced increase in the DPH fluorescence anisotropy has been observed in previous studies and was explained by a rigidifying effect of Aβ on the membranes [12,13].

The effect of Aβ on the anisotropy of membrane-embedded fluorescent probes was also examined with DPHpPC which is a phospholipid

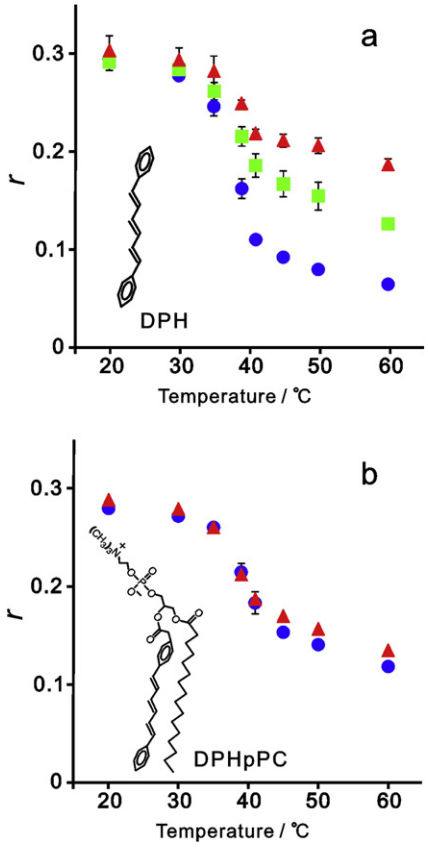

Fig. 1. Temperature dependences of fluorescence anisotropy (r) of DPH (a) and DPHpPC (b) in DPPC bilayer membranes in the presence of 0 (circle), 5 (square; for DPH only), and 10 μM (triangle) Aβ1–42. Concentrations of DPPC and DPH (DPHpPC) in the liposome suspension (pH 7.4) were 500 and 10 μM, respectively. Each data point represents average of three measurements with standard deviations indicated by the error bars.

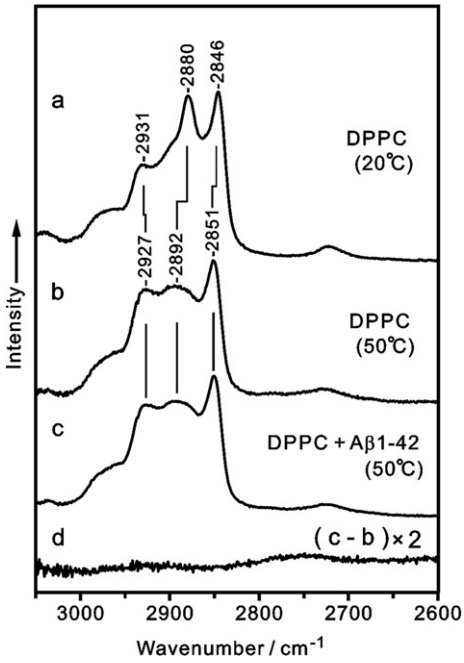

Fig. 2. Raman spectra of DPPC liposomes at a lipid concentration of 5 mM (pH 7.4); (a) liposome alone at 20 °C, (b) at 50 °C, (c) after 1 h of co-incubation with 0.1 mM Aβ1–42 at 50 °C, and (d) the difference (c)–(b). The difference spectrum was magnified by a factor 2.0. Strong bands in the 3000–2800 cm^{-1} interval are due to the C–H stretching vibrations of the methylene and methyl groups of the saturated hydrocarbon chains. Raman bands of peptide can be observed neither in the raw spectrum (c) nor in the difference spectrum (d) at the peptide-to-lipid ratio of 1:50.

derivative of DPH (inset of Fig. 1b). A plot of the fluorescence anisotropy of DPHpPC versus temperature produces a sigmoidal curve like that of DPH (Fig. 1). A higher anisotropy of the DPHpPC fluorescence compared to that of DPH at temperatures higher than the T_m of DPPC is attributable to a more restricted motion of the fluorophore in DPHpPC due to a covalent linkage of the fluorophore to the glycerol moiety [30]. Another significant difference between DPH and DPHpPC is the sensitivity of their anisotropies to Aβ. In contrast to DPH, the fluorescence anisotropy of DPHpPC is much less sensitive to the addition of Aβ when the membranes are in the liquid crystalline phase (Fig. 1b). Aβ is judged to have no significant effect on the fluidity of the DPPC membrane from the insensitivity of the DPHpPC anisotropy to the addition of Aβ.

3.2. Effects of Aβ on the membrane order probed by Raman spectroscopy

The effect of Aβ on the membrane order was also examined by Raman spectroscopy. The top two traces in Fig. 2 are Raman spectra of DPPC liposomes at 20 and 50 °C, respectively, in the absence of Aβ. The strong and sharp bands at 2880 and 2846 cm^{-1} in the spectrum obtained at 20 °C are assigned to the methylene antisymmetric and symmetric C-H stretching vibrations of the saturated hydrocarbon chains, respectively [31]. Both a significant broadening and a high-wavenumber shift of the 2880 cm^{-1} band are observed upon increasing temperature from 20 to 50 °C. These spectral changes can be ascribed to changes in the intermolecular vibrational coupling in addition to the *trans-gauche* conformational changes in the hydrocarbon chains on the gel-to-liquid phase transition of the membrane [32]. In contrast, the 2846 cm^{-1} band is less sensitive to the melting transition. Thus the intensity ratio of Raman bands at 2880 and 2846 cm^{-1} can be used as a sensitive probe for the lateral packing of the hydrocarbon chains. Raman spectrum of the DPPC liposomes after 1 h of co-incubation with Aβ1–42 at 50 °C (Fig. 2c) is nearly identical to the corresponding spectrum of the liposomes alone (Fig. 2b). No difference is seen between Raman spectra of the liposomes in the absence and presence of Aβ even in the difference spectrum (Fig. 2d). This indicates that Aβ1–42 does not increase the packing order of the hydrocarbon chains in the liquid-crystalline membranes at the peptide-to-lipid ratio of 1:50, at which Aβ1–42 significantly increases the fluorescence anisotropy of DPH (Fig. 1a).

3.3. Aβ-induced CD of DPH

In order to examine whether the Aβ-induced increase in the fluorescence anisotropy of DPH properly reflects a change in the rotational mobility of DPH molecules in the membrane bilayers, we examined the possibility that DPH could translocate from the membranes to Aβ by using CD spectra of DPH. Although DPH itself is achiral, ICD arises when DPH is embedded in membranes of chiral phospholipid and when the membranes are in the gel phase [33]. For example, positive ICD bands appear in the 340–380 nm interval of CD spectrum of DPH-containing liposomal membranes of the naturally occurring L-isomer of DPPC at 20 °C (inset of Fig. 3a). These CD bands are assignable to the lipid-induced CD of DPH, because a vertical mirror-image spectrum with a negative sign is obtained when L-DPPC is replaced by D-DPPC (inset of Fig. 3b).

Next, we measured CD spectra of DPH-containing L-DPPC liposomes at 50 °C where the membranes are in the liquid crystalline phase. In the absence of Aβ, no CD is given from the liposomes at this temperature (inset of Fig. 3a). This is consistent with the previous observation that the ICD of DPH is given by the gel membranes but not by the liquid crystalline membranes [33]. However, ICD bands appeared during co-incubation of the DPH-containing L-DPPC membranes with Aβ1–42 at 50 °C (Fig. 3a). After addition of Aβ1–42 to the liposomes, weak negative

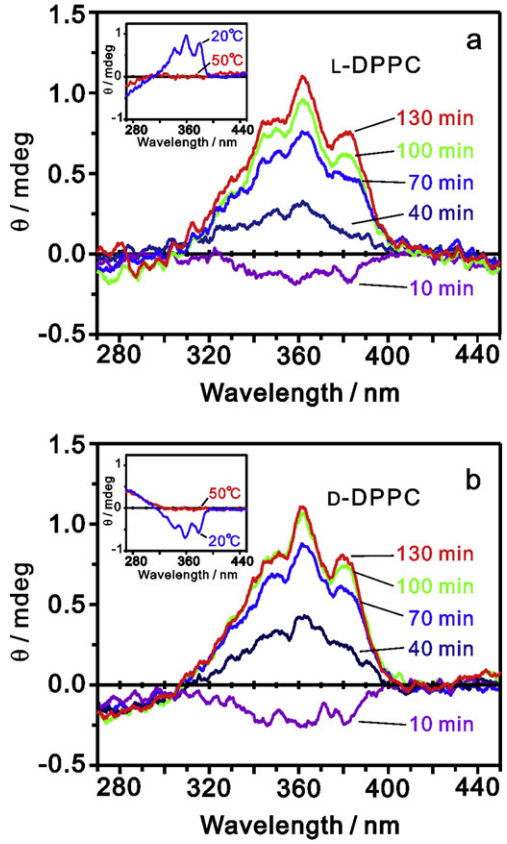

Fig. 3. ICD spectra of DPH-containing L-DPPC (a) and D-DPPC (b) membranes after incubation at 50 °C with Aβ1–42 at incubation periods of 10, 40, 70, 100, and 130 min. Concentrations of lipid, DPH, and peptide were 500, 10, and 10 μM, respectively. Insets are ICD spectra of DPH embedded in L-DPPC (a) and D-DPPC (b) membranes in the gel phase (20 °C) and in the liquid crystalline phase (50 °C) in the absence of Aβ1–42.

CD bands of DPH initially appear in the spectrum. The direction of the CD growth is reversed about 30 min after addition of Aβ and positive ICD bands gradually increase in intensity.

A similar experiment was performed by using DPHpPC instead of DPH. In the absence of Aβ, lipid-induced CD bands are also observed for DPHpPC in L-DPPC membranes only at a temperature where the membranes are in the gel phase (Fig. 1S, Supplementary material). Unlike DPH, however, no significant ICD of the membrane-embedded DPHpPC appears after incubation with Aβ1–42 at 50 °C (Fig. 1S, Supplementary material).

The appearance of ICD of DPH on addition of Aβ1–42 seems to be consistent with that Aβ1–42 has a rigidifying effect on the DPPC membranes. If Aβ binds to the liquid-crystalline membranes and yields highly ordered regions, something like microdomains, in the vicinity of the binding sites, the DPH molecules that are distributed within the regions possibly show a lipid-induced CD in the chiral microenvironment of the membrane. However, this hypothesis is inconsistent with the results obtained from the Raman spectra of lipids (Fig. 2). Alternatively, chiral environments of peptide or its assemblies may induce CD of DPH, if DPH translocates from the membrane to Aβ. In order to elucidate the origin of the ICD of DPH, we measured CD spectra of DPH-containing liposomal membranes which were prepared from the optical isomer of the naturally occurring DPPC. As shown in Fig. 3b, positive CD bands of DPH eventually appear after co-incubation of DPH-containing membranes with Aβ1–42 at 50 °C even when L-DPPC is replaced by D-DPPC. We have also confirmed that a similar result was obtained with the racemic mixture of L-DPPC and D-DPPC (Fig. 2S, Supplementary material), which by itself will never show CD of DPH even in the gel phase. Taken together, it can be summarized that the ICD of DPH with the positive sign appears regardless of the chirality of DPPC in the presence of Aβ. The findings of our study clearly show that the CD bands of DPH observed at 50 °C are not induced by the lipid bilayers. DPH molecules may at least partly translocate from the DPPC membranes to Aβ peptides, and a chiral environment of peptides is most likely to be the origin of the ICD of DPH.

3.4. Temperature dependence of DPH transfer from the membrane to Aβ

The evidence for the translocation of DPH from the membrane to Aβ was obtained from the appearance of the induced CD of DPH at 50 °C (Fig. 3). Next, we examined the DPH transfer at a lower temperature near the physiological range. The DPPC membrane undergoes the phase transition from the liquid crystalline to the gel phase at 41 °C on lowering temperature, but the latter phase will not appear in the cellular membranes. Since the gel phase membrane is not appropriate for a model of the cellular membranes, the following results were obtained with the use of POPC that forms the liquid crystalline membrane at temperatures > −2.5 °C [29]. Aβ was co-incubated with the POPC bilayers that contain DPH, and then the CD spectra of the sample solution were measured after various incubation intervals. Fig. 4 shows time-dependent changes of the peak intensity (~360 nm) of the ICD of DPH. No CD appeared in the absence of Aβ1–42 (data not shown), indicating that the observed CD bands were induced by Aβ1–42. The ICD of DPH gradually increases in intensity and reaches a plateau after about 100 min of incubation at 50 °C. At 37 °C, on the other hand, the ICD initially grows toward the negative direction and then turns back to the positive direction. Although the time course of the ICD growth at 37 °C is different from that at 50 °C, the appearance of the ICD bands of DPH provides evidence that the translocation of DPH from lipid membranes to Aβ occurs at a physiological temperature. The appearance of a transient negative ICD suggests a possibility that at least two structurally different arrangements of Aβ assemblies occur during incubation. The time course of the ICD intensity may not simply reflect the transfer of DPH from the membrane to Aβ, but is contributed by structural changes of the peptides that are responsible for the ICD of DPH.

3.5. Secondary structure and aggregation state of Aβ that promotes DPH release from lipid bilayers

The secondary structure of Aβ1–42 peptide has been investigated by measuring CD spectra of the peptide in the 190–260 nm interval under the experimental condition identical to that was employed for the fluorescence anisotropy (Fig. 1) and ICD (Fig. 3) measurements (10 μM peptide in the presence of DPH-containing DPPC membranes, at 50 °C). A pair of positive and negative bands at 195 and 217 nm characteristic

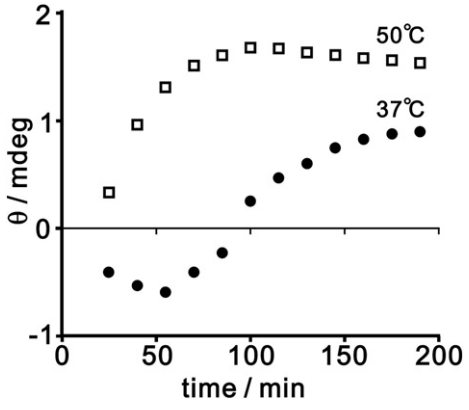

Fig. 4. Peak intensity of the 360-nm ICD band of DPH as a function of incubation time. The DPH-containing POPC membranes were co-incubated with Aβ1–42 at 37 °C (solid circle) and 50 °C (open square). The membranes were in the liquid crystalline phase at both temperatures. Concentrations of lipid, DPH, and peptide were 500, 10, and 10 μM, respectively.

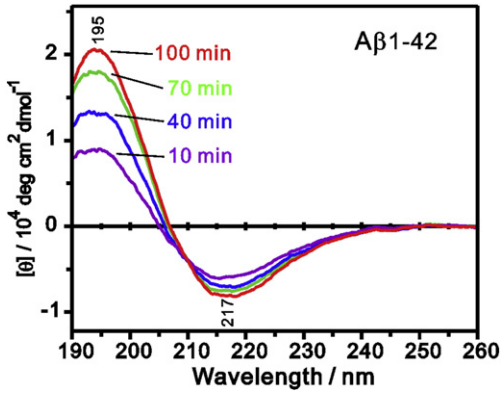

Fig. 5. CD spectra of Aβ1–42 incubated at 50 °C with DPH-containing DPPC membranes at incubation periods of 10, 40, 70, and 100 min. Concentrations of lipid, DPH, and peptide were 500, 10, and 10 μM, respectively.

of β-sheet structure increases in intensity during incubation (Fig. 5) [34]. The CD signature of β-sheet structure is already seen after 10 min of incubation, indicating that the Aβ1–42 peptide readily forms a β-sheet-rich structure at 50 °C. This indicates that the Aβ1–42 peptide, which causes both the increase in the fluorescence anisotropy and the ICD of DPH, is primarily in an aggregated form. In order to examine whether the peptide aggregation is essential for the extraction of DPH from lipid bilayers, we measured CD spectra of DPH-containing DPPC membranes in the presence of Aβ1–40 which has a weaker propensity to aggregate than Aβ1–42. The CD spectrum in the 190–260 nm region is dominated by a negative peak at 198 nm, indicating that Aβ1–40 is structurally disordered in the unaggregated form (Fig. 3S(a), Supplementary material) [34]. ICD bands of DPH did not appear upon co-incubation with the unaggregated Aβ1–40 (Fig. 3S(b), Supplementary material). We also confirmed that the unaggregated Aβ1–40 did not alter the fluorescence anisotropy of DPH in DPPC membranes (Fig. 3S(c), Supplementary material).

4. Discussion

4.1. The cause of Aβ-induced increase in the fluorescence anisotropy of DPH

In the present study, we examined the effect of Aβ on the anisotropy of membrane-embedded fluorescence probes, DPH and its phospholipid derivative. The addition of Aβ to DPPC membranes increases the fluorescence anisotropy of DPH in a concentration-dependent manner when the membranes are in the liquid crystalline phase (Fig. 1a). In sharp contrast, the fluorescence anisotropy of DPHpPC is insensitive to the addition of Aβ regardless of the phases of the membrane where DPHpPC is incorporated (Fig. 1b). Although both DPH and DPHpPC have been widely used to measure the membrane fluidity, there are some notable differences between these probes. For example, DPHpPC is preferentially accommodated in relatively fluid regions of lipid membranes, whereas DPH partitions equally between the fluid and the solid membranes [35]. The partition preference for the fluid membrane seems to explain the diminished effect of Aβ on the fluorescence anisotropy of DPHpPC, because DPHpPC may stay in the fluid regions of the membranes even if solid-like domains were generated only in the vicinity of the sites where Aβ deposits. However, the results obtained from Raman spectroscopy suggest that Aβ may have no effect on the packing order of the hydrocarbon chains of the DPPC membrane and provide no evidence for the presence of the Aβ-induced solid-like domains (Fig. 2).

The Aβ-phospholipids interaction has been studied in previous works by using fluorescence of either the phenol group of Tyr10 [8] or an artificial probe that is covalently attached to the peptide or lipid molecules [36,37]. Since the membrane-associated Aβ often undergoes a conformational transition from an unfolded to a folded structure rich in α-helix or β-sheet, CD spectra of Aβ have also been used to make an estimate of the Aβ-membrane binding [38–41]. These previous studies have shown that Aβ interacts preferentially with acidic membranes such as those consisting of anionic phospholipids or containing gangliosides. In contrast, Aβ does not bind to neutral membranes consisting of zwitterionic phosphatidylcholine from egg yolk [36], bovine brain [38], or synthetic phosphatidylcholine [39]. In our more detailed study, Aβ was found to have an affinity for the electrically neutral phosphatidylcholine membranes only when the membranes are in the gel phase [8]. A strong interaction that could lead to the membrane rigidification is unlikely to occur between Aβ and membranes in the liquid crystalline phase. As shown in the present study (Fig. 1a) and by others [13], however, the Aβ-induced increase in the fluorescence anisotropy of DPH was observed at temperatures above the T_m of the membrane. As a consequence of the above considerations, the observed changes in the fluorescence anisotropy may not be attributable to the rigidifying effect of Aβ on lipid membranes.

Another notable property of DPHpPC distinct from DPH is its inability to exchange between membranes through solution [30]. Based on the lack of exchangeability between membranes, the translocation of DPHpPC from the membrane to Aβ is also considered to be unlikely to occur. This assumption is supported by our findings that no significant peptide-induced CD of DPH appears after co-incubation of the DPHpPC-containing liposomal membranes with Aβ1–42 (Fig. 1S, Supplementary material). The difference in the exchangeability of these two fluorescent probes provides important insights into the mechanism of the Aβ-induced changes in the fluorescence anisotropy of DPH. Because a significant increase in the fluorescence anisotropy on addition of Aβ was observed only when the exchangeable fluorophore was used as the probe, the observed change in anisotropy is consistently explained by the transfer of the probe molecules from the membrane to Aβ, but cannot be ascribed to an environmental change of the probe molecules which stay in the membrane. The fluorescence anisotropy of DPH associated with aggregated Aβ under lipid-free condition is as high as that in the gel phase membrane (~0.29, Supplementary material), which also supports our interpretation of the Aβ-induced increase in the DPH fluorescence anisotropy.

4.2. Structural requirements of Aβ for the extraction of DPH from lipid membranes

It has been pointed out previously that the aggregated Aβ, but not the monomeric Aβ, causes an increase in the steady-state fluorescence anisotropy of DPH in POPC membranes [12]. We have observed in the present study that the increase in the fluorescence anisotropy of DPH in the DPPC membranes is induced by aggregated Aβ1–42 but not by unaggregated Aβ1–40 (Figs. 1 and 1S). The different effects of aggregated and unaggregated Aβ on the DPH anisotropy were previously explained by a stronger rigidifying effect of the aggregated Aβ on the membranes compared with the unaggregated one [12]. We propose an alternative explanation based on the findings from the CD spectra of Aβ and DPH. The Aβ1–42 peptide that is responsible for the ICD of DPH is in an aggregated form rich in β-sheet structure as shown by CD spectra of the peptide (Fig. 5). In contrast, the ICD of DPH did not appear on incubation with unaggregated Aβ1–40 (Fig. 3S, Supplementary material). These results suggest that aggregation of Aβ is essential not only for the elevation of the fluorescence anisotropy but also for the extraction of DPH from membranes. Therefore, a greater ability of the aggregated peptide to extract DPH from membranes can consistently account for the greater effect of the aggregated peptide on the DPH anisotropy.

As indicated by the appearance of a transient negative ICD and the subsequent growth of a positive ICD of DPH (Figs. 3 and 4), at least two structurally different arrangements of Aβ assemblies may occur during the incubation period. This assumption seems to be consistent with the finding that an oligomeric and a fibrillar Aβ adopt antiparallel

and parallel β-sheet structures, respectively [42]. Although the structural details of the aggregates that are responsible for the negative and positive ICD of DPH are still under investigation, our findings suggest a possibility that the Aβ-induced CD of DPH can be used for detection of transient species on the amyloid-forming pathway of Aβ.

4.3. The limitations of DPH fluorescence anisotropy as a method for estimating the effect of Aβ on the membrane fluidity

Fluidity of cell membranes is thought to play an important role in various cellular functions including enzyme activity [20,21], signal transduction [22], and hormone activity [23]. It is now well established that factors perturbing membrane fluidity can have profound effects on the activity of membrane proteins that are essential for cell viability [43,44]. Hence, binding of Aβ to the plasma membrane offers a potential explanation for its neurotoxicity if Aβ has a distinct perturbing effect on the membrane fluidity. A number of studies have examined the effects of Aβ on the fluidity of not only liposomal membranes [12,13] but also natural membranes extracted from mouse or human brains [5,9–11,14–16] by measuring the fluorescence anisotropy of DPH. These previous works concluded that Aβ rigidifies the membranes especially in the aggregated form based on the increased fluorescence anisotropy of the membrane-embedded DPH. However, the appearance of Aβ-induced CD of DPH demonstrates in this study that DPH molecules are at least partly transferred from a lipid to a peptide environment on addition of Aβ to the DPH-containing membrane. Although the fluorescence anisotropy of DPH is a convenient and useful tool to study membrane fluidity, the extraction of DPH by aggregated Aβ can disturb the estimation of membrane fluidity. The fluorescence anisotropy of DPH has also been used to investigate the effect of other amyloid proteins such as transthyretin [43] and lysozyme [45] on the fluidity of lipid membranes.

Pyrene is also frequently used as a fluorescent probe for study of membrane fluidity. The intensity ratio of the excimer/monomer fluorescence of pyrene decreases with increasing pressure, content of cholesterol or decreasing temperature, all of which reduce the rate of lateral diffusion of pyrene in membranes [24,25]. The membrane-fluidity dependences of the excimer/monomer ratio of pyrene and anisotropy of DPH were compared in a previous study, and a close correlation was found between these parameters [25]. However, several works, in which pyrene fluorescence was applied to the investigation of the effect of Aβ on membranes, have shown that Aβ has a fluidizing effect [17–19]. This conclusion contrasts with that of the DPH anisotropy studies [9–16]. Based on our findings, the translocation of DPH from the membranes to Aβ might be considered as a potential cause of this discrepancy, although the possibility of a direct interaction between pyrene and Aβ is also required to examine. Resistance of the membrane-embedded probes to the extraction by additives is an important requirement for the fluorescence techniques to be applied to investigation of the effect of proteinaceous aggregates on the membrane fluidity.

5. Conclusions

The results obtained from Raman spectroscopy of lipid membranes and fluorescence anisotropy of DPHpPC demonstrate that neither monomeric nor aggregated Aβ affects the fluidity of phosphatidylcholine membranes. Our results also provide insight into the reason for the Aβ-induced increase in the fluorescence anisotropy of DPH in a DPH/lipid/Aβ mixture. The Aβ-induced CD bands of DPH appear during incubation of DPH-containing membranes with Aβ1–42, which provides evidence for the transfer of DPH molecules from the membrane to Aβ. The extraction of DPH from lipid membranes by Aβ is a potential cause of changes in the fluorescence anisotropy of DPH. The present findings do not rule out the possibility that Aβ affects the fluidity of natural membranes with higher affinity for Aβ compared to phosphatidylcholine membranes. However, the possibility of a membrane-to-peptide transfer of DPH has to be taken into account no matter what kind of lipid membrane is used.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.12.003.

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