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Study of β-Amyloid Peptide (Aβ40) Insertion into Phospholipid Membranes Using Monolayer Technique

S.-R. Ji, Y. Wu, and S.-f. Sui*

Department of Biological Sciences and Biotechnology, State-Key Laboratory of Biomembranes, Tsinghua University, Beijing 100084, People's Republic of China; fax: +8610-62784768; E-mail: suisf@mail.tsinghua.edu.cn

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Abstract—β-Amyloid peptide (Aβ), a normal constituent of neuronal and non-neuronal cells, has been proved to be the major component of extracellular plaque of Alzheimer's disease (AD). The interaction of Aβ with lipid membranes may be essential for its neurotoxicity. Our previous study revealed that membrane insertion may provide a possible pathway by which Aβ prevents itself from aggregation and fibril formation. In the present work we studied the membrane insertion of Aβ and the factors that affect its insertion ability using a monolayer approach. The results show that Aβ is surface active and can insert into lipid monolayers. When a high level of cholesterol is present, Aβ40 can insert into the phospholipid mixtures simulating physiological membrane composition. Acidic pH enhances Aβ insertion, while the effect of ionic strength is rather complex. Aβ insertion ability may be ultimately relative to cholesterol-rich domains in the monolayers, which indicates strong interaction between Aβ and cholesterol.

Key words: β-amyloid peptide, monolayer, membrane insertion, cholesterol

Alois Alzheimer first reported Alzheimer's disease (AD) in 1907. AD is a degenerative disorder of the human brain characterized as neuritic plaques (also called senile plaques) and cerebrovascular amyloid deposits [1]. All the extracellular plaques are made primarily of beta-amyloid peptide (Aβ) with antiparallel β-sheet conformation in the core of plaques. [2]. As the AD-related neurotoxic protein, Aβ, with 40 or 42 amino acid residues and a molecular mass of approximate 4 kD, is a cleavage product of a larger transmembrane amyloid precursor protein (APP). APP can be cleaved by β-secretase and γ-secretase together to generate the intact Aβ molecule, or only by α-secretase to release a larger, soluble fragment, sAPP. Aβ is an amphiphilic peptide with a hydrophilic N-terminal domain and a hydrophobic C-terminal. The latter locates in the transmemebrane domain of APP [3].

As reported in many papers, Aβ is toxic to cultured neuronal cells [4, 5]. To understand the neurotoxic action of Aβ, it is essential to identify specific cellular components that interact with the peptide and mediate a biological response of the affected cells. As demonstrated in a number of observations, a likely primary target of Aβ is the neuronal membrane because the peptide may alter important physical and biological properties of the membrane [6, 7]. Our previous work [8] revealed the interaction between Aβ and membrane, membrane insertion of Aβ, may provide a possible pathway that Aβ avoids aggregation and fibril formation. In this paper we specifically studied the membrane insertion of Aβ and various factors that affect its insertion ability using the monolayer approach.

Abbreviations: Aβ) β-amyloid peptide; AD) Alzheimer's disease; APP) amyloid precursor protein; PC) phosphatidylcholine; PG) phosphatidylglycerol; PS) phosphatidylserine; SA) stearic acid; DPPC) 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine; DPPG) 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoglycerol; DPPS) 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoserine; DMPC) 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine; DLPC) 1,2-didodecadecanoyl-*sn*-glycero-3-phosphocholine; DSPC) 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine; π_i) initial surface pressure; $\Delta\pi$) increase in surface pressure; π_c) critical insertion pressure.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials. 1,2-Dihexadecanoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoglycerol (DPPG), 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoserine (DPPS), 1,2-ditetradecanoyl-*sn*-

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glycero-3-phosphocholine (DMPC), 1,2-didodecadecanoyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine (DSPC), and cholesterol were all purchased from Sigma Chemical Co. (USA). β-Amyloid peptides (1-40) and (1-28) (Aβ) were purchased from AnaSpec Co. (USA), and if not stated, Aβ refers to Aβ(1-40). All the other chemicals used were of analytical grade and manufactured in China. The water was deionized.

Usually the subphase buffer was 0.05 M Tris-HCl containing 0.025 M NaCl with pH 7.4. But in pH change experiments, Na_2HPO_4-citric acid buffer was used to provide sufficient range of pH.

Experimental procedures. We used a NIMA 9000 (England) microbalance to measure the monolayer surface pressure (π), defined as the change of the surface tension after spreading a monolayer on a water surface. A filter paper of 1.0 cm width was employed as the Wihelmy plate. A magnetic bar was used to help the diffusion of protein, and the data were automatically collected and recorded by a computer.

To measure protein insertion, a circular Teflon trough with a volume of 4.0 ml and surface area of 10 cm^2 was used. The trough was specifically designed by Dr. X. H. Han of this laboratory [9]. The trough can convert the vortex flow driven by the magnetic stirring bar into a laminar flow at the subphase surface, and therefore the Wihelmy plate will not be disturbed by the flow of the subphase solution. In addition, it has a smaller subphase volume and a larger surface area compared with the usual one, so that the sample consumption for each measurement can be much reduced.

In the experiments, the circular trough used for insertion assay was first filled with 4.0 ml of buffer. Then the phospholipid monolayers were prepared by carefully spreading the lipid solution on to the buffer surface. The lipid monolayers were spread from a chloroform–methanol (3 : 1 v/v) solution of phospholipid (1.0 mg/ml) to the desired initial surface pressure (π_i). After the film pressure stabilized at a constant value, Aβ was injected into the subphase through a side sample hole. The pressure changes were monitored until the surface pressure increase (Δπ) had reached a maximal value, usually within 2 h. The measurements were performed at temperature of 24.0 ± 1.0°C.

For each example, the value of Δπ was a function of various π_i values. The plot of Δπ versus π_i yields a straight line with negative slope which intersects the abscissa at a limiting surface pressure. The limiting surface pressure is defined as the critical insertion pressure (π_c) of Aβ for the corresponding lipid monolayer. If the π_i of the monolayer is kept at or above π_c, the protein cannot insert into the membrane. Therefore, the value of π_c may quantitatively reflect the ability of the protein to insert into a certain phospholipid monolayer.

RESULTS

Self-penetration of Aβ into an air–water interface. The self-penetration of Aβ into an air–water interface without phospholipid monolayer was measured and the results are shown in Fig. 1 which shows the surface pressure variation as a function of time. Measurements were performed in which the bulk concentrations of Aβ were 200, 400, 500, 600, and 800 nM, respectively. The experimental results show that Aβ can significantly increase the surface pressure, indicating a strong surface activity. From the curves of Fig. 1 we can see that the increasing rate of Δπ is greater when more Aβ was injected into the subphase, which may be the result of quicker diffusion speed of the protein. Since the maximum Δπ induced by the self-penetration of Aβ was found to be 13.7 mN/m with the lowest Aβ concentration of 500 nM, the π_i values of the phospholipid monolayers in the following protein insertion experiments were kept at or above 15.0 mN/m and the peptide concentration was 500 nM. In addition, from the curves of $\Delta\pi-t$ we observe that the surface pressure increases rapidly at first, then gradually levels off and remains stable during 2 h after protein injection. Thus the duration of the following insertion measurements was chosen to be 2 h.

Interactions of Aβ with single component monolayers. The effect of different lipids on the insertion of Aβ into monolayers was measured for DPPC, DPPG, DPPS, and stearic acid (SA). The $\Delta\pi-\pi_i$ curves are shown in Fig. 2.

Figure 2 shows that the injected Aβ induces an increase in all measured samples. No change in surface pressure was observed when only pure buffer control was injected (data not shown). A comparison among the $\Delta\pi-\pi_i$ curves shows that the π_c for the DPPG, DPPS, and SA is slightly higher than for DPPC: the numerical value of π_c for DPPC is 26.6 mN/m, those for negatively charged monolayers DPPG and DPPS are 28 and 28.3 mN/m, respectively, whereas it is 27.2 mN/m for the positively charged monolayer SA.

In the following experiments we chose phosphatidylserine (PS), not phosphatidylglycerol (PG), as the negatively charged phospholipid, which was based on the following understandings: the amount of PS in the inner plasma membrane is significant in common [10]; the binding and internalization of peptide is more efficient when the bilayer contains PS, not PG [11]; and PS in model membranes bind with monovalent and divalent ions more efficiently than PG [12].

Effect of cholesterol on Aβ insertion. The insertion ability of Aβ into DPPC, DPPS, and SA monolayers containing different cholesterol content was studied. Figure 3 shows the relation between π_c and cholesterol for the system of SA and cholesterol (a) and for the system of PS and cholesterol (b) (the system of phosphatidylcholine (PC) and cholesterol was shown in [8]). From Fig. 3 we can see that there is a notable increase in π_c when the content of cholesterol is increased to 33%. Also, there are

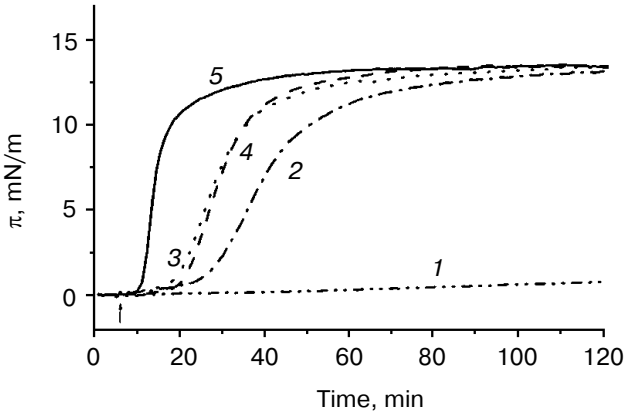

Fig. 1. The π–*t* curves of Aβ reflecting the property of self-penetration with the concentration of Aβ of 200 (*1*), 400 (*2*), 500 (*3*), 600 (*4*), and 800 nM (*5*). The buffer used is 0.05 M Tris-HCl containing 0.025 M NaCl with pH 7.4. Aβ is added directly after surface pressure stabilization without phospholipid monolayer. The arrow represents the point of Aβ addition.

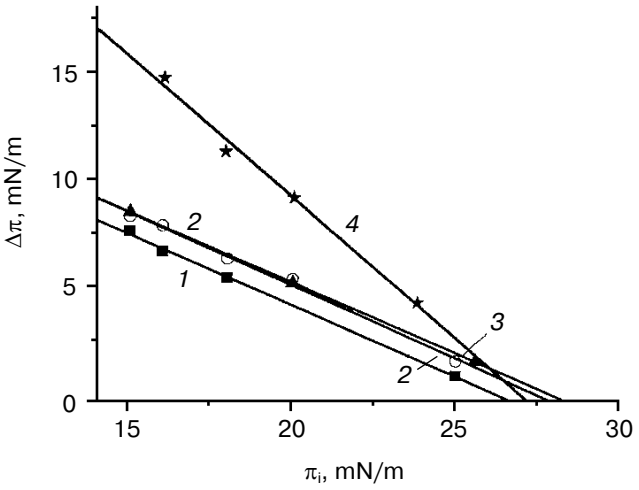

Fig. 2. The Δπ–π_i curves of Aβ inserting into phospholipid monolayers with single component, where the phospholipids are DPPC (*1*), DPPG (*2*), DPPS (*3*), and SA (*4*). A certain amount of phospholipid solution (dissolved in chloroform–methanol, 3 : 1) is layered on the surface of buffer to acquire a stabilized initial surface pressure, and then Aβ is added through a side sample hole. The plot of Δπ versus π_i yields a straight line with negative slope which intersects the abscissa at a limiting surface pressure that is defined as the critical insertion pressure (π_c) of Aβ for the corresponding lipid monolayer. The value of π_c may quantitatively reflect the ability of the protein to insert into a certain phospholipid monolayer.

cholesterol domains in monolayers studied by π–A isotherms [13-19]. In addition, when the content of cholesterol is increased to 33% the π_c values of Aβ to the monolayers are all above 31 mN/m. It is well known that the packing density of lipid monolayer with a surface pressure of 30-32 mN/m can be assumed to be comparable to that of the bilayer [20, 21]. So, Aβ has a capacity for insertion into such lipid mixtures. Since the physiological content of cholesterol in plasma membranes of normal aged brain is about 30% (detailed in [8]), in the following experiments we used 33% cholesterol containing phospholipid system to simulate physiological condition.

Also, we carried out experiments to test the effect of other lipids in brain, for example, cerebroside and sphingomyelin (data not shown). Control experiments (data not shown) were also carried out for other short peptides such as Aβ42 (4.5 kD) and Cecropin B (3.8 kD), and we found no such phenomenon. Therefore, cholesterol has a stronger interaction with Aβ.

Effect of the tail length of phospholipid on Aβ insertion. Figure 4 shows the insertion ability (measured by π_c) of Aβ into phospholipid monolayers with different tail length. From Fig. 4 one can see that π_c value increases with increase in tail length. This result indicates that the hydrophobic interaction between insertion fragment of Aβ and the tail of phospholipids contributes partly to the insertion of Aβ.

Effect of subphase pH value on Aβ insertion. The insertion of Aβ into 33% cholesterol containing DPPC monolay-

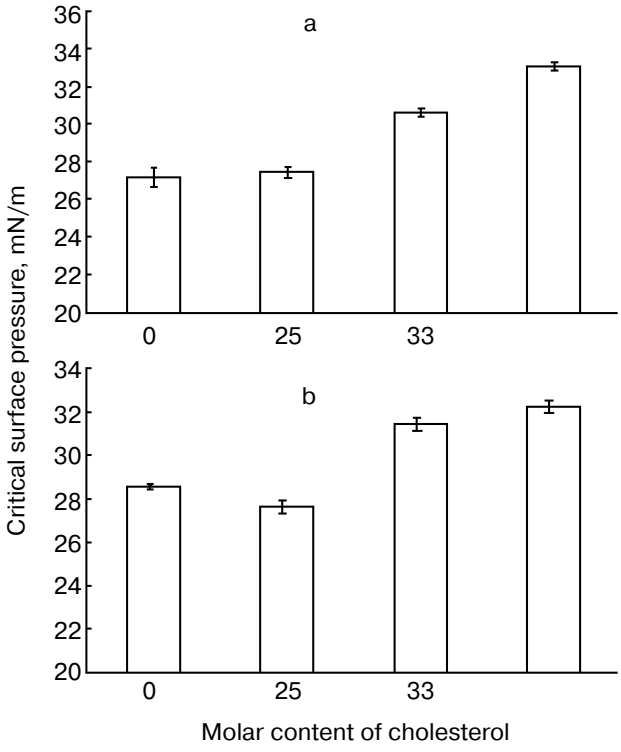

Fig. 3. Fitted π_c–cholesterol content curves for SA (a) and PS (b), respectively. In the experiments phospholipid solution containing different components was used to produce certain monolayers. The figure shows that π_c value increases distinctly when the content of cholesterol is above 33%, which may be due to the presence of cholesterol domains.

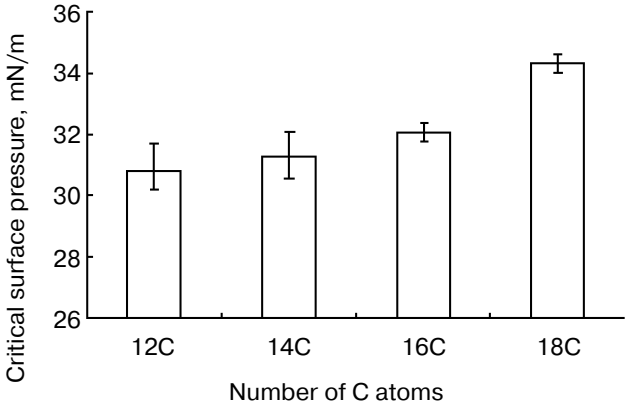

Fig. 4. Effect of the tail length of phospholipid on Aβ insertion using 33% cholesterol containing PC system. Phospholipid molecules with tail length of 12C, 14C, 16C, and 18C were used.

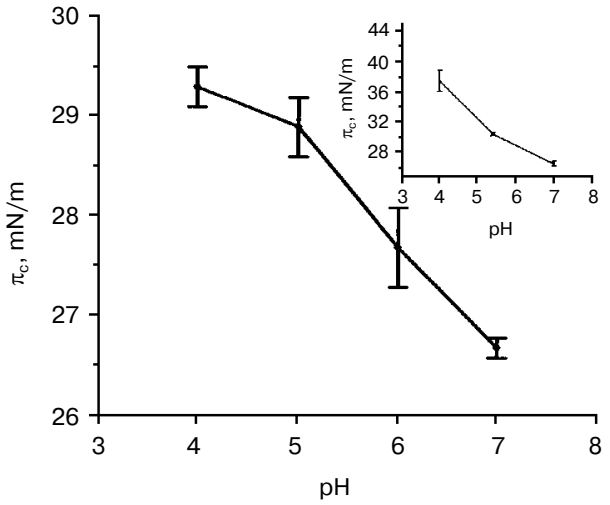

Fig. 5. Effect of pH on Aβ insertion. The buffer used here is Na_2HPO_4-citric acid buffer. The main part of the figure is π_c–pH curves for 33% cholesterol containing PC monolayers. Inset, π_c–pH curves for 33% cholesterol containing PS monolayers.

er under various pH values adjusted by Na_2HPO_4 and citric acid was studied. Figure 5 exhibits the π_c–pH curves, from which we can know clearly that π_c decreases along with the increase in pH, indicating that the ability of Aβ insertion becomes lower at high pH. For 33% cholesterol containing DPPS monolayer similar results were obtained as shown in the inset of Fig. 5. But the effect of pH on negatively charged monolayer is much stronger than on a neutral monolayer.

Effect of ionic strength on Aβ insertion. We used electrolyte NaCl and $CaCl_2$ to change the ionic strength in the subphase. Figure 6a shows the features of Aβ insertion into 33% cholesterol containing DPPC and 33% cholesterol containing DPPS monolayer at different Na^+ concentrations. In the case of the 33% cholesterol containing DPPS monolayer, π_c decreases gradually as the bulk concentration of Na^+ increases from 0.025 to 0.1 M. But when the Na^+ concentration continued to increase from 0.1 to 0.5 M, we found that π_c increases. In contrast with the negatively charged monolayer, the 33% cholesterol containing DPPC monolayer curve of Fig. 6a shows that the π_c value is not sensitive to the Na^+ concentration. The influence of Ca^{2+} is shown in Fig. 6b, which exhibited a similar effect to that of Na^+.

Insertion ability study of Aβ28. We carried out experiments similar to those for Aβ40 to study the property of Aβ28. The self-penetration is shown in Fig. 7a and its insertion into DPPC and DPPG monolayers is in Fig. 7b (the corresponding curves for Aβ(1-40) are also exhibited

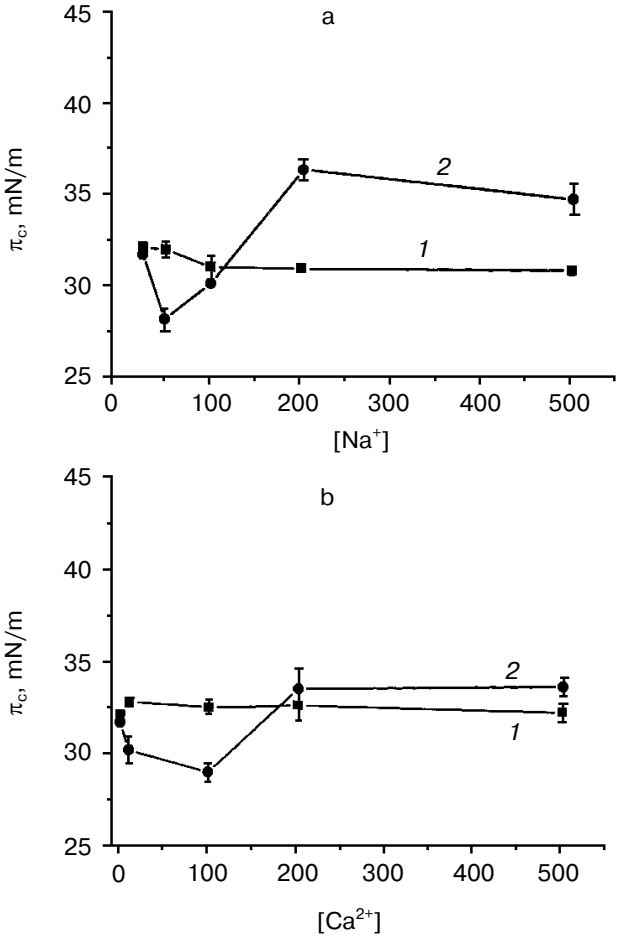

Fig. 6. Effect of ionic strength on Aβ insertion. The figure shows the π_c–[Na^+] (a) and π_c–[Ca^{2+}] (b) curves of Aβ inserting into 33% cholesterol containing PC (*1*) and 33% cholesterol containing PS monolayer (*2*) under various Na^+ and Ca^{2+} concentrations. Tris-HCl buffer (0.05 M) containing the desired amount of NaCl and $CaCl_2$ at pH 7.4 was used.

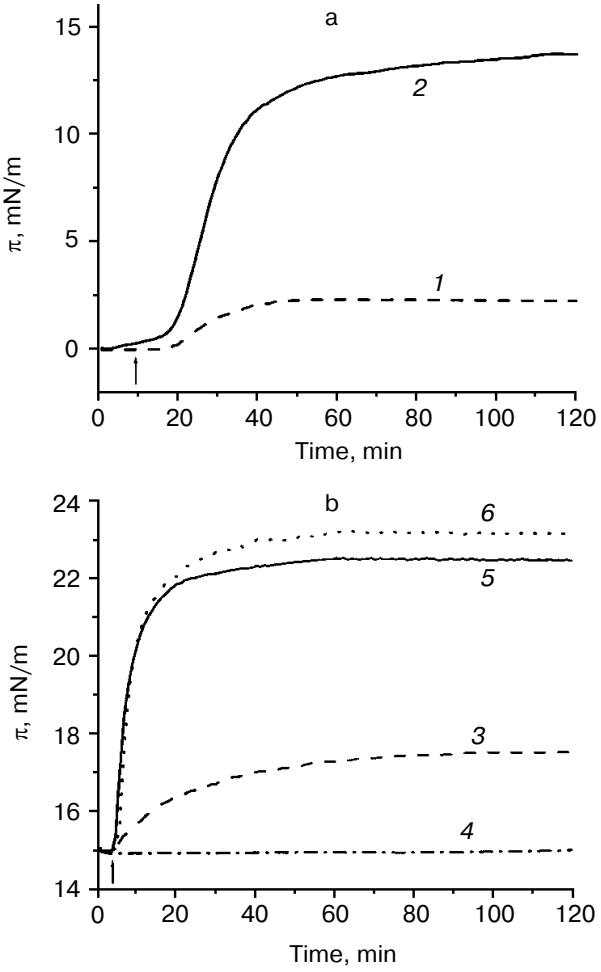

Fig. 7. Self-penetration of Aβ28 (a, *1*) and its insertion into DPPC and DPPG monolayers (b, *3* and *4*, respectively) together with the corresponding curves of Aβ40 for comparison ((a, *2*) and (b, *5* and *6*, respectively)). The conditions for Aβ28 were identical with those for Aβ40 and the concentration of peptide is 500 nM. The arrow is the point of peptide addition.

for comparison). The results show that Aβ28 is hydrophilic and has nearly no surface activity.

DISCUSSION

Considering the fact that the relationship of Aβ with membrane is very important in its neurotoxicity [6, 8, 22-25], we specifically investigated the interaction of Aβ with monolayer. The results of the present study show that Aβ has a strong surface activity. It is believed that the molecules that interact only with the head groups of the lipid monolayer will not increase the surface pressure of the monolayer [26]. Thus, when protein molecules are injected into the subphase, the corresponding $\Delta\pi$ can be interpreted to be the result of the protein inserting into the monolayer. In such studies π_c value is a very useful parameter. In fact that the protein cannot insert into the membrane if the π_i of the monolayer is kept at or above π_c. So the value of π_c may quantitatively reflect the ability of the protein to insert into a certain phospholipid monolayer. Therefore, in the experiments we used the parameter of π_c to compare the Aβ insertion ability into different phospholipids monolayer.

Figure 2 shows that the π_c values of Aβ insertion into the charged lipid monolayers are slightly higher than that into the neutral monolayer. As we have shown, Aβ contains six positive and six negative amide acids at about pH 7.4, so the whole molecule of Aβ is electronically neutral. And Terzi et al. [1, 3] proved that the conformation of Aβ in buffer is mainly random coil, which means that Aβ may expose appropriate charge in the buffer. So for charged monolayers, the concentration of Aβ near the interface may be slightly higher than that in bulk owing to electrostatic interaction. This may explain to a certain extent why the π_c for charged lipid is slight higher than that of neutral lipid. As well as the electrostatic interaction, the tail-length dependence of π_c (as shown in Fig. 4) suggests that hydrophobic interaction is also involved in the membrane insertion of Aβ.

Figure 3 indicates that π_c values of Aβ increase distinctly when the content of cholesterol goes up to 33%. Such phenomena may be due to the presence of cholesterol-rich domains in the mixtures. From our separated experiments (data not shown), we found that there is nearly no change in π_c value of Aβ by adding other phospholipids, such as sphingomyelin and cerebroside. Other short peptides, such as Aβ42 and Cecropin B, do not exhibit similar interaction with cholesterol, maybe due to either inappropriate secondary conformation (cannot expose needed interaction sites) or different sequence (have no interaction with needed amino acid residues), and perhaps due to both. So we speculate that Aβ has a stronger interaction with cholesterol, especially present in the form of cholesterol-rich domains, on which Morishima-Kawashima also reported [27]. In addition, cholesterol and sphingomyelin are components of raft which is believed to play an essential role in transferring apical proteins to apical membrane. Amyloid precursor protein (APP) is an apical protein, which finally localizes on presynaptic membrane. So perhaps cholesterol also has an effect on the localization of Aβ, but this still needs definite experiment to determine.

A change in the subphase pH value and ionic strength can change the degree of the ionization of the protein and the lipids, thus affecting the interaction of Aβ with lipid membrane. From Fig. 5 we can see that π_c value increases as the subphase pH value decreases gradually from 7.0 to 4.0. Such variation in membrane insertion behavior of Aβ may be due to the influence of pH on both membrane and protein. The π–A isotherms of 33% cholesterol containing DPPC monolayer at different pH values were measured. And the results showed that the mean molecule area for DPPC molecules at lower pH just before collapse was

larger than that at neutral pH (data not shown), indicating more area occupied by cholesterol molecules in the monolayer. Such effect of pH on lipid membrane may influence the membrane insertion of Aβ, which may still be relative with cholesterol. In addition, the effect of pH on Aβ itself was investigated in separate measurement by ANS fluorescence which was used to measure the relative exposure of hydrophobic sites. The results showed that the fluorescence intensity increased as pH decreased (data not shown), indicating more hydrophobic groups were exposed under acidic environment. Therefore, the hydrophobic interaction between Aβ and monolayer would be enhanced as pH decreased, which also relates to the membrane insertion ability of Aβ. From the insert of Fig. 5 we can see that for 33% cholesterol containing PS monolayer the pH effect on π_c looks much stronger than that of the PC monolayer. Concretely, in the pH region from 7.0 to 4.0 for neutral lipid membrane the π_c change is 2.6 mN/m, while for negatively charged membrane it is 10.8 mN/m. Such a remarkable difference may be caused by a change in the electrostatic interaction: the whole molecule of Aβ has six positive charges at pH 4.0.

As the subphase Na^+ concentration increased from 0.025 to 0.5 M, the π_c values have nearly no change for neural lipid membranes (33% cholesterol containing PC monolayer). But for negatively charged monolayer (33% cholesterol containing PS monolayer), more complex results are obtained. There are at least two types of changes when increasing Na^+ concentration. First, a high Na^+ concentration can change the surface potential of the system and as a result, the electrostatic interaction between the protein and the lipid membrane will be weakened. Second, the increase in Na^+ concentration may decrease the quantity of H^+ in the diffuse double layer, thus the amount of dissociated PS molecules increases [28, 29]. For separate DPPS system, PS molecules tend to cluster as a result of intermolecular charge interactions ([10], also proven by our experiment using a microfluorescence film balance, data not shown). Therefore PS molecules prefer the formation of domains, which finally results in the increase in the cholesterol domains. Such two opposite effects may result in the trend of π_c values: first decrease and then increase. Concentrated bivalent ions such as Ca^{2+} can affect the intermolecular electrostatic repulsion directly by binding to the lipid head groups and bridging a pair of lipid molecules [29] and then condenses monolayers [30], so more cholesterol domains are generated. Therefore, the attenuation of electrostatic interaction and the increase of cholesterol domains may cause the trend of π_c values: first decrease and then increase.

Since the interaction between Aβ and membrane has been revealed to be an important factor that affects the formation of plaques, the study on the membrane insertion behavior of Aβ under various conditions may be potentially useful for understanding of interaction between Aβ and membrane and the pathogenesis of AD.

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