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Interaction between Alzheimer's Aβ(25–35) peptide and phospholipid bilayers: The role of cholesterol

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ABSTRACT

There is mounting evidence that the lipid matrix of neuronal cell membranes plays an important role in the accumulation of β-amyloid peptides into senile plaques, one of the hallmarks of Alzheimer's disease (AD). With the aim to clarify the molecular basis of the interaction between amyloid peptides and cellular membranes, we investigated the interaction between a cytotoxic fragment of Aβ(1–42), i.e., Aβ(25–35), and phospholipid bilayer membranes. These systems were studied by Electron Paramagnetic Resonance (EPR) spectroscopy, using phospholipids spin-labeled on the acyl chain. The effect of inclusion of charged phospholipids or/and cholesterol in the bilayer composition was considered in relation to the peptide/membrane interaction. The results show that Aβ(25–35) inserts in bilayers formed by the zwitterionic phospholipid dilauroyl phosphatidylcholine (DLPC), positioning between the outer part of the hydrophobic core and the external hydrophilic layer. This process is not significantly influenced by the inclusion of the anionic phospholipid phosphatidylglycerol (DLPG) in the bilayer, indicating the peptide insertion to be driven by hydrophobic rather than electrostatic interactions. Cholesterol plays a fundamental role in regulating the peptide/membrane association, inducing a membrane transition from a fluid-disordered to a fluid-ordered phase. At low cholesterol content, in the fluid-disordered phase, the insertion of the peptide in the membrane causes a displacement of cholesterol towards the more external part of the membrane. The crowding of cholesterol enhances its rigidifying effect on this region of the bilayer. Finally, the cholesterol-rich fluid-ordered membrane looses the ability to include Aβ(25–35).

1. Introduction

In the century which has passed since 1907, when Alois Alzheimer published the first report on the neurodegenerative disorder that now bears his name [1], the incidence of Alzheimer disease (AD) has enormously increased [2]. The main reason for this probably lies in the increase in average human life expectancy. Nowadays AD is the most common form of senile dementia, affecting up to 15 million individuals worldwide. Because of the ongoing increase in life expectancy, by 2050 we can expect approximately 25% of people living in the Western hemisphere to be over 65 years of age, one third of whom are likely to develop AD.

AD is characterised by progressive memory deficit, cognitive impairment and personality changes. From a morphological viewpoint, the main features of AD are [3]: i. accumulation of extracellular amyloid plaques, triggered by the aggregation of the β-amyloid peptides (Aβ), in the brain of affected individuals; ii. presence of intracellular neurofibrillary tangles, which consist mainly of aggregated forms of protein tau.

The Aβ peptides present a length ranging between 39 and 43 amino acids; they are derived by cleavage of the amyloid precursor protein (APP) in the transmembrane region by a γ-secretase and at its N-terminus by a β-secretase [4]. The patho-physiological role of these peptides in causing AD is not yet assessed. In the past years amyloid plaques were generally regarded as the cause of the cognitive disorder. More recently, the relevance of soluble protofibrillar oligomeric forms of Aβ has been recognised [5]. Particularly, it has been shown in vivo that the neurotoxic effect of Aβ(1-42) is independent of plaques formation [6] and that protofibrillar intermediates of Aβ induce progressive neurotoxicity in cortical neurons [7].

In the Aβ peptides sequence, the residues from 29 to the C-terminus belong to the transmembrane domain of APP, and consequently these peptides are expected to favourably interact with lipid membranes, both as monomers and in aggregated forms [8,9]. Various studies have been conducted in the attempt to explore the molecular basis of interactions between Aβ peptides and cellular membranes [10,11]. Particularly, researchers have focused their attention on the

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interaction between a cytotoxic fragment of Aβ(1–42), i.e., Aβ(25–35), and phospolipid bilayers of different composition and net charge. However, the conclusions are still controversial and, to some extent, contradictory. Terzi et al., by analysing circular dichroism and calorimetry data, proposed the adsorption of the peptide on the surface of anionic bilayer, driven by electrostatic interactions, to be the predominant phenomenon; according to the same authors no interaction would occur between Aβ(25–35) and uncharged membranes [11]. A similar conclusion was drawn by Martínez-Senac et al., on the basis of infrared spectroscopy evidences [12]. In contrast, the analysis of X-ray diffraction pattern led Mason et al. to the conclusion that Aβ(25–35) inserts into the hydrocarbon core of uncharged lipid bilayers [13]. Successively, Dante et al. performed a very accurate and detailed analysis of the interaction between Aβ(25–35) and lipid bilayers by using the neutron diffraction technique. These authors evidenced the partitioning of the peptide in two populations, one inserted quite deeply in the bilayer core and the other one adsorbed on the bilayer surface. The relative ratio of these two populations is determined by the phospholipids net charge, in the direction that an excess negative charge increases the fraction of external peptides [14,15]. More recently, Lau et al. pointed out that, in *in vitro* experiments employing model membranes, the Aβ(25–35) location could depend on the procedure through which the samples are prepared: when added after liposome formation, the peptide preferentially interacts with the lipid head-groups; when included during liposome formation, it inserts deeper into the bilayer [16].

Another aspect that has attracted the researchers' interest is the effect of cholesterol on the Aβ insertion into a lipid bilayer. Cholesterol is an essential component of animal cellular membranes and is well known for influencing the membrane fluidity, permeability and dielectric properties. A large body of data has been collected in an attempt to clarify how enrichment or depletion of membrane cholesterol relates to AD. The results, reviewed in Puglielli et al. [17], Wolozin [18] and Ledesma et al. [19], are highly controversial. From one side, the cholesterol content in neuronal cells of AD patients has been found to be low [20]. In contrast, an inhibiting effect of cholesterol on channel formation of Aβ in lipid bilayers, thus preserving the membrane functionality, has been reported [21]. Concerning the fragment Aβ(25–35), a recent work by Dante et al. has put in evidence, by neutron diffraction, that high levels of cholesterol inhibit the insertion of Aβ(25–35) in lipid bilayers [22].

Electron Paramagnetic Resonance (EPR) spectroscopy, by using spin-labeled substances, has proved to be a fruitful experimental approach to the study of the interactions between peripheral as well as integral proteins and membranes. Both the protein, or the polypeptide, and the lipid can be labeled, so that the systems can be studied by different "points of view". In a previous work we investigated the interaction of spin-labeled Aβ(25–35) with surfactant micelles and phospholipid liposomes, finding that the C-terminal portion of the peptide is highly associated to the aggregates, while the N-terminal part extends into the external aqueous medium with occasional contacts with the lipid head-group region [23]. Particularly, the interaction of the C-terminal portion of the peptide is enhanced with charged bilayers.

An alternative approach is the study of peptide–membrane interaction by using spin-labeled lipids. By this way, surface association can be distinguished from membrane penetration and transmembrane insertion by the characteristic effects on the lipid chain mobility that are registered in the spin-label spectrum [24,25]. EPR is well suited to this kind of investigation, because its dynamic sensitivity is optimally matched to the timescale of the rotational motions of the lipids in biological membranes. In this work, we investigate the Aβ(25–35)–membrane interaction by using phospholipid EPR probes that are labeled on the acyl chain. The effect of both the bilayer charge and the presence of cholesterol have been investigated, in the attempt to clarify the molecular bases of the peptide–membrane interaction.

2. Materials and methods

2.1. Materials

Dilauroyl phosphatidylcholine and phosphatidylglycerol (DLPC and DLPG) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Spin-labeled phosphatidylcholine (1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphocholine, *n*-PCSL) with the nitroxide group at two different positions, $n=5$ or $n=12$, in the *sn*-2 acyl chain, were also purchased from Avanti Polar Lipids. The spin-labels were stored at −20 °C in ethanol solutions at a concentration of 1 mg/mL. Cholesterol (CHOL) was obtained from Sigma (St. Louis, MO, USA).

2.2. Peptide synthesis

The Aβ(25–35) amyloid peptide, GSNKGAIIGLM, was manually synthesized by conventional solid-phase chemistry using the Fmoc/tBu strategy. The TentaGel S RAM (0.20 mmol/g capacity) resin was purchased from Fluka (St. Louis, MO, USA). After deprotection of the 9-fluorenylmethoxycarbonyl (Fmoc) group with 30% piperidine in *N*,*N*-Dimethylformamide (DMF), the amino acids in 4 fold excess were coupled with the growing peptide chain, using DMF solution with an equimolar excess of 1-Hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazol-1-yl-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). Peptide–resin cleavage and side chain deprotection reactions were carried out in 95% Trifluoroacetic acid (TFA), 2.5% water and 2.5% Triisopropylsilane (TIS). The resin was filtered, and the solution added drop wise to cold *tert*-butylmethyl ether in order to precipitate the peptide. All the reagents were from Novabiochem (Darmstadt, Germany) or Sigma Aldrich; solvents used in peptide synthesis were obtained from Carlo Erba (Milano, Italy) and used without further purification.

Analytical HPLC was carried out with a System Gold 125 s model (Beckmann Coulter, Fullerton, CA, USA) equipped with a UV166 detector. Preparative HPLC was carried out with a Waters 600E system controller equipped with a 486 UV detector (Waters, Milford, MA, USA). A Jupiter (Phenomenex, Torrance, CA, USA) C18 column (25×4.6 cm, 5 μm, 300 Å pore size) was used for analytical runs and a Vydac (Hesperia, CA, USA) C18 column (25×10 cm, 5 μm, 300 Å pore size) was used for peptide purification. Analytical separations were performed with a linear gradient (5–50% in 45 min) of CH_3CN in water containing 0.1% TFA. Flow rate: 1 ml/min. UV detection: 210 nm. The sample purity was >98%. The peptide was characterized by mass spectra analysis with a Finnigan LCQ-Deca ion trap instrument equipped with an electrospray source (LCQ-Deca Finnigan, Milano, Italy); samples were directly infused in the ESI source by using a syringe pump at a flow rate of 5 μL/min. Data were analyzed with Xcalibur software.

Unstructured peptide aggregates, which are often present in dry samples from synthesis, can severely hamper solubility [26]. For this reason, peptide samples to be used in EPR experiments were treated with trifluoroacetic acid immediately before being dissolved in the final mixture.

2.3. Sample preparation

Samples of DLPC multi lamellar vesicles (MLV) for EPR spectroscopy were prepared as follows: 20 μg of DLPC, dissolved in a CH_2Cl_2–methanol mixture (2:1 v/v), and 1% (wt/wt) of the spin-label, dissolved in ethanol, were thoroughly mixed, and a thin lipid film was produced by evaporating the solvents with dry nitrogen gas. Final traces of solvents were removed by subjecting the sample to vacuum

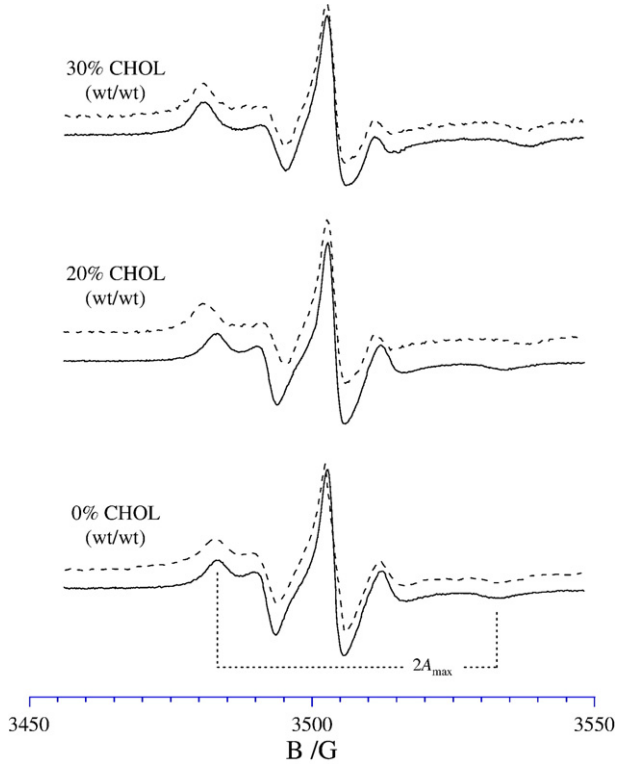

Fig. 1. EPR spectra of 5-PCSL in DLPC/CHOL bilayer membranes, in the absence (solid line) and in the presence (dashed line) of 1:1 wt/wt Aβ(25–35).

desiccation for at least 3 h. The samples were then hydrated with 20 μL of 10 mM phosphate buffer, gently warmed at ~35 °C, and repeatedly vortexed. The lipid suspension thus obtained was transferred into a 25 μL glass capillary.

MLV's of DLPC including also DLPG and/or CHOL were prepared by the same procedure, mixing appropriate amounts of lipid or sterol solutions in CH_2Cl_2–methanol before evaporation of the organic solvents.

Samples containing the peptide–lipid complex were prepared in a similar manner, except that the lipid film was hydrated directly with the peptide solution in phosphate buffer.

2.4. EPR measurements

EPR spectra were recorded with a 9 GHz Bruker Elexys E-500 spectrometer (Bruker, Rheinstetten, Germany). Samples were placed in 25 μL glass capillaries and flame sealed. The capillaries were placed in a standard 4 mm quartz sample tube containing light silicone oil for thermal stability. All the measurements were performed at 25 °C. Spectra were recorded using the following instrumental settings: sweep width, 120 G; resolution, 1024 points; time constant, 20.48 ms; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; incident power, 6.37 mW. Several scans, typically 16, were accumulated to improve the signal-to-noise ratio. Values of the outer hyperfine splitting, $2A_{max}$, were determined by measuring the difference between the low-field maximum and the high-field minimum, through a home-made, MATLAB-based software routine. The main source of error on the $2A_{max}$ value is the uncertainty in composition of samples prepared by mixing few microliters of mother solutions. For this reason, reproducibility of $2A_{max}$ determination was estimated by evaluating its value for selected independently prepared samples with the same nominal composition. It was found to be ±0.2–0.3 G. In general, $2A_{max}$ is dependent on both the amplitude (i.e., order) and rate of chain rotational motion [27], and is therefore a useful parameter for characterising chain dynamics in phospholipid membranes.

3. Results

3.1. Effect of inclusion of charged lipids and cholesterol in phospholipid membranes

As a preliminary part of our research, we investigated DLPC membranes incorporating phosphatidylcholine spin-labeled on the 5 C-atom of the *sn*-2 chain (5-PCSL). This label presents the nitroxide group close to its hydrophilic headgroup. The EPR spectrum of 5-PCSL in DLPC membranes, shown in Fig. 1 (lower spectrum, solid line), presents a clearly defined axially anisotropic lineshape, with a value of the outer hyperfine splitting, $2A_{max}$, equal to 49.6±0.1 G. We also investigated DLPC membranes including phosphatidylcholine spin-labeled on the 12 C-atom of the *sn*-2 chain (12-PCSL), in which the nitroxide group is positioned close to the terminal methyl region of the chain. In this case a narrow three-line, quasi-isotropic spectrum is obtained, see Fig. 2 (lower spectrum, solid line). The higher isotropy of the 12-PCSL spectrum with respect to that obtained for 5-PCSL indicates a flexibility increase in segmental chain mobility in going from the polar headgroups to the inner hydrophobic core, which is a characteristic hallmark of the liquid-crystalline state of fluid phospholipid bilayers [28].

The effect of the inclusion of charged phospholipid on the bilayer fluidity, as monitored by 5-PCSL, has been investigated by substituting a fraction of the zwitterionic phospholipid DLPC with its anionic analogue, DLPG. Inspection of Fig. 3, open circles, shows that the $2A_{max}$ values very slightly increase with the DLPG content (note, however, the expanded ordinate axis of the figure). This indicates that the electrostatic repulsion among the charged DLPG headgroups on the bilayer surface induces a weak structuring effect.

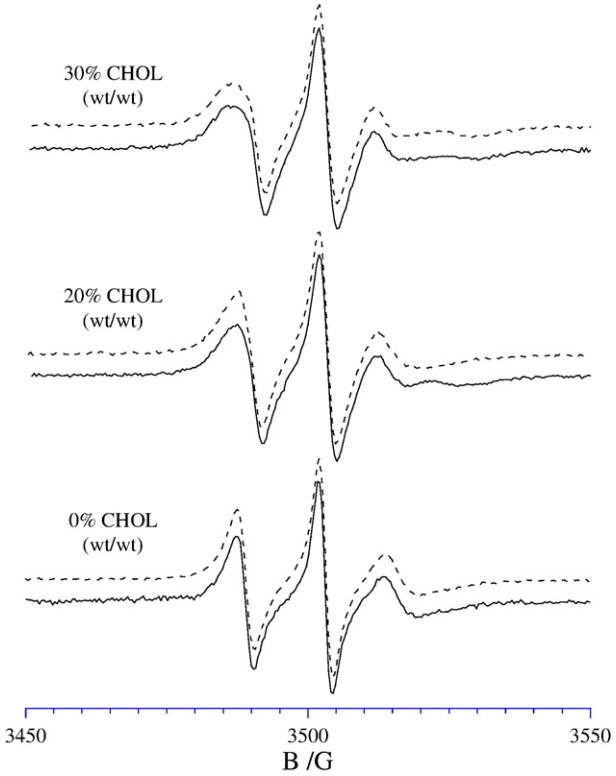

Fig. 2. EPR spectra of 12-PCSL in DLPC/CHOL bilayer membranes, in the absence (solid line) and in the presence (dashed line) of 1:1 wt/wt Aβ(25–35).

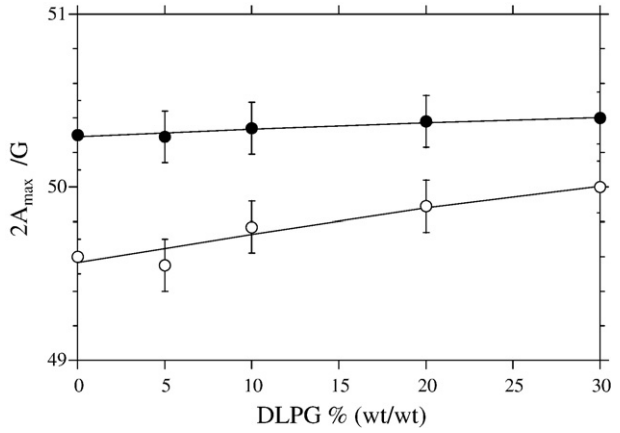

Fig. 3. Outer hyperfine splitting, $2A_{max}$, of 5-PCSL in DLPC/DLPG bilayers as a function of the DLPG content, in the absence (open circles) and in the presence of 1:1 wt/wt Aβ(25–35) (solid circles).

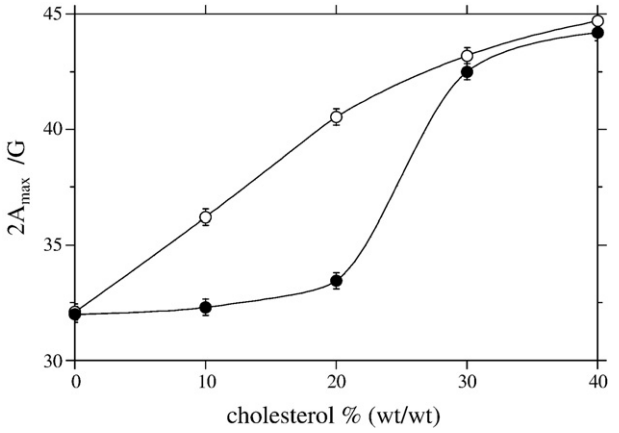

Fig. 5. Outer hyperfine splitting, $2A_{max}$, of 12-PCSL in DLPC/CHOL bilayers as a function of the cholesterol content, in the absence (open circles) and in the presence of 1:1 wt/wt Aβ(25–35) with respect to total lipids (solid circles).

We also investigated DLPC and DLPC/DLPG (70:30 wt/wt) membranes, at various concentrations of cholesterol. Selected EPR spectra of 5-PCSL in DLPC/CHOL lipid samples are shown in Fig. 1, solid lines; for all investigated samples, a clearly defined axially anisotropic spectrum is obtained. Inspection of the figure shows significant perturbations due to the presence of cholesterol. The dependence of the outer hyperfine splitting, $2A_{max}$, on the CHOL content in the bilayer is reported in Fig. 4, open circles. A sharp increase in $2A_{max}$ is evident at ca. 20% wt/wt cholesterol on total lipids, putting in evidence a decrease in lipid chain mobility; addition of cholesterol above 30% wt/wt results in a more limited $2A_{max}$ increase. This evidence should be related to the well-established transition of the lipid bilayer structure from a CHOL-poor fluid lamellar phase, Lα, to a CHOL-rich fluid-ordered phase, L_{01} [29,30].

Interestingly, the corresponding change in the 12-PCSL spectrum is more gradual than that observed in the 5-PCSL one, see Fig. 2. In an attempt to easily quantify this evidence, also in the case of 12-PCSL spectra in DLPC/CHOL membranes we determined the outer hyperfine splitting, $2A_{max}$. The results are shown in Fig. 5, open circles, and confirm that no sharp transition can be detected with this label.

Addition of CHOL to the partially charged DLPC/DLPG membranes leads to an increase in the $2A_{max}$ value of included 5-PCSL. However, the trend, reported in Fig. 6, shows a linear $2A_{max}$ increase, different from the sigmoid registered in DLPC bilayers (see Fig. 4). Thus, the quite abrupt transition from disordered to ordered fluid phases is substituted by a gradual bilayer structuring.

3.2. Effect of the bilayer charge on the interaction of Aβ(25–35) with phosphatidylcholine and phosphatidylcholine/phosphatidylglycerol membranes

The association of Aβ(25–35) with lipid membranes can be detected by the perturbation of the chain mobility of spin-labeled lipids, by using EPR spectroscopy as found for classical water-soluble peripheral membrane proteins and derived peptides [31,32]. Addition of Aβ(25–35) to pure DLPC bilayers, at a 1:1 wt/wt ratio, significantly affects the 5-PCSL spectrum, see Fig. 1, dashed line. In fact, the peptide causes a slight but significant $2A_{max}$ increase (from 49.6± 0.1 G to 50.4±0.2 G), i.e., the mobility of the spin-labeled chains decreases by interaction of the peptide with the membrane. Restriction in the mobility of phospholipid acyl chains interacting with the peptide was detected also by other experimental techniques, such as solid-state NMR [16]. In contrast, the addition of Aβ(25–35) to pure DLPC bilayers does not causes any change in the 12-PCSL spectrum, see Fig. 2.

The electrostatic contribution in driving the association of Aβ(25–35) with lipid membranes has been monitored by substituting a fraction of the zwitterionic phospholipid DLPC with its anionic analogue, DLPG. At all the considered DLPG concentrations, addition of 1:1 wt/wt Aβ(25–35) with respect to total lipids leads to a $2A_{max}$ increase, see Fig. 3. This variation is almost unaffected by the DLPG concentration, i.e. by the bilayer surface charge.

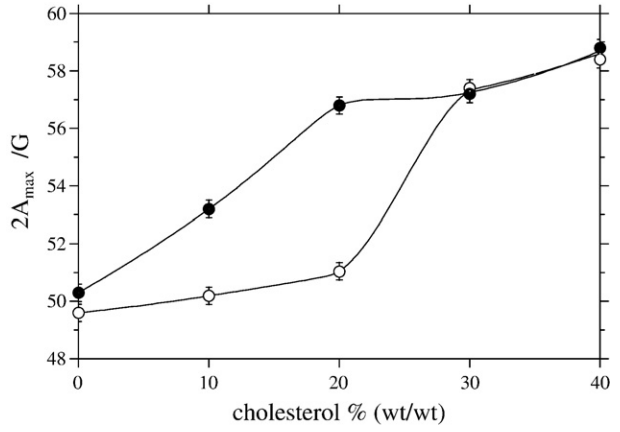

Fig. 4. Outer hyperfine splitting, $2A_{max}$, of 5-PCSL in DLPC/CHOL bilayers as a function of the cholesterol content, in the absence (open circles) and in the presence of 1:1 wt/wt Aβ(25–35) with respect to total lipids (solid circles).

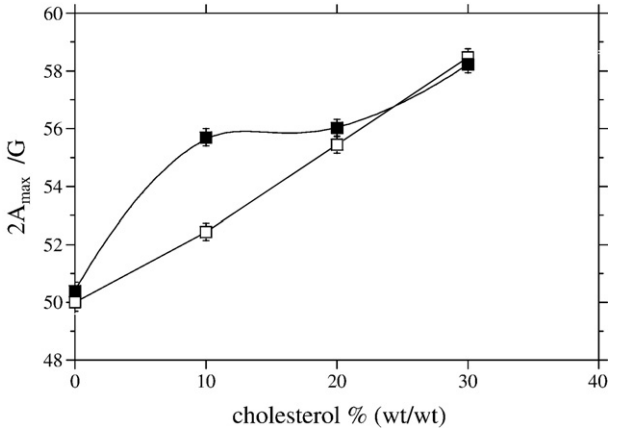

Fig. 6. Outer hyperfine splitting, $2A_{max}$, of 5-PCSL in DLPC/DLPG/CHOL bilayers as a function of the cholesterol content, in the absence (open squares) and in the presence of 1:1 wt/wt Aβ(25–35) (solid squares). In all samples the DLPC/DLPG ratio is 70:30 wt/wt.

3.3. Effect of cholesterol on the interaction of Aβ(25–35) with phosphatidylcholine and phosphatidylcholine/phosphatidylglycerol membranes

Addition of 1:1 wt/wt Aβ(25–35) with respect to total lipids significantly affects the 5-PCSL spectra in DLPC/CHOL membranes (Fig. 1) and the consequent $2A_{max}$ trend with cholesterol content (Fig. 4, solid circles). With introducing cholesterol in the membrane, the variation of the $2A_{max}$ value due to the presence of the peptide increases, reaching a maximum at a cholesterol content equal to 20% wt/wt, see Figs. 1 and 4. However, at higher cholesterol content the 5-PCSL spectrum is not affected by the presence of the peptide.

The effect of the addition of Aβ(25–35) on the 12-PCSL spectra in DLPC/CHOL membranes is shown in Fig. 2. At a 20% wt/wt CHOL content, a quasi-isotropic spectrum is obtained in the presence of the peptide, as opposed to the clearly defined axially anisotropic spectrum obtained in its absence. This is particularly revealed by the loss of the second high-field minimum. At higher cholesterol content the spectrum lineshape is not affected by the presence of the peptide anymore. The $2A_{max}$ trend confirms what is inferred from the spectra. In fact, at a CHOL content<30% wt/wt the presence of the peptide causes a significant $2A_{max}$ lowering with respect to that registered in its absence, while at higher CHOL content the $2A_{max}$ value is not perturbed by the peptide.

In the presence of Aβ(25–35), the introduction of CHOL in DLPC/DLPG (70:30 wt/wt) membranes causes an initial increase in the $2A_{max}$ value due to the presence of the peptide. This variation reaches a maximum at a CHOL content equal to 10% wt/wt, see Fig. 6. At higher cholesterol content the 5-PCSL spectrum is affected no more by the presence of the peptide.

3.4. Peptide-lipid titration

Fig. 7 shows the outer hyperfine splitting, $2A_{max}$, of 5-PCSL in DLPC/CHOL membranes, at 0, 10, and 20% wt/wt cholesterol on total lipids, with increasing Aβ(25–35) concentration. In all curves, a typical binding saturation trend is registered: the decrease in lipid chain mobility saturates at a weight ratio of added peptide to total lipids of approximately 20% wt/wt peptide on total lipids. In the case of pure DLPC liposomes, this corresponds to ca. 8 lipids per peptide. The evidence that the saturation value does not decrease with increasing the fraction of cholesterol in the bilayer, suggests that some cholesterol molecules come in direct contact with the peptide, substituting the DLPC ones.

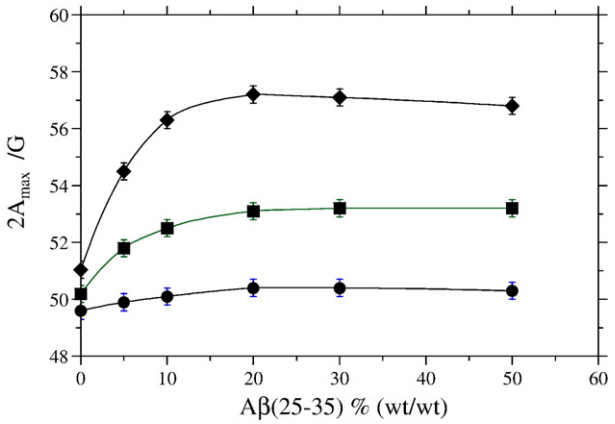

Fig. 7. Dependence of the outer hyperfine splitting increase, $2A_{max}$, of 5-PCSL on Aβ(25–35)/total lipid ratio for DLPC/CHOL bilayers at 0 (solid circles), 10 (solid squares) and 20% cholesterol (solid diamonds).

4. Discussion

Alzheimer's disease is a member of the heterogeneous family of diseases referred to as amyloidosis, characterized by deposits mainly formed by amyloid peptides in a variety of organs [33]. There is now an increasing amount of evidence that amyloid toxicity involves interaction with plasma membranes. As an example, this has been shown in the case of the human Islet Amyloid PolyPeptide (IAPP), a type-2-diabetes-related peptide that interacts in monomeric form with the plasma membrane of pancreatic β-cells [34–36].

In the same way, it is now widely accepted that Aβ peptide neurotoxicity is mediated by direct interaction between the peptide and the cellular membrane, through a still controversial mechanism. However, the effect of this interaction, i.e. whether it favours or not AD evolution, is unclear. In fact, from one side, retention of monomeric peptide in the membrane should hinder its accumulation in the external medium and, consequently, fibrils and plaques formation. On the other side, intercalation of Aβ oligomers in the membrane could result in ion-permeable channels formation, and thus in cell death [9].

With the aim to explore the molecular basis of the interaction between amyloid peptides and cellular membranes, we investigated the interactions between Aβ(25–35) and phospholipid bilayers. Obvious cautions have to be paid when generalising conclusions drawn from study involving a peptide fragment to biological systems in which the full-length peptide is present. Nevertheless, some indications can be obtained. Indeed, Aβ(25–35) is generally considered as the biologically active region of Aβ because it represents the shortest fragment that exhibits large β-sheet aggregated structures and because it retains the toxicity of the full-length peptide [37]; furthermore, it is also able to induce ion-channel formation in phospholipid membranes [38].

Particularly, in our work the effect of the peptide presence on the bilayer structure and dynamics has been monitored by spin-label EPR spectroscopy. Liposomes of the zwitterionic phospholipid DLPC have been initially considered as biomembrane-mimicking systems. At the temperature at which our investigation has been performed (25 °C), DLPC acyl chains are in the fluid-phase state, which may amplify interaction with guest molecules [32]. By using two spin-labels presenting the nitroxide group in different position along the hydrophobic alkyl chain, it was possible to investigate the depth of peptide penetration into the bilayer. The results show that the mobility of the spin-label 5-PCSL is significantly affected by the presence of the peptide, while that of 12-PCSL is almost unperturbed. Results similar to those obtained for 12-PCSL were seen with 14-PCSL (data not shown). This evidence indicates that Aβ(25–35) inserts in DLPC positioning between the outer part of the hydrophobic core and the external hydrophilic layer. These results are in only partial agreement with those reported by Dante et al. [14], who proposed a deeper penetration of Aβ(25–35) in the bilayer core. However, our findings are in agreement with those of a previous EPR study by some of us [23], in which, using two spin-labeled Aβ(25–35) analogues containing the nitroxide group of the amino acid TOAC as a paramagnetic probe at the N- or the C-terminus of the peptide sequence, respectively, it was found that the C-terminal portion of the peptide is embedded in the interior of the membrane, while the N-terminal part extends into the aqueous phase with occasional contacts with the lipid head-group region.

Indeed, the Aβ(25–35) three-dimensional structure in a membrane-mimicking environment has been solved by NMR study [39,40] revealing the propensity of the seven amino acids residues in the hydrophobic C-terminal region of the peptide to assume a turn-helical structure, most likely embedded in the membrane, whereas the four residues of the N-terminus, including a positively charged lysine, would be in the vicinity of the hydrophilic environment. From the NMR-derived structure, it can be valuated that the length of the hydrophobic region inserting in the apolar core of the bilayer is about

11 Å. By applying the Tanford rule for computing the length of saturated alkyl chains, it is possible to estimate that this length corresponds to about 8 methylenes; i.e. it is expected that the peptide insertion can directly perturb the motion of the alkyl chain down to the position 8, which is in agreement with our result.

In order to discriminate the driving force of the Aβ(25–35)/membrane interaction, we investigated how increasing amounts of the anionic phospholipid DLPG influence the peptide insertion. It was found that the membrane net charge only slightly affects the 5-PCSL spectrum variation due to the presence of the peptide, see Fig. 3. This evidence indicates that the hydrophobic interactions play a major role in driving the peptide/membrane interaction. It is to be noted that our experimental approach is tailored to detect the penetration of a guest molecule in the bilayer. Consequently, we cannot exclude that a fraction of Aβ(25–35) molecules adsorbs onto the bilayer surface, as recently reported by Dante et al. [14,15], and that this process is somehow influenced by the bilayer net charge. However, the similarity of the 5-PCSL spectra in DLPC and DLPC/DLPG membranes in the presence of the peptide leads us to suggest that the eventual electrostatic interaction between the bilayer external surface and the peptide is not markedly effective in causing a massive "extrusion" of the peptide from its location in the bilayer structure.

In this connection, Lau et al. have recently pointed out that the Aβ(25–35) positioning with respect to lipid bilayers could depend on the sample preparation procedure, i.e., it interacts with the head-groups if added after liposome preparation, while it inserts in the bilayer if inserted during liposome preparation [16]. We tested samples prepared through different procedures, finding negligible differences in the EPR spectra (data not shown). Indeed, it is possible that the procedure could affect an eventual partitioning of the peptide in two populations, one adsorbed on the bilayer surface and the other one inserted quite deeply in the bilayer core [14,15], and that our experimental approach, for the reasons discussed above, is not sensitive to this change.

The presence of cholesterol modulates the Aβ(25–35)/bilayer interaction. Cholesterol is a major component of mammalian cells and is known to influence membrane thickness and fluidity. In the case of DLPC bilayers at room temperature, the presence of cholesterol amounts ≥20% wt/wt induces a transition from a fluid-disordered to a fluid-ordered phase, as clearly detected by 5-PCSL EPR spectra. In turn, the fluidity of the membrane, as modulated by the cholesterol content, regulates the insertion of the Aβ(25–35) peptide. In the fluid-disordered phase the presence of cholesterol enhances the changes in the 5-PCSL spectrum due to the presence of the peptide. This indicates that the peptide is still able to penetrate the bilayer and that cholesterol and peptide exert a synergistic rigidifying action on the bilayer structure. At the same time, in the co-presence of cholesterol and peptide the 12-PCSL spectrum shows that the inner part of the hydrophobic tail is more flexible than in the presence of cholesterol alone. This evidence indicates that the insertion of the peptide causes a re-positioning of cholesterol closer to the hydrophilic external layer. A similar effect has been recently reported in the literature for the full-length Aβ peptide [41]. However, as the cholesterol content exceeds 30% wt/wt, i.e. in the fluid-ordered bilayer, the peptide insertion appears to be inhibited completely. This is consistent with the literature data indicating that cholesterol preserves the bilayer integrity from the disrupting effect of the amyloid peptide [42].

In the presence of DLPG, i.e. for charged membranes, the transition of lipid membrane from the fluid-disordered to the fluid-ordered phase, induced by increasing the cholesterol content in the bilayer, is much broader, as indicated by the gradual change in the 5-PCSL spectrum. Also in this case, low amounts of cholesterol seem to favour the peptide penetration while a further increase of the cholesterol content results in peptide-depleted membranes. Comparison between uncharged and charged bilayer systems, indicates that exclusion of the peptide from the membrane occurs at lower cholesterol content (20% rather than 30% wt/wt) in the latter case, suggesting a certain degree of synergism of cholesterol and DLPG in disfavouring Aβ(25–35) insertion in the membrane. Intriguingly, even though the bilayer used in this work is a simplified model system of cellular membranes, the cholesterol content necessary for peptide release is not much higher than that present in normal neurons (15–20% w/w [43]).

It is interesting to put the results of the present work in the framework of the scientific debate on the subject. The role played by cellular membranes in the Aβ peptides formation and self-aggregation is still highly controversial, even if an increasing number of papers strongly supports its relevance [44]. Indeed, it is likely that membranes play different roles in different steps of the neurodegenerative process. Aβ peptides sequence includes a segment of the transmembrane domain of the precursor APP; moreover, the cleavage of the precursor in the transmembrane region is performed by the transmembrane enzyme γ-secretase. Thus, the membrane plays a fundamental role in the Aβ peptides formation. Since the peptide self-aggregation finally resulting in plaques formation occurs in the extracellular medium, the factors favouring the peptide release from the membrane are relevant. Furthermore, the peptide self-aggregation occurs close to the membrane surface, so that it is well possible that interaction between the peptides and the membrane surface could catalyse the aggregation process [45]. Furthermore, peptides' oligomers could intercalate in the membrane, causing, as reported in the literature, a loss of the membrane functionality and the consequent cell death [9].

Our work focused on the interaction between Aβ(25–35) in monomeric form and phospholipid bilayers. Consequently, our results shed light on the factors influencing the process by which the peptide, initially positioned in the more external region of the lipid bilayer hydrophobic core is released to the external aqueous medium. This process is not significantly influenced by the membrane charge, being the peptide/membrane association driven fundamentally by hydrophobic interactions. In contrast, cholesterol plays a crucial role in regulating the peptide release. Actually, this is a feature shared with other peptides, e.g. the antimicrobials ones [46,47], even if details of the mechanism through which cholesterol works are not completely understood. In the case under consideration in this work, this mainly occurs through an indirect mechanism by which cholesterol induces a transition from a fluid-ordered to a fluid-disordered phase. In turn, fluid-ordered membranes loose the ability to include Aβ(25–35). Interestingly, our results suggest also some direct cholesterol–peptide interactions to the involved in the process. In fact, at low cholesterol content, the insertion of the peptide in the membranes causes a displacement of cholesterol towards the more external part of the membrane. The crowding of cholesterol enhances its rigidifying effect on this region of the bilayer, finally resulting in the peptide exclusion. Because of the fundamental role played by cholesterol in ruling the membrane plasticity, permeability and functionality, its specific membrane location can be more important than the absolute amount; thus, it can be suggested that the crowding of cholesterol molecules in the more external part of the hydrophobic core of the phospholipid bilayer due to the insertion of the amyloid peptide can significantly affect the neuronal cell function.

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