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AβP1-42 incorporation and channel formation in planar lipid membranes: the role of cholesterol and its oxidation products

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Abstract Amyloid beta peptide (AβP) is a natural peptide, normally released into the cerebrospinal fluid (CSF), that plays a key role in Alzheimer's disease. The conversion of the peptide from a native soluble form to a non-native and often insoluble form, such as small and large aggregates, protofibrils and fibrils of AβP appears to be implicated in the pathogenesis of AD. Although the molecular mechanisms of AβP neurotoxicity are not fully understood, a large body of data suggests that the primary target of amyloid peptides is the cell membrane of neurons, that may modulate the structural and functional conversion of AβP into assemblies involved in pathological processes. In our study, we provide a systematic investigation of AβP1-42's ability to incorporate and form channel-like events in membranes of different lipid composition and focus on cholesterol and its oxidation products. We propose that cholesterol and its oxidation products can be considered neuroprotective factors because a) by favouring AβP1-42 insertion into membranes, the fibrillation/clearance balance shifts toward clearance; b) by shifting channel selectivity toward anions, the membrane potential is moved far from the threshold of membrane excitability, thus decreasing the influx of calcium into the cell.

Keywords AβP · Ion channel · Cholesterol · Zwitterionic lipids · Ion selectivity

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Introduction

Amyloid beta peptide (AβP) is a natural peptide, normally released in various body fluids including plasma and cerebrospinal fluid (CSF), that plays a key role in the pathogenesis of the most common and devastating neurodegenerative disorder, Alzheimer's disease (AD). Biochemical analysis has demonstrated that the AβP peptide has a great propensity towards aggregation and deposition into senile plaques, one of the main pathological features assumed to occur in the brains of patients with AD. Converging lines of evidence suggest that the aggregation and progressive accumulation of AβP is a crucial event in the onset of Alzheimer's disease.

The AβP is a product of cleavage of the Amyloid Precursor Protein (APP), a transmembrane protein, by β-secretase and γ-secretase (Masters et al. 1985; Kang et al. 1987; Thinakaran and Koo 2008; Selkoe 2001a). Several isoforms of AβP are generated and have been isolated in vivo including AβP1-38, AβP1-39, AβP1-40, AβP1-42 and AβP1-43. AβP1-40 is the predominant species isolated from cerebrospinal fluid (Haass et al. 1992), while AβP1-42 is the principal species associated with senile plaques in parenchyma (Iwatsubo et al. 1994; Miyashita et al. 2009).

AβP1-42 is a small amphiphilic peptide whose N-terminal segment (1DAEFRHDSGYEVHH14) is very hydrophilic, whose middle segment (15QKLVFFAEDVGSNK28) has similar numbers of hydrophobic and hydrophilic residues, and whose C-terminal segment (29GAIIGLMVGGVVIA42) is very hydrophobic with no polar side chains (Selkoe 2004; Shafrir et al. 2010).

Although the specific role of AβP in the neurodegenerative processes of AD is not fully understood and the molecular mechanisms of AβP neurotoxicity remain a matter of debate, several studies suggest that AβP may have toxic properties, particularly in its aggregated state. Sequential

oligomerization into larger aggregates leads to different physical forms—monomers, oligomers, annular assemblies, protofibrils and fibrils (Bitan et al. 2003; Lambert et al. 1998; Walsh et al. 1999; Lesné et al. 2006; Haass and Selkoe 2007). It is well established that AβP1-42 is the most toxic form of AβP, owing to its much stronger aggregation propensity (Kirkitadze and Kowalska 2005; Sato et al. 2006; Choucair et al. 2007). It has been suggested that pentamer and hexamer paranuclei form the basic units of the protofibrils and this may be caused by the hydrophobic isoleucine and alanine at the C-terminus (Williams and Serpell 2011). Therefore the conversion of the peptide from a native soluble form to a non-native often insoluble form, such as small and large aggregates, protofibrils and fibrils of AβP enriched in β-sheet structures, appears to be implicated in the pathogenesis of AD (Selkoe 2001a, b).

Recent evidence, both in vitro and in vivo, suggests that AβP toxicity resides primarily with soluble oligomeric and intermediary forms of the peptide rather than AβP monomers or mature fibrils (Valincius et al. 2008; Walsh et al. 2002; Kayed et al. 2003, 2004; Sokolov et al. 2006) and these forms of AβP correlate with the severity of the disease better than the presence of amyloid plaques.

These different assemblies of AβP, on interacting with cell membrane, may differentially contribute to AD pathogenesis at various stages of this disorder, causing synaptic dysfunction and neuronal death (Di Paolo and Kim 2011).

The fluidic nature, structure asymmetry and heterogeneity of membranes are functionally important factors that influence biomolecular processes, peptide–lipid interactions and movement of ions across the membrane. In particular, the cholesterol (Ch) content of membrane plays a significant role in modulating peptide–membrane interaction and incorporation (Meleleo et al. 2009, 2011).

Considerable amounts of Ch are found in neuronal plasmalemma and in lipid rafts (small platforms composed of sphingolipids and Ch in the outer exoplasmic leaflet, connected to phospholipids and Ch in the inner cytoplasmic leaflet of the lipid bilayer). Ch partitions between the raft and non-raft phase, having higher affinity to raft sphingolipids than to unsaturated phospholipids. Ch is thought to serve as a spacer between the hydrocarbon chains of the sphingolipids and to function as a dynamic glue that keeps the raft assembly together (Simons and Toomre 2000). Ch is to a large extent concentrated in the myelin sheath and its role is mainly thought to be to decrease ion conductance; the human brain contains as much as 25 % of the total pool of cholesterol (Bohr 2005). Ch is not uniformly distributed in biological membranes: in brain synaptic plasma membranes in young people, ~87 % of total plasma membrane cholesterol is contained in the cytofacial leaflet, whereas during the aging process, the distribution of cholesterol tends to level off in the cytofacial and exofacial leaflet (Shinitzky 1993).

In AD, the Ch content of certain regions of the brain can be markedly different from the same regions in nondemented brains. As found by some authors, in the grey matter of the superior temporal gyrus, the cholesterol to phospholipid mole ratio in AD brains is lower than that found in nondemented brains (Williams and Serpell 2011; Mason et al. 1992). Although Ch has been identified as a major risk factor for AD, its role in AβP production, aggregation and toxicity is controversial (Yu and Zheng 2011). It has been reported that the presence of Ch promotes AβP generation by APP processing and aggregation to form soluble and toxic oligomers binding to the cell membrane. On the other hand, the physiological concentrations of cholesterol are reported to be neuroprotective factors against β-amyloid toxicity in neuroblastoma cells (Granzotto et al. 2011). It seems that Ch can inhibit or promote AβP aggregation on or penetration into the cell membrane, depending on the molar ratio of lipid/cholesterol in the cell membrane.

Some authors have found that membrane components, such as Ch and gangliosides, alter the affinity of AβP for phospholipid membranes. Ch and gangliosides, once associated with phospholipid membranes, lead to an increase in β-sheet content and/or the rate of aggregation of AβP (Choo-Smith et al. 1997). On the other hand, recent studies have suggested that Ch promotes the transition from β-sheet to α-helix and random coil of many proteins and peptides (Ji et al. 2002; Qiu et al. 2011).

In this study, we investigated AβP1-42 incorporation and channel-like event formation in planar lipid membranes (PLMs) made up of different lipids, focusing on PLMs made up of Oxidized Cholesterol (OxCh), that is considered a mixture containing cholesterol (60–70 %) and other oxidation products which can be negatively charged, due to the evidence of a cholesterol-rich domain in both eukaryotic plasma membranes and aged membrane.

Materials and methods

Peptide preparation

AβP1-42 was purchased from Sigma. A stock solution of AβP1-42 was prepared by dissolving AβP1-42 powder (0.1 mg) in 48 μL of bidistilled sterile water under stirring for 3 min to obtain a concentration of 4.6×10^{-4} M. From this solution, 5 μL were withdrawn and diluted in 45 μL of bidistilled sterile water under stirring for 3 min to obtain a concentration of 4.6×10^{-5} M. Both solutions were stored at −20 °C until use.

Electrophoresis and Western blotting

From a stock solution of 4.6×10^{-4} M AβP1-42, one aliquot was removed and incubated at 23 °C for 24 h (T24) and one

aliquot was removed before analysis (T0) to be examined using non-denaturing gel electrophoresis. Samples were mixed with Laemmli loading buffer 1× without SDS and loaded (5 μg per lane) on 4–20 % polyacrylamide gel (TGX precast gel, Biorad) without SDS using TGS1X as running buffer with SDS (0.1 %). Electrophoresis was performed for 45 min at 130 V. Samples were not boiled, so as to preserve the structure of potential oligomeric AβP1-42 species. After electrophoresis, the gel was silver stained.

In addition, AβP1-42 was detected by Western blotting, which proved more sensitive for the detection of higher molecular mass complexes. Aliquots of peptide (5 μg per lane) were separated by non-denaturing gel electrophoresis and peptides electrophoretically transferred from gel onto polyvinylidene difluoride (PVDF) membrane at a constant current of 80 V for 90 min. Membrane was then blocked with 5 % milk in TBS-tween 0.05 % buffer, with gentle agitation for 1 h at room temperature. The blocking solution was replaced with the primary monoclonal anti-amyloid β-peptide antibody (Sigma), produced in mouse (1:5,000 dilution in TBS-tween 0.05 % buffer) and incubated with gentle agitation overnight at 4 °C. The blot was then probed with the secondary polyclonal anti-mouse IgG antibody conjugated to horseradish peroxidase (1:20,000 dilution in TBS-tween 0.05 % buffer) with gentle agitation for 1 h at room temperature and then developed by the ECL detection system. The blot image was acquired through the ChemiDoc system (Biorad). To verify the presence of fibrils in samples, the same membrane was incubated with the primary monoclonal conformational antibody OC. Preliminarily, in order to eliminate the previous primary antibody, the same membrane was incubated with heated acidic stripping solution (3 mM SDS, 200 mM glycin, 1 % tween 20, pH 2.2) for 20 min with vigorous agitation and, after three washes, re-blocked with 10 % milk in TBS-tween 0.01 % with gentle agitation for 1 h at room temperature. The membrane was then incubated with OC antibody produced in rabbit (1:10,000 dilution in 5 % milk dissolved in TBS-tween 0.01 % buffer) under gentle agitation overnight at 4 °C and subsequently with the secondary polyclonal anti-rabbit IgG antibody conjugated to peroxidase (1:20,000 dilution in 5 % milk dissolved in TBS-tween 0.01 % buffer) with gentle agitation for 1 h. The blot was developed using the ECL detection system.

Single channel measurement

Channel activities were recorded in a lipid bilayer membrane composed of dioleoyl-phosphatidylserine:dioleoyl-phosphatidylethanolamine (DOPS:DOPE=50:50, w:w) or palmitoyl-oleoyl-phosphatidylcholine (POPC) (Avanti Polar Lipid, Alabaster, AL), or POPC:Ch (70:30, w:w) in 1 % of n-decane or OxCh in n-decane (1:1, v:v) (Fluka). The OxCh was obtained following the method of Tien et al. (1966). Bilayers were formed across a 200 μm hole in a Teflon partition separating two Teflon chambers which held symmetrical 50 mM KCl solutions, pH=7, temperature 23± 1 °C. The salts used in the experiments were of analytical grade. The Müller-Rudin or painted technique (Müller et al. 1962; Tien 1974; Tien et al. 1977) was used to form PLMs with lipids solubilised in n-decane. Briefly, a small volume of 0.5–1 μL of lipid solution is applied through a micropipette directly onto the hole of the Teflon set; a PLM forms in ~10 min after draining the excess solvent into the aqueous bathing solution.

The final concentration of AβP1-42 used in single channel experiments was 5×10^{-8} M.

In single-channel experiments, the membrane current was monitored with an oscilloscope and recorded on a chart recorder for further analysis by hand. The cis and trans chambers were connected to the amplifier head stage by Ag/AgCl electrodes in series with a voltage source and a highly sensitive current amplifier (OPA 129). The single-channel instrumentation had a time resolution of 1–10 msec depending on the magnitude of the single-channel conductance. The polarity of the voltage was defined according to the side where AβP was added (the cis-side). A trans-negative potential (indicated by a minus sign) means that a negative potential was applied to the trans side, the compartment opposite the one where AβP was added.

Data analysis

Membrane capacitance was calculated using a calibration curve obtained by simulating the membrane capacitance with a discrete set of capacitances of known values, Cn, and measuring the corresponding output voltage, Vlh. The data obtained were fitted by the formula:

$$Y = \frac{A \times X}{(B + X)}$$

in which Y and X correspond to Vlh and Cn respectively, while A and B are free parameters to be estimated by the fitting procedures. The values of parameters A and B were used to transform the Vlh value into capacitance data, as described by Micelli et al. (2002).

The single channel data, filtered at 300 Hz as reported by other authors (Arispe et al. 1993a), were obtained from at least two and sometimes four experiments (more than 100 single channels per experiment) performed on different days.

To determine conductance, we measured the amplitude of channel-like events by hand. A histogram of the conductance amplitude distribution for each experiment was constructed and fitted by a Gaussian distribution function.

Results are expressed as central conductance ± standard error (Λ_c ± SE) and were evaluated by analysis of variance (ANOVA-Tukey test) and Student t test. A value of $P<0.05$ was considered significant. The Gaussian distribution function, ANOVA test, Student t test and the fitting procedures were performed using the GraphPad Prism 3 software (GraphPad Prism™ version 3.0).

To define channel lifetime, from records extending over prolonged periods, channel duration was measured considering the time between the opening and closing of each channel. The average lifetime of the conductance unit was estimated by the formula:

$$N = A_1 e^{\left(\frac{-t}{\tau_1}\right)} + A_2 e^{\left(\frac{-t}{\tau_2}\right)}$$

where N is the number of channels that remain open for a time equal to or greater than a certain time t, A_1 and A_2 are the zero time amplitudes, and τ_1 and τ_2 are related to the fast and slow components of the time constant, respectively. The single-exponential distribution is included in the formula ($A_2=0$). To choose between the two models, we performed an appropriate statistical test (F-test, Graph Pad Prism™ version 3.0).

To identify the charge on the ion carrying the current, we measured the shift in the reversal potential induced by a change from a symmetrical to an asymmetrical salt solution system. When the membrane conductance reached a virtually stable value, a concentration gradient was set, with 100 mM on the *cis* side and 50 mM on the *trans* side. The reversal potential was determined by an I-V curve, when the measured amplitude of the channel events at each potential was used.

The permeability ratio was calculated by means of the equation (Hodge and Colombini 1997):

$$V = \left(\frac{RT}{F}\right) \times \frac{(p_{Cl^-} - p_{K^+})}{(p_{K^+} + p_{Cl^-})} \ln \frac{a_c}{a_t} \quad (1)$$

where a_c/a_t is the activity ratio of the ion species in the *cis* and *trans* compartments, respectively; where $p_{Cl}-$ and p_K+ are the permeabilities of the Cl^- and K^+, respectively; where R, T and F are respectively molar gas constant, thermodynamic temperature and Faraday constant.

Results

Analysis of AβP1-42 by electrophoresis and Western blot

Preliminarily, we checked the aggregation state of the AβP1-42 peptide used in this study by electrophoresis and Western blotting (Fig. 1). Analysis of AβP1-42 by non-denaturing PAGE in the sample at time zero showed monomers, trimers and tetramers with an apparent molecular weight (MW) of 4.5, 13.5 and 18 kDa, respectively. After 24 h of incubation, an increase in 13.5 and 18 kDa species was observed (Fig. 1a). After electrophoresis, the two samples were blotted onto PVDF membrane, which was stained for AβP1-42 immunoreactivity. Western blot analysis of AβP1-42 samples with anti-amyloid β-peptide antibody showed peptide immunoreactive bands with apparent molecular masses of 4.5, 13.5 and 18 kDa (Fig. 1b). To verify the presence of fibrils in the samples, Western blot analysis of AβP1-42 was carried out with OC antibody, which is able to recognize the presence of fibrils and to follow their formation during incubation. Analysis showed AβP1-42 immunoreactive bands with an apparent MW higher than 250 kDa and growing intensity during the time course, starting from time 0 up to 24 h (Fig. 1c).

Effect of membrane different lipid composition on AβP1-42 channel activity

In our work, preliminarily, PLM stability was tested by applying a voltage of ±120 mV for 10–15 min under stirring and monitoring constant values for PLM conductance and capacitance. Neither conductance nor capacitance ever exceeded 25 pS and 0.32 μF/cm^2, respectively, and neither showed channel-like activity. The capacitance value measured for the PLMs used in this work is in accordance with that found by other authors for the lipid bilayer (White 1970; Tien and Ottova-Leitmannova 2000).

In many different experiments on DOPS:DOPE, POPC and POPC:Ch PLMs, the addition of 5×10^{-8} M of AβP1-42 to the *cis*-side of the medium facing the membrane did not determine any conductance variation for a long period of time, upon application of voltages as high as 120 mV. However, after PLM breakage and withdrawal, AβP1-42 channel-like activity appears, after different lag times depending on PLM composition, as non-random discrete current fluctuations, compatible with channel-type openings and closures with different conductance levels, lifetimes and frequency. The 300 Hz detection system used in this study could result low for the resolution of the all conductance states caused by AβP1-42 peptide, differently from those reported by other authors (Hirakura et al. 1999). In this study, although many applied potentials were investigated, only those potentials that led to channel formation are reported.

In the DOPS:DOPE PLMs, the channel-like activity of AβP1-42 appears after about 2 h' lag time at the minimal applied voltage of 60 mV (Fig. 2a). After the first channel-like event formation, the applied voltage can be lowered down as far as 20 mV and channel amplitude can be monitored. No channel-like activity was observed at negative applied voltages.

In the POPC PLMs, the lag time was not less than 24 h and the minimal potential at which channel-like activity can

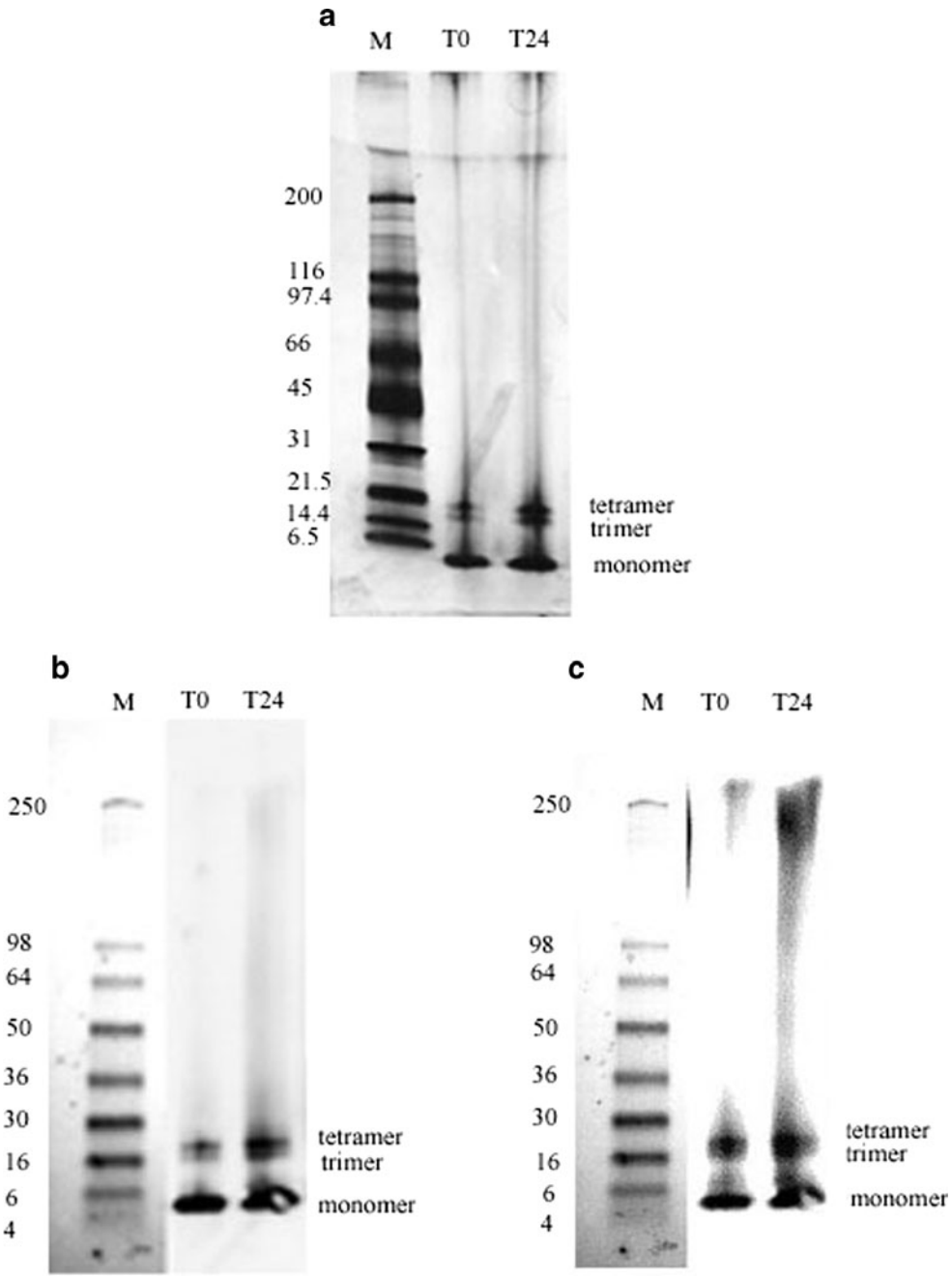

Fig. 1 Aggregation states of AβP1-42 peptide analyzed with non-denaturing Electrophoresis and Western blot. **a** Non-denaturing PAGE of AβP1-42 samples: *M*, protein molecular weight marker; *T0*, AβP1-42 sample removed from stock solution and analysed; *T24*, AβP1-42 sample after 24 h of incubation at 23 °C. Molecular masses are indicated in kDa. **b** Western blot analysis of AβP1-42 oligomers separated on a 4–20 % non-denaturing polyacrylamide gel with the monoclonal anti-amyloid β-peptide antibody; **c** Western blot analysis of AβP1-42 fibrils separated on a 4–20 % non-denaturing polyacrylamide gel with the monoclonal conformational antibody OC

be observed was 100 mV (Fig. 2b). After the first channel-like event formation, the applied voltage can be lowered to 80 mV. No channel-like activity was observed at negative applied voltages.

The addition of 30 % of Ch to POPC PLMs (weight ratio) decreased lag time to 6 h. In POPC:Ch (70:30, w:w) PLMs, channel-like activity appeared as sudden, non random current fluctuations when a constant voltage of 100 mV was applied across the membrane (Fig. 2c). After the first channel-like event formation, the applied voltage can be lowered to ±60 mV and channel amplitude can be monitored.

All single channel-like events were used to calculate the channel-like structure amplitudes that revealed the existence of one main conductance level at different applied voltages. A histogram of amplitude distribution was constructed and fitted by a Gaussian distribution function and gave the central value of the single channel-like event conductance (Gallucci et al. 2003; Stipani et al. 2001) (Fig. 3).

Table 1 summarizes the characteristic values of some parameters, such as central conductance ± standard error ($\Lambda_c \pm SE$), frequency ± standard deviation (i.e. the number of openings in a period of 1 min, obtained from the total number of records), open-state lifetime of AβP1-42

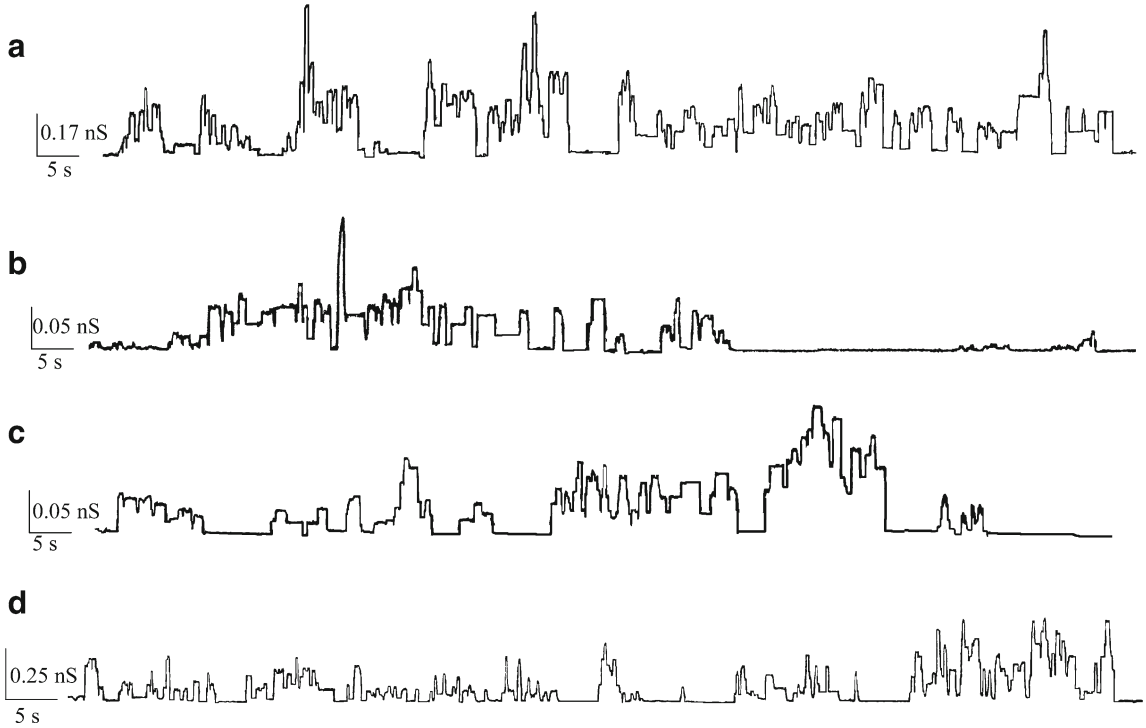

Fig. 2 AβP1-42 channel-like activity in different PLMs used. Representative traces illustrating channel-like activity of AβP1-42 in membranes made up of DOPS:DOPE (50:50,w:w) (**a**), POPC (**b**), POPC:Ch (70:30,w:w) (**c**), OxCh (**d**) recorded after an average of 30 min from first channel formation, when channel-like activity is substantial and lasting. Applied voltage was set to 60 mV (**a**) and 100 mV (**b**, **c** and **d**). Experiments were performed in the presence of AβP1-42 (5×10^{-8} M) added to the *cis* side, while the aqueous phase contained 50 mM KCl (pH 7) and $T=23\pm1$ °C

channel-like events as a function of applied voltage in the different PLMs used. It is worth noting that in DOPS:DOPE PLMs, the AβP1-42 channel-like activity was observed at applied voltages lower than those in POPC:Ch and POPC PLMs, suggesting that anionic lipids favour peptide interaction with the membrane and channel-like structure formation as found by other authors (Hirakura et al. 1999).

In all membranes used, excluding POPC, central conductance seems to be dependent on the applied voltages, decreasing as the voltage is increased. Furthermore, in POPC:Ch and OxCh PLMs, where it was possible to monitor channel-like fluctuations at positive and negative voltages, channel-like event conductance shows symmetric behaviour. It is worth noting that the central conductance value for the same applied voltage (60 mV), in DOPS:DOPE PLMs, is significantly higher than that in POPC:Ch PLMs ($P<0.0001$, Student t test). At applied voltages of 80 and 100 mV, where it was possible to make a comparison between POPC and POPC:Ch, the central conductance values are not different ($P=0.1775$, $P=0.2640$ respectively; Student t test).

The frequency values (Table 1) in DOPS:DOPE and POPC PLMs seem to be independent of the applied voltage, suggesting that channel-like event turnover is not modulated by voltage. In POPC:Ch PLMs, the frequency shows higher values than those in POPC PLMs.

Channel-like event current recordings with a conspicuous number of channel-like events were analysed to obtain cumulative open-state lifetime distributions, as reported in Table 1. The results indicate that: 1) in DOPS:DOPE PLMs, the fast channel lifetime component prevails ($P<0.05$) at applied voltages of 60 and 40 mV, whereas at an applied voltage of 20 mV there seems to be a channel stabilization because the channel-like event manifests both the fast and slow components of lifetime ($P<0.05$); 2) in POPC:Ch PLMs, the channel-like event manifests both the fast and slow components of lifetime ($P<0.05$) at all applied voltages except at an applied voltage of 60 mV for which the fast channel lifetime component prevails ($P<0.05$); 3) in POPC PLMs, the number of channel-like events is not conspicuous enough to provide a reliable analysis of open-time distribution.

In OxCh PLMs, the membrane utilized as a model of old brain or Alzheimer disease, AβP1-42 channel activity appears spontaneously after 2 h' lag time from its addition in the medium facing the membrane. The incorporation of

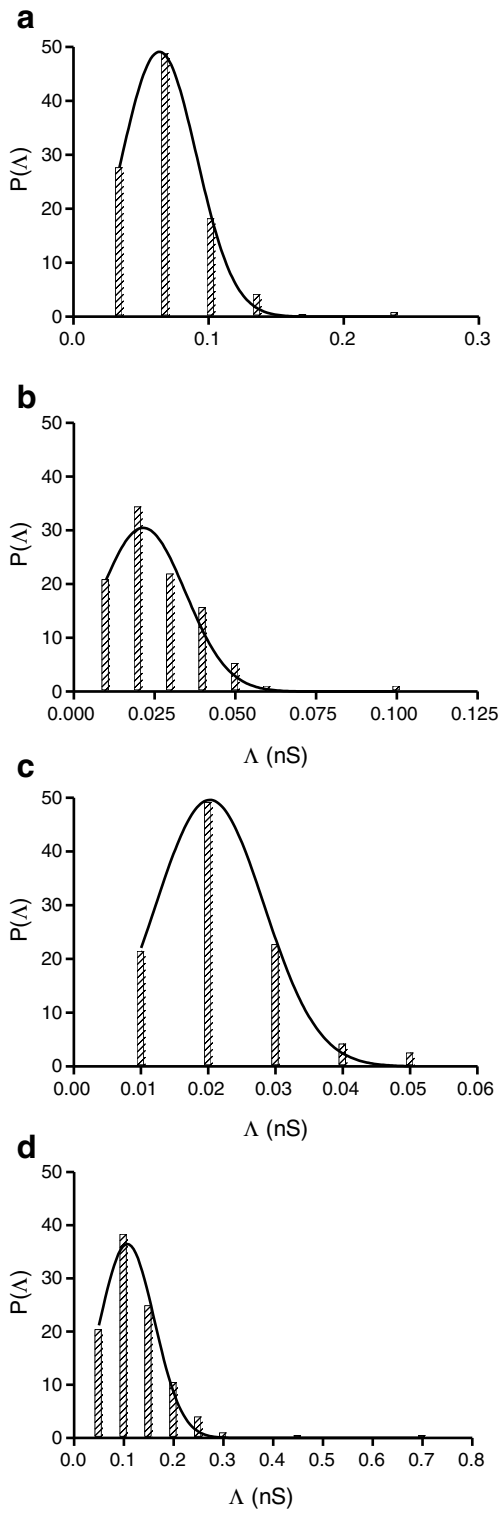

Fig. 3 Histograms of AβP1-42 conductance amplitude distribution. The histograms of the probability, P(Λ), for the frequence of a given conductivity unit, relative to each trace reported in Fig. 1, were fitted by a Gaussian which is shown as a *solid curve*

AβP1-42 into OxCh PLMs leads to non-random discrete current jumps that fluctuate between conductive and non-conductive states, compatible with channel-type opening and closure with different levels, lifetime and frequencies. Figure 2d shows an example of chart recording of AβP1-42 channel-like event formation in OxCh PLMs at the minimal potential of 100 mV, at which the first channel-like activity was observed. After the first channel-like event formation, the applied voltage can be lowered to ±20 mV.

Figure 3 reports the amplitude histogram of AβP1-42 channel-like event conductance for the example shown in Fig. 2.

The central conductance values are symmetrical for negative and positive applied voltages except at 20 mV for which the central conductance value is higher than −20 mV ($P<0.0001$, Student t test). The central conductance values are significantly higher ($P<0.0001$; ANOVA-Tukey test and Student t test) at all applied voltages than those observed in other kinds of membrane studied.

Frequency values as a function of applied voltage are quite constant in the range between −100 and 40 mV while they increase with applied voltage in the range between 60 and 100 mV. In OxCh PLMs, the analysis of open-time distributions gives a statistically significant better description ($P<0.05$) of the two-exponential function at all applied voltages, except at an applied voltage of 20 mV, where it does not clearly distinguish between single and double exponentials ($P=0.13$).

The prevalence of dual-channel populations clearly indicates that the AβP1-42 channel-like structure is more stabilized in membranes containing cholesterol than in those containing anion lipids.

Another parameter that characterizes a channel is its ion selectivity. The ion selectivity of AβP1-42 channel-like structures in POPC:Ch and OxCh PLMs was determined by means of an I-V relationship at different transmembrane potentials under asymmetrical solution conditions (see Materials and methods section). This analysis was impossible for POPC PLMs.

The reversal potential was determined using the I-V curves, when the measured amplitude of the channel-like events at each potential was used. In OxCh PLMs, owing to presence of negative charges (40 %), the I-V curve has been constructed considering the surface potential. The surface potential (ψ) was calculated by means of the formula: $4A^2\sigma^2/C = \exp(-ze\psi kT)$ where k is the Boltzmann constant, T is temperature, e is electronic charge, z is the valence of the symmetrical electrolyte solution, σ is the charge density, ψ is the surface potential, C is the bulk aqueous electrolyte concentration and $A=1/(8N\varepsilon_r\varepsilon_0 kT)^{1/2}$, where N is Avogadro's number, ε_r the dielectric constant and ε_0 the permittivity of free space (Mclaughlin 1977). The reversal potential was 2.24 and 26.90 mV in POPC:Ch and OxCh PLMs, respectively (Fig. 4). The permeability ratio (Pcation/Panion), calculated using the Eq. (1), was 0.78/0.12 in POPC:Ch/ OxCh PLMs, respectively, indicating that the ion selectivity of the AβP1-42 channel shifts from neutral to anions, in relation to the lipid composition of the PLM.

Table 1 Characteristic parameters of AβP1-42 channel-like event in different PLMs used

PLM	Vs (mV)	$\Lambda_c \pm$ SE (nS)	Frequency ± SD	τ_1 (s)	τ_2 (s)
DOPS:DOPE	60	0.064±0.001	4.12±0.25	3.14	
	40	0.091±0.002	3.65±0.23	2.85	
	20	0.194±0.001	5.26±0.37	0.45	3.35
POPC	100	0.021±0.001	3.32±0.33	–	–
	80	0.021±0.011	3.51±0.46	–	–
POPC:Ch	100	0.020±0.0004	7.12±0.94	0.29	3.90
	80	0.029±0.0015	7.25±0.63	0.97	5.77
	60	0.034±0.0007	5.60±0.40	2.08	
	−60	0.035±0.0005	4.40±0.26	0.11	2.98
	−80	0.026±0.0006	3.80±0.29	0.11	4.45
	−100	0.019±0.0005	4.72±0.36	0.11	2.34
ColOx	100	0.107±0.004	7.50±0.53	0.23	3.00
	80	0.119±0.002	7.10±0.35	1.16	4.65
	60	0.160±0.002	5.70±0.22	0.12	3.50
	40	0.236±0.002	3.90±0.15	0.10	4.92
	20	0.506±0.009	3.90±0.22	2.26	
	−20	0.431±0.005	2.90±0.14	0.11	3.39
	−40	0.227±0.004	3.90±0.13	0.14	4.00
	−60	0.157±0.002	4.10±0.15	0.11	3.20
	−80	0.118±0.003	4.20±0.17	0.11	3.30
	−100	0.110±0.002	4.30±0.24	0.17	2.36

The mean conductance fitted by Gaussian distribution ($\Lambda_c \pm$ SE), the frequency (frequency ± SD), the channel lifetime (τ) of the AβP1-42 channel-like events in different PLMs at different applied voltages. The minimum and maximum number of channel-like events considered (N) out of a total number of channels considered (Nt) was: DOPS:DOPE, 202<N<264, Nt=712; POPC, 172<N<196, Nt=368; POPC:Ch, 190<N<337, Nt=1,570; OxCh, 201<N<864, Nt=5,238

Discussion

It is a widespread belief among researchers that in the case of many proteins and peptides their conformational structure depends on the lipids surrounding these molecules. Specific phospholipids, such as cardiolipin (bis-phosphatidylglycerol), are essential for the function of the cytochrome bc_1 complex (Hayer-Hartl et al. 1992).

AβP1-42 is one of the pathological features of Alzheimer's disease and together with AβP1-40 is among the major components of senile plaques. Notably, the AβP1-42 peptide is more neurotoxic than AβP1-40, owing to its much stronger aggregation propensity (Sato et al. 2006; Mobley et al. 2004). AβP is present at very low concentrations ($<1\times10^{-8}$ M) in biological fluids, including plasma and CFS (Galasko et al. 1998; Mehta et al. 2001), and its physiological role is unknown. An important concept in the thermodynamics of amyloidogenesis is the critical concentration, i.e. the concentration below which amyloid cannot form. It has been reported that the physiological concentration of AβP1-42 in CFS is in the range of 1×10^{-10} M and its critical concentration is >100-fold higher than its physiological concentration (Usui et al. 2009). The conversion of soluble, non-toxic AβP to aggregated toxic AβP rich in β-sheet structures ignites the neurotoxic cascade of AβP.

Several observations indicate that the primary target of amyloid peptides is the cell membrane of neurons, which may be where the neurotoxic cascade is initiated through direct and indirect mechanisms (Williams and Serpell 2011). Some researchers have proposed that biological membranes may modulate the pathological conversion of structural and functional proteins, such as AβP and α-synuclein, into amyloidogenic assemblies involved in pathological processes (Selkoe 2004; Haass and Selkoe 2007; Aisenbrey et al. 2008).

As a consequence of its lipophilicity, amyloid peptide can interact strongly with specific membrane lipids (Ji et al. 2002; Terzi et al. 1997; Curtain et al. 2003), altering the physical and biological properties of membranes (Kayed et al. 2004; Arispe et al. 1993a, b; Ambroggio et al. 2005). In disturbing the structure and integrity of the plasma membrane, AβP promotes the increased leakage of ions, particularly calcium, into the cell, leading to an ion imbalance and neuronal cytotoxicity, which are considered to play a causative part in Alzheimer's disease. In particular, it has been demonstrated that AβP forms slightly cation-selective,

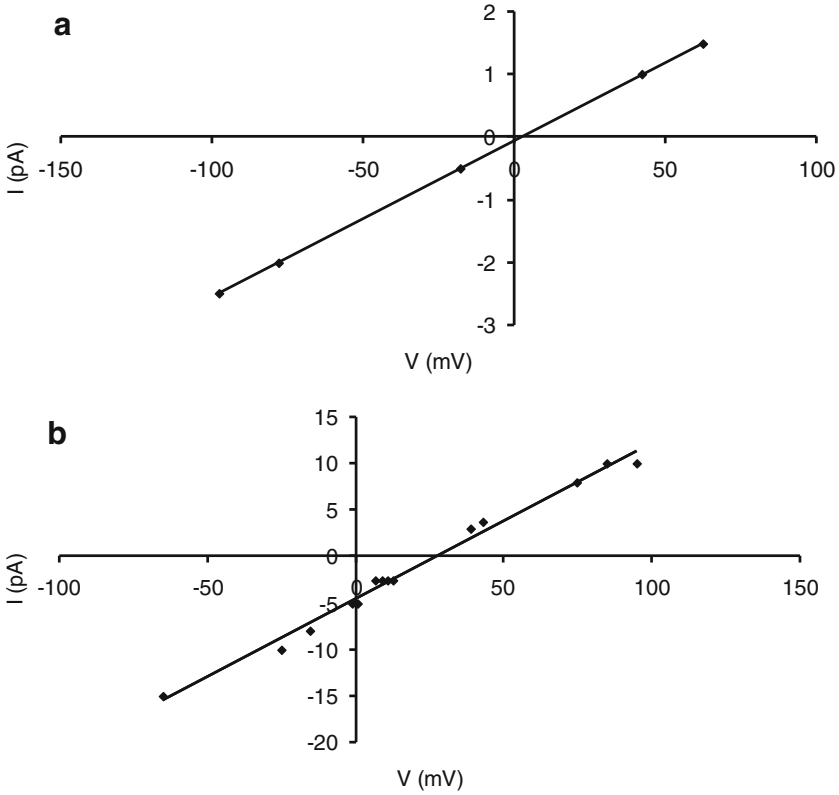

Fig. 4 AβP1-42 channel-like event ion selectivity in different PLMs used. AβP1-42 channel selectivity in POPC:Ch (**a**) or OxCh (**b**) PLMs. The amplitude of channel current (pA) is plotted as a function of the transmembrane potential (mV). Each point along the I-V curve represents the mean value of at least three readings at the amplitude of the current at the potential indicated. Conductance was determined by linear regression of the current values in asymmetrical solutions 100 mM/50 mM KCl cis/trans. Intercept was used to calculate P_{K^+}/P_{Cl^-}

voltage-independent ion channels in PLMs containing anionic phospholipids (Hirakura et al. 1999) and exhibits ionophore-like activity and induces calcium influx into neurons (Simmons and Schneider 1993) and neuron-like cells (Sanderson et al. 1997).

In the present study, we evaluated the ability of the AβP1-42 peptide to incorporate and to form ion channel-like structures in membranes of different lipid composition. Our results demonstrate that AβP1-42 interacts with all kinds of membranes used at a concentration of 5×10^{-8} M near to critical concentration of this peptide.

Its C-terminal isoleucine and alanine residues make the AβP1-42 molecule more hydrophobic than AβP1-40, forming stable oligomers (Haass and Selkoe 2007), prone to interact and incorporate into the membrane rather than monomers. We have observed that the AβP1-42 and AβP1-40 monomers does not show channel activity within 48 h (data not shown). The results obtained with electrophoresis and Western blotting indicate that monomers, trimers and tetramers are present in our AβP1-42 sample and that after 24 h of incubation an increase in trimers and tetramers was observed. It is possible to hypothesize that the time between AβP1-42 addition to the cis side of the medium facing the DOPS:DOPE, POPC and POPC:Ch membrane and PLM breakage and withdrawal, in which no conductance variation was observed, could be the timescale needed for the formation of a critical amount of oligomers. Furthermore, the lag time for the onset of AβP1-42 channel-like activity in phospholipid PLMs depends on lipid composition. The addition of negatively charged lipid (50 %, weight ratio) or cholesterol (30 %, weight ratio) to membranes made up of zwitterionic lipids increases the channel-like activity of AβP1-42, favouring the appearance of a conspicuous number of channel-like events after a shorter lag time and at minimal applied potential (24/2 h, 100/60 mV in POPC/DOPS:DOPE PLMs, respectively and 24/6 h in POPC/POPC:Ch PLMs, respectively). Additionally, AβP1-42 oligomers seem to be more prone to interacting with POPC PLMs and to incorporating into membranes than AβP1-40 oligomers because of their high stability (Micelli et al. 2004). Our results are in line with previous observations of other authors who, using atomistic molecular dynamics (MD), showed that the AβP1-40 insertion efficiency in PC bilayers is different than that of AβP1-42. AβP1-40 exhibited complete insertion into the bilayer only for 25 % of replicates, whereas in the case of AβP1-42 this rose to 50 % of replicates (Qiu et al. 2011; Zhao et al. 2011).

These results suggest that negatively-charged lipids favour the binding of AβP1-42 to the membrane surface via electrostatic interaction and its incorporation into the membrane to form channel-like structures. Our findings support those of Hirakura et al. (1999) that anionic lipids are relevant for the interaction of AβP1-42 with the membrane. Given that the phosphatidylserine levels in AD-affected brain are higher than the control brain (Wells et al. 1995), AβP1-42's capacity to perturb the membrane, by forming

ion channels (Hirakura et al. 1999; Lin et al. 2001) or by perturbing liposome membranes (McLaurin and Chakrabartty 1996) or by increasing the membrane permeability of liposomes (Rhee et al. 1998), may be involved in its toxicity in vivo, due to cation-selectivity of channel in this kind of membrane. It is suggested that the Lys28 of Aβ seems to provide an anchor for the phosphate groups of lipids, while aliphatic amino acids, at the C-terminus of AβP, interact with fatty acids of phospholipids (Chauhan et al. 2000). Moreover, this pattern is also followed by other peptides such as AβP1-40 (Arispe et al. 1993a; Micelli et al. 2004), human islet amyloid polypeptide (hIAPP) (Engel et al. 2008) and human calcitonin (Stipani et al. 2001). Some authors have shown that acidic phospholipids alter the structure of AβP1-42; in fact, AβP1-42 is unstructured in a solution of NaCl/Pi, pH 7 and in the presence of PE vesicles, while in the presence of acidic phospholipid vesicles it possesses a prevailing β structure (McLaurin and Chakrabartty 1996).

The results obtained with POPC:Ch PLMs confirm the previous observations of other authors who demonstrated that the addition of Ch to a phospholipid bilayer increases the area expansion modulus in order to accommodate peptides or proteins into the membrane; the increase in peptide-membrane affinity was a result of the involvement of cholesterol in altering membrane fluidity and structure (Subasinghe et al. 2003). Studies of MD simulations show that the incorporation of cholesterol into the bilayer increases surface hydrophobicity, alters lipid ordered packing, and reduces lipid mobility. The changes to the physico-chemical properties of the lipid bilayer enable AβP to bind to the high cholesterol-rich regions more than the pure POPC bilayer (Yu and Zheng 2011). Some authors have shown that, when AβP was added to a 33 % cholesterol-containing DMPC vesicle, the structure of AβP was drastically altered; i.e. the β-sheet structure decreased to zero while the α-helix increased to 58.8 % (Ji et al. 2002). Ashley and colleagues (2006) have demonstrated that the auto-insertion of soluble oligomers, but not monomers, of AβP1-42 into POPC/Ch membranes is accompanied by relocation of the sterol towards the bilayer surface. In addition, Qiu and colleagues have shown that AβP1-42 exhibited complete insertion into the PC/Ch bilayers for 100 % of replicates whereas in the case of AβP1-40 this dropped to 50 % of replicates (Qiu et al. 2011).

The inclusion of 30 % of cholesterol in POPC PLMs does not modify the channel-like event conductance compared with that obtained in POPC membranes, while the channel frequency is higher, suggesting that cholesterol increases the turnover and stability of the channel-like events. This finding indicates that the cholesterol molecule may be considered a target of AβP1-42. Recent studies show that after Aβ binds to raft membranes containing cholesterol, the peptide can be translocated to PC membranes to which monomeric soluble Aβ does not bind (Kakio et al. 2003), thus supporting a direct link between the level of membrane cholesterol, the incorporation of AβP into the membrane and its ability to form a calcium-permeant pore (Abramov et al. 2011).

On the other hand, this membrane presents a channel frequency and lifetime comparable to those found in DOPS:DOPE membranes, while its central conductance is lower. The difference in Λ_c found in different PLMs could suggest a modulation of AβP molecule assembly induced by membrane composition. By means of conductance value, and assuming a water-filled channel with an ion mobility similar to that of the bulk solution, knowing the thickness of the bilayer and the equivalent conductivity of the ions, the channel diameter can be calculated. In our case, in POPC:Ch/DOPS:DOPE membranes (with an average thickness of 5 nm) and at an applied voltage of 60 mV, the channel diameter was 5.8 / 8.0 Å, respectively. It is worth underlining, however, that the calculation of pore area by means of conductance, assuming the channel to be a water-filled hole, is not a straightforward rule (Smart et al. 1997). However, considering the experimental error, these results seem to be not far from the values found in the theoretical calculation by Durell et al. (1994).

Oxidized cholesterol, whose main component is cholesterol, typically contains other products characteristic of aged cholesterol, which also can be found under physiological conditions (Weiner et al. 1972). In addition, some authors have found that the naturally-occurring small molecule oxidation products derived from cholesterol modify AβP, increasing its amyloidogenicity (Usui et al. 2009; Bieschke et al. 2006). For these reasons, we used planar lipid membranes of sterols such as OxCh that could be considered a model of aged membranes useful for clarifying some aspects of AD.

Our results highlight AβP1-42's ability to *spontaneously* interact with OxCh PLMs and to form channel-like structures within 2 h of addition to the medium facing the membrane, which suggests that AβP1-42 molecules have greater affinity for cholesterol and its oxidation products than for phospholipids. In fact, the channel-like activity presumably appears when the amount of AβP1-42 oligomers (trimers and tetramers) is lower at time zero than at 24 h, as the results obtained by electrophoresis and western blot would seem to indicate. Incorporation into OxCh membranes seems to be driven by hydrophobic interaction, as would be expected given the hydrophobic amino acid content in the C-terminus of AβP1-42. By applying the same calculation to OxCh membranes, with an average thickness of 4 nm (Tien et al. 1966) and at applied voltages of 20 and 60 mV, the channel diameter was ~20 and 11 Å, respectively. Considering the experimental error, these results seem to be quite close to the values found in the theoretical calculation of a channel made up of six and four units, respectively, by Durell et al. (1994) and Lin et al. (2001). The central

conductance and diameter values seems to indicate that the AβP1-42 channel-like structure is formed by a greater number of AβP1-42 subunits in OxCh membranes than in phospholipid membranes, supporting the claim that the membrane structure is responsible for the differences in the biophysical properties of the AβP1-42 channel. Our results seem to indicate that membrane structure affects the ion selectivity of the AβP1-42 channel, which is toward anions in OxCh PLMs, whereas it appears to be non-selective in POPC:Ch PLMs. This finding could be of considerable importance during the turnover of the plasma membrane during the life cycle. In fact, during the aging process, when the homogeneous distribution and the presence of oxidation products of cholesterol occurs, it can be speculated that channels with ion selectivity toward anions will hyperpolarize the membrane, thus moving the membrane potential further from the threshold of membrane excitability. In this condition, the contribution to calcium conductance is presumed to be very small. This is important because it has been hypothesized that the increased calcium permeability through AβP channels determines degeneration of neurons (Arispe et al. 1993a; Abramov et al. 2011).

In our previous study, we found that AβP1-40 does not form ion channels in POPC PLMs, while Ch addition (30 % weight ratio) determines channel activity and AβP1-40 spontaneously interacts with OxCh PLMs to form ion channels with anion selectivity (Micelli et al. 2004) similar to those found for AβP1-42 in the present work. Furthermore, using monolayer surface pressure measurements, it has been shown that AβP1-40 spontaneously inserts into monolayers containing a 30 mol% cholesterol to phospholipid ratio and adopts an α-helical structure (Williams and Serpell 2011; Ji et al. 2002).

These experimental observations indicating the affinity of AβP1-42 and AβP1-40 for cholesterol and its oxidation products raise the hypothesis that sterols could be considered neuroprotective factors.

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Conflict of interest The authors declare that they have no conflict of interest.

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