Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1665 (2004) 40–47

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

http://www.elsevier.com/locate/bba

The αM1 segment of the nicotinic acetylcholine receptor exhibits conformational flexibility in a membrane environment

Maurits R.R. de Planque[a,*,1], Dirk T.S. Rijkers[b], Jamie I. Fletcher[c], Rob M.J. Liskamp[b], Frances Separovic[a,*]

[a]*School of Chemistry, University of Melbourne, Melbourne VIC 3010, Australia*
[b]*Department of Medicinal Chemistry, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*
[c]*Department of Biochemistry and Molecular Biology, University of Melbourne, Melbourne VIC 3010, Australia*

Received 14 April 2004; received in revised form 18 June 2004; accepted 24 June 2004
Available online 25 July 2004

Abstract

The transmembrane domain of the nicotinic acetylcholine receptor (nAChR) is predominantly α-helical, and of the four distinctly different transmembrane M-segments, only the helicity of M1 is ambiguous. In this study, we have investigated the conformation of a membrane-embedded synthetic M1 segment by solid-state nuclear magnetic resonance (NMR) methods. A 35-residue peptide representing the extended αM1 domain 206–240 of the *Torpedo californica* nAChR was synthesized with specific ^{13}C- and ^{15}N-labelled amino acids, and was incorporated in different phosphatidylcholine model membranes. The chemical shift of the isotopic labels was resolved by magic angle spinning (MAS) NMR and could be related to the secondary structure of the αM1 analog at the labelled sites. Our results show that the membrane-embedded αM1 segment forms an unstable α-helix, particularly near residue Leu18 (αLeu223 in the entire nAChR). This non-helical tendency was most pronounced when the peptide was incorporated in fully hydrated phospholipid bilayers, with an estimated 40–50% of the peptides having an extended conformation at position Leu18. We propose that the conserved proline residue at position 16 in the αM1 analog imparts a conformational flexibility on the M1 segments that could enable membrane-mediated modulation of nAChR activity.

Keywords: Nicotinic acetylcholine receptor; αM1 transmembrane segment; Conformation; Phosphatidylcholine model membranes; Solid-state NMR

1. Introduction

The nicotinic acetylcholine receptor (nAChR) is the most prominent member of the superfamily of ligand-gated ion channels that are involved in neurotransmission [1,2]. The nAChR from the electric organ of the electric ray *Torpedo californica* consists of a ring of four different subunits that are arranged as a $\alpha_2\beta\gamma\delta$ pentamer around a central axis perpendicular to the membrane, forming a cation-selective ion channel which regulates nerve impulse transduction [3]. Each subunit traverses the membrane with four hydrophobic polypeptide segments, M1–M4, and significant sequence homology exists between analogous M-segments of the α, β, γ and δ chains [4]. The five M2 segments line the aqueous channel pore and are surrounded by the M1, M3 and M4 segments, which are in contact with the lipid matrix of the synaptic membrane [5,6].

Abbreviations: nAChR, nicotinic acetylcholine receptor; CD, circular dichroism; NMR, nuclear magnetic resonance; MAS, magic angle spinning; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine; DTPC, 1,2-ditetradecyl-*sn*-glycero-3-phosphatidylcholine

* Corresponding authors. Maurits R.R. de Planque is to be contacted at Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom. Fax: +44 1865 275234. Frances Separovic is to be contacted at Tel.: +61 3 8344 6464; fax: +61 3 9347 5180.

E-mail addresses: maurits.deplanque@bioch.ox.ac.uk (M.R.R. de Planque), fs@unimelb.edu.au (F. Separovic).

[1] Present address: Biomembrane Structure Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom.

0005-2736/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2004.06.021

In the absence of a high-resolution structure of the nAChR, the conformation of the transmembrane M-segments, originally predicted to constitute four-helix bundles [4,7], has remained controversial. From cryoelectron microscopy images, Unwin [3] initially constructed a 9-Å in resolution structure in which the M2 segments were identified as α-helices and the other M-segments seemed part of a β-barrel. However, infrared spectroscopy indicated that the transmembrane nAChR domain consists mainly of α-helices and not of β-sheets [8,9]. Also Trp and Cys substitution and labelling experiments with membrane-or pore-delivered probes identified M2, M3 and M4 as α-helices [10–13]. Only for the (α)M1 segment was the labelling pattern inconsistent with a well-defined helical conformation [10,12], and it was postulated that M1 is kinked or otherwise deformed in the region of the proline residues [14]. Most recently, Unwin presented a refined structure of the transmembrane domain with a resolution of 4 Å, in which all the M-segments appear as undistorted α-helices [5].

The secondary structure of individual transmembrane nAChR segments also has been investigated using isolated or synthetic M-segments instead of the entire protein. Membrane-reconstituted proteolytic digests, containing one or more M-segments, were shown to be predominantly (>80%) helical by infrared and circular dichroism (CD) spectroscopy, with the exception of αM1, for which only an overall helicity of about 60% was apparent [15]. Furthermore, a synthetic δM2 peptide was demonstrated by solid-state nuclear magnetic resonance (NMR) methods to be an undistorted α-helix in model membranes of phosphatidylcholine lipids in which tetramers of M2, but not of the other M-segments, form functional cation-selective pores [16]. Solution NMR studies have shown that synthetic M3 and M4 peptides also form well-defined α-helices in membrane-mimicking environments [16–19].

It thus appears that of the four distinctly different transmembrane segments, only M1 may contain non-helical elements or form an unstable helix, which would render M1 sensitive to conformational changes that could play a role in the regulation of channel activity. Therefore, we studied a 35-residue peptide corresponding to the extended M1 sequence of the α subunit of the *T. californica* nAChR (Fig. 1) in phospholipid membranes. We demonstrated previously that this αM1 analog strongly interacts with model membranes, ordering lipid acyl chains and inducing

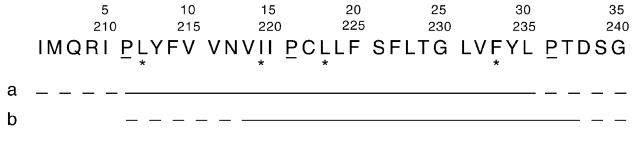

Fig. 1. Amino acid sequence of the 35-residue αM1 peptide with residue numbering as in the nAChR α subunit; labelled residues are marked with an asterisk and proline residues are underlined. Residues in contact with the hydrophobic membrane core (continuous line) and with the lipid headgroups (dotted line) are indicated according to (a) sequence analysis and (b) the structural model of the nAChR pore [5].

the formation of small vesicles, possibly through modification of the bilayer lateral pressure profile. However, some lipid compositions, e.g., phosphatidylcholine/cholesterol, were found to retain a multilamellar vesicle morphology in the presence of this αM1 peptide [20]. Lipid acyl chain ordering and a lipid-dependent modulation of conformational equilibria have also been observed in reconstitution studies of the entire nAChR [21], highlighting the benefits of studying transmembrane protein segments in a bilayer rather than a micellar environment.

In the present study, we investigated the secondary structure of a specifically labelled αM1 analog incorporated in different phosphatidylcholine model membranes. ^{13}C and ^{15}N magic angle spinning (MAS) NMR spectra indicated that several labelled sites in the peptide can have either a helical or a non-helical conformation, most notably at Leu18 (αLeu223 in the nAChR). This residue is in proximity to the conserved central proline at position 16 of the αM1 segment, and we propose that the presence of this membrane-embedded Pro residue confers a conformational flexibility on M1 that distinguishes it from the other M-segments of the *T. californica* nAChR, which, with the exception of δM4 [5], do not contain Pro residues in the transmembrane stretch.

2. Materials and methods

The specifically labelled amino acids L-leucine-1-^{13}C and L-phenylalanine-3-^{13}C were obtained from Cambridge Isotope Laboratories (Andover, MA), L-leucine-2-^{13}C from Isotec (Miamisburg, OH) and L-isoleucine-^{15}N from Spectra Stable Isotopes (Columbia, MD). All phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL), and the anaesthetic halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) from Aldrich (St. Louis, MO).

2.1. Peptide synthesis

The peptide sequence, H-Ile-Nle-Gln-Arg-Ile-Pro-Leu[2-^{13}C]-Tyr-Phe-Val-Val-Asn-Val-Ile[^{15}N]-Ile-Pro-Cys-Leu[1-^{13}C]-Leu-Phe-Ser-Phe-Leu-Thr-Gly-Leu-Val-Phe[3-^{13}C]-Tyr-Leu-Pro-Thr-Asp-Ser-Gly-OH, representing the αM1-segment amino acid sequence 206–240 (230–264 when including the signal sequence) of the nAChR of *T. californica* [7], was synthesized using Fmoc chemistry as described earlier [20]. However, the labelled amino acid derivatives (two equivalents) were coupled with *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5]-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate/*N*-hydroxy-7-azabenzotriazole [22,23], in the presence of four equivalents of *N*,*N*-diisopropylethylamine in *N*-methylpyrrolidone. After the applied coupling time of 16 h, no free amino functionalities could be observed by the Kaiser test [24]. Following size exclusion chromatography, peptide purity as determined by analytical HPLC [20] exceeded 90%. The

calculated average $(M+H)^+$ mass of the peptide is 3977.86, and a value of 3977.23 was found by mass spectrometry.

2.2. Sample preparation

The αM1 peptide was dissolved in 20 μl of trifluoroacetic acid per milligram of peptide. Subsequently, the excess solvent was evaporated by a stream of nitrogen, and the resulting peptide film was redissolved in 0.5 ml of trifluoroethanol. This peptide solution was added to 0.5 ml of a phospholipid solution in trifluoroethanol, followed by addition of 15 ml of distilled water and overnight lyophilization to remove the organic solvents. This method ensures that hydrophobic peptides and lipids are mixed on a molecular level and that multilamellar vesicles are formed prior to lyophilization [25]. Normally, 3 μmol of peptide and 90 μmol of phospholipid were used for NMR experiments. Control samples with only peptides (20 μmol) or only lipids (80 μmol) were also prepared following this procedure.

2.3. Circular dichroism spectroscopy

Samples with a 1:30 peptide/lipid molar ratio (containing 0.3 μmol of peptide) were prepared as described above and were hydrated in 1 ml of distilled water. Subsequently, samples were sonicated (2 min, 25% duty cycle, input power of 40 W, Branson 250 tip sonicator) to obtain unilamellar vesicles, and centrifuged (5 min, 12,000×*g*) to pellet down titanium particles and any residual multilamellar structures. Part of the clear supernatant fraction was diluted with distilled water, resulting in an αM1 concentration of 0.1 mM. CD spectra of these diluted samples were recorded on a Jasco J-810 spectropolarimeter (Jasco International Co. Ltd., Tokyo, Japan) equipped with a CDF-426S temperature controller (1-mm path length cell, 0.5-nm data pitch, 0.5-s response time, 20 nm/min scan speed, 1-nm bandwidth, 34 °C). To obtain aligned multilayers, 150 μl of undiluted supernatant fraction was spread out on the side of a quartz cuvette and the excess water was removed with a nitrogen flow. Oriented CD spectra were recorded to confirm a transmembrane peptide topology [26]. Spectra were corrected for the contribution of the lipids and smoothed.

2.4. NMR measurements

Lyophilized peptide/lipid powders (containing 3.0 μmol of peptide) were packed into 5-mm silicon nitride rotors (Doty Scientific, Columbia, SC); during this procedure, samples with POPC, DMPC or DTPC formed a gel-like paste due to the absorption of atmospheric water. After initial NMR measurements, samples were explicitly hydrated by adding 75–100 μl of distilled water to the material in the rotor, resulting in an approximate membrane/water ratio of 1:1 (w/w), and followed by homogenization (manual stirring) and 10 cycles of freeze-thawing. Halothane was added by injecting 25 μl of a 0.4 M solution of halothane in water into an already hydrated sample, followed by rapid homogenization, air-tight closure of the rotor, and repeated freeze-thawing. Solid-state NMR measurements were performed on a Varian Inova 300 (Varian, Palo Alto, CA) equipped with a 5-mm-diameter Doty Scientific MAS probe. The operating frequencies were 30.41 MHz for ^{15}N, 75.45 MHz for ^{13}C, 121.46 MHz for ^{31}P, and 300.04 MHz for ^{1}H. ^{13}C NMR spectra were obtained under cross polarization and MAS conditions (6-kHz spin rate, 4096 complex data points, 3-s delay time, 50-kHz spectral width, proton 4.4 μs π/2 pulse with a decoupling power of ~70 kHz, 2-ms contact time). Typically, 20 000 scans were accumulated and a 10-Hz line broadening was applied after zero-filling to 8192 points and prior to Fourier transformation. ^{15}N MAS NMR spectra were recorded under similar conditions (6-kHz spin rate, 4-s delay time, 30-kHz spectral width, 1.25-ms contact time, proton 6.5 μs π/2 pulse with a decoupling power of ~40 kHz), with accumulation of 15 000–20 000 scans and a 40-Hz line broadening. To characterize the macroscopic lipid organization, broadline ^{31}P NMR spectra were obtained from static samples (3.5 μs π/2 pulse, 4096 complex data points, 1.5-s delay time, 50-kHz spectral width, ~70-kHz proton decoupling), typically with accumulation of 3000 scans, zero-filling to 8192 points, and a 100-Hz line broadening. ^{13}C MAS NMR spectra were referenced to hexamethylbenzene powder (17.0 and 131.9 ppm), ^{15}N MAS NMR spectra to L-isoleucine-^{15}N powder (41.4 ppm, corresponding to 0 ppm for liquid $^{15}NH_3$), and ^{31}P NMR spectra to an aqueous solution of 85% H_3PO_4 (0 ppm).

3. Results

3.1. Conformation and topology of αM1 in model membranes

The conformational behavior and membrane-association properties of αM1 (Fig. 1) were characterized by CD spectroscopy. In the absence of lipid, no significant signal was observed because the hydrophobic peptide is insoluble in aqueous solution, and consequently, the spectrum of αM1 in the presence of DTPC vesicles (Fig. 2, curve a) corresponds exclusively to membrane-associated peptide. The line shape of this spectrum, with minima near 222 and 210 nm, a maximum near 196 nm and a cross-over point at 204 nm, is indicative of a peptide that is predominantly α-helical [27]. Similar spectra were obtained for αM1 in the presence of DMPC (curve b) and POPC (curve c). It can be estimated from theoretical reference spectra [27] that αM1 incorporated in these PC bilayers consists of 60–70% α-helical elements and 30–40% β-extended elements. The considerable percentage of extended conformation most likely originates from unstructured N- and C-termini, as observed in high-resolution NMR structures of extended transmembrane peptides in micelles (e.g., Ref. [28]), and as

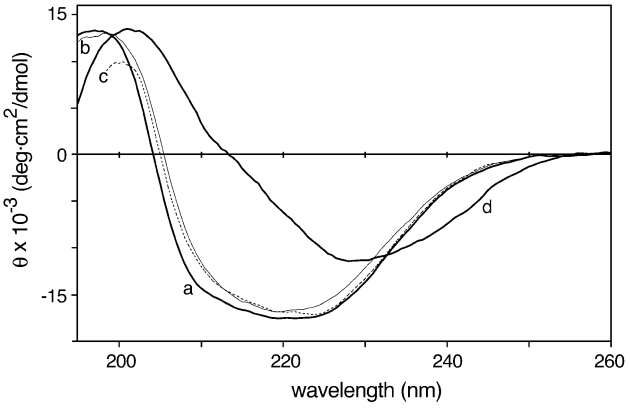

Fig. 2. CD spectra of αM1 in sonicated vesicles of (a) DTPC, (b) DMPC (feint line), and (c) POPC (dotted line) in excess water, and (d) of αM1 in aligned multilayers of DTPC, with a 1:30 molar ratio of peptide to lipid and at 34 °C.

suggested from preliminary 2D ^{1}H NMR data of micelle-embedded αM1 (results not shown). The CD spectrum of αM1 in aligned multilayers of DTPC (curve d) does not show a minimum around 210 nm, and the other minimum, the maximum and the cross-over point, have all shifted to longer wavelengths. This line shape is characteristic for a helix with its long axis parallel to the incoming light [29], implying a transmembrane orientation of the αM1 peptide in PC bilayers and a helical conformation of the residues that are embedded in the bilayer hydrophobic core.

3.2. Conformation of αM1 at specific sites

To investigate the local conformation of the peptide in PC bilayers, the 35-residue αM1 peptide employed in this study was specifically labelled at four positions. Sequence analysis indicates that the labelled residues $^{13}C_{\alpha}$-Leu07 and $^{13}C_{\beta}$-Phe28 would be expected to be in contact with the membrane–water interface on either side of the bilayer, whereas the residues ^{15}N-Ile14 and ^{13}C=O-Leu18 should be deeply buried in the hydrophobic membrane core (Fig. 1). ^{13}C and ^{15}N NMR spectra were acquired using MAS, to remove the chemical anisotropy and to suppress the dipole–dipole interaction inherent in NMR of membrane systems, in combination with cross polarization from protons to enhance the signal intensity of ^{13}C and ^{15}N nuclei [30,31].

^{15}N MAS NMR spectra of αM1 in the absence and presence of lipids are depicted in Fig. 3. The spectrum of αM1 as a lyophilized powder without lipids (Fig. 3A) was characterized by two overlapping signals of about equal intensity at 118.7 and 126.7 ppm. Since the peptide contains only a single ^{15}N-label, the observation of two distinct resonances most likely reflects the presence of two peptide populations, which differ in secondary structure at the position of the ^{15}N-labelled Ile14 residue. Shoji et al. [32] investigated the relationship between ^{15}N chemical shift and peptide conformation for a series of homopolypeptides with different secondary structures. For powder of poly-Leu, values of 117.7 and 127.7 ppm (w.r.t. liquid $^{15}NH_3$) were observed for the α-helix and β-sheet conformation, respectively, while a value of 126.8 ppm was found for powder of poly-Ile in a β-sheet conformation [32]. These data indicate that the two resonances observed for αM1 powder correspond to ^{15}N-Ile14 in α-helix and β-extended conformations, implying that the secondary structure of αM1 in the absence of lipid is not defined, as may be expected for a hydrophobic peptide. In the presence of the lipids DSPC or POPC, however, the resonance near 118 ppm dominates the spectrum (see Fig. 3B–C), demonstrating that the lipid matrix imposes an α-helical conformation on αM1. Note that the αM1/DSPC sample is a dry lyophilized powder, but that the lyophilized αM1/POPC (unsaturated lipid) sample has absorbed a considerable amount of atmospheric water. The higher water content results in an increase in molecular motion, but reduces the efficiency of cross polarization [30,31], which is reflected in the lower signal-to-noise ratio of the αM1/POPC and αM1/DMPC (not shown) spectra in comparison to the αM1/DSPC spectrum.

^{13}C MAS NMR spectra of the αM1 systems discussed above are shown in Fig. 4. The pure peptide sample gives rise to three main resonances near 175, 55 and 40 ppm, corresponding to the ^{13}C=O-Leu18, $^{13}C_{\alpha}$-Leu07 and $^{13}C_{\beta}$-Phe28 labels, respectively (see Fig. 4A). Analogous to the situation with the ^{15}N-Ile14 residue, the ^{13}C=O-Leu18 and $^{13}C_{\alpha}$-Leu07 resonances are each split into two overlapping peaks, which correspond to different conformations of αM1 at the labelled sites. Saitô [33] reported chemical shifts of 175.8 (C=O) and 55.8 ppm (C_{α}) for the α-helical, and 171.3 (C=O) and 51.2 ppm (C_{α}) for the β-sheet conformation of lyophilized poly-Leu peptides. These values correspond well to the observed resonance positions of the ^{13}C=O-Leu18 label (176.6 and 172.7 ppm) and the $^{13}C_{\alpha}$-

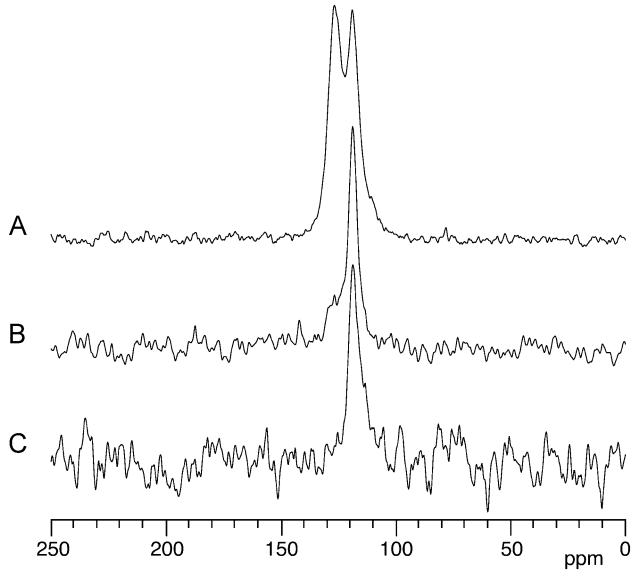

Fig. 3. ^{15}N MAS NMR spectra of (A) αM1 powder, and lyophilized vesicles of (B) αM1/DSPC and (C) αM1/POPC, at a 1:30 peptide/lipid ratio and at 34 °C. Spectra are indirectly referenced to liquid $^{15}NH_3$.

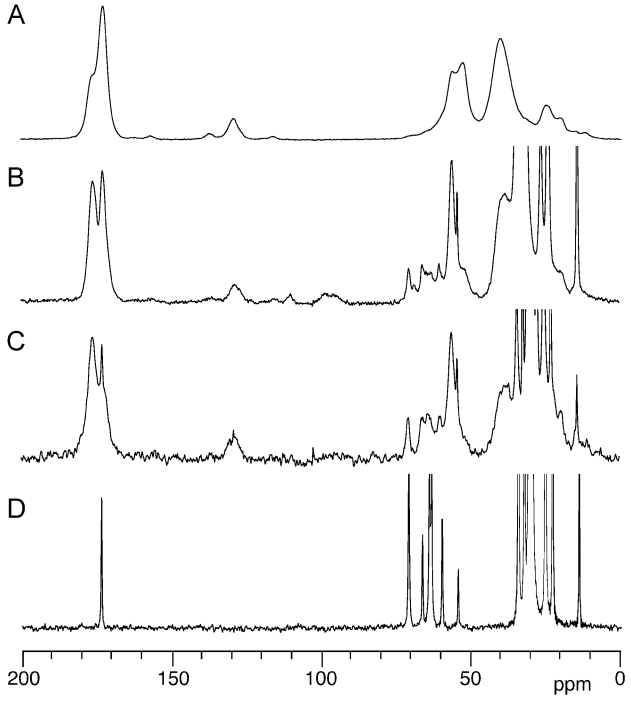

Fig. 4. ^{13}C MAS NMR spectra of (A) αM1 powder, and lyophilized vesicles of (B) αM1/DSPC and (C) αM1/POPC at a 1:30 peptide/lipid molar ratio, and (D) a hydrated sample of DMPC alone, acquired at 34 °C.

Leu07 label (56.3 and 52.1 ppm) of the αM1 peptide, confirming the notion that in the absence of lipids, αM1 does not adopt a well-defined secondary structure. The $^{13}C_{\beta}$-Phe28 residue gave rise to only a single broad signal around 39.4 ppm, and although a value of 39.3 ppm has been reported for α-helical poly-Phe peptides [33], due to spectral overlap, only the C=O and C_{α} resonances were used to probe the local secondary structure of membrane-incorporated αM1. The ^{13}C MAS NMR spectra of αM1/DSPC and αM1/POPC (Fig. 4B–C) are dominated by the natural abundance signals from the lipid components, which partially overlap with the resonances of ^{13}C=O-Leu18 and $^{13}C_{\alpha}$-Leu07. However, peptide and lipid resonances can be distinguished since the lipid resonances are narrower because of the relatively fast axial reorientation of the lipids, especially in the more fluid POPC and DMPC samples with a comparatively high water content. By comparison to the spectra of peptide-only and lipid-only (Fig. 4D) samples, we estimate that only the α-helical spectral component of the $^{13}C_{\alpha}$-Leu07 label significantly contributed to the αM1/PC spectra. However, in the αM1/POPC and αM1/DMPC (not shown) spectra, the peaks around 175 ppm can be interpreted as *two* relatively broad signals from ^{13}C=O-Leu18, with greater intensity at 176 than at 172 ppm, in combination with a relatively narrow resonance from the lipid carbonyls at 172 ppm. It thus seems that upon association with PC bilayers, αM1 attains a well-defined α-helical conformation around Leu07 (and Ile14), but that Leu18 belongs to a region that can also exist in a more extended conformation.

3.3. Conformation of αM1 in fully hydrated multilamellar vesicles

We reported in an earlier wide-line ^{31}P and ^{2}H NMR study that the αM1 analog induces a slow conversion from large multilamellar vesicles to significantly smaller vesicles, an effect that becomes more pronounced upon increased water content [20]. After performing the ^{15}N and ^{13}C MAS NMR measurements described above, we therefore assessed the macroscopic lipid organization of the αM1/PC samples by wide-line ^{31}P NMR [34,35]. The corresponding spectra (not shown) indeed indicated that some lipids were not present as large multilamellar vesicles, as evident from spectral components with a reduced chemical shift anisotropy. The increased curvature of lipid bilayers in vesicles with a significantly reduced size can be reflected in a different bilayer lateral pressure profile, which may affect the conformation of a transmembrane peptide. Consequently, the αM1 analog was studied in more detail in vesicles of DTPC for the reason that DTPC bilayers retain the multilamellar morphology in the presence of αM1 [20]. As an additional advantage, this ether-linked lipid enables estimation of the relative intensities of the ^{13}C=O-Leu18 signals at 176 and 172 ppm without the complicating spectral contribution from the lipid ester carbonyl moieties [36].

As shown in Fig. 5A, in the ^{13}C MAS NMR spectrum of lyophilized DTPC powder, which has absorbed some atmospheric water, lipid resonances are indeed observed only between 0 and 80 ppm. Consequently, the resonances at 176 and 172 ppm in the spectrum of lyophilized vesicles of αM1/DTPC (Fig. 5B) correspond exclusively to the different conformations of ^{13}C=O-Leu18. As judged from the relative intensities of these signals, 60–70% of the peptides are α-helical near Leu18, while the remaining

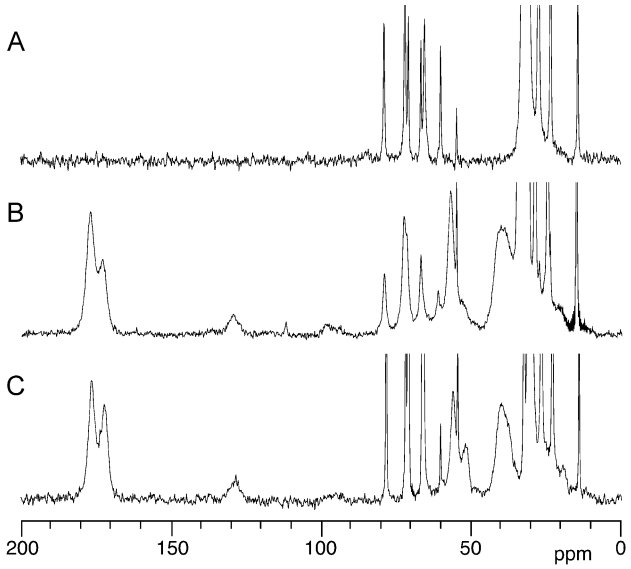

Fig. 5. ^{13}C MAS NMR spectra of lyophilized vesicles of DTPC (A) without and (B) with αM1, and (C) of hydrated αM1/DTPC vesicles, at a 1:30 peptide/lipid molar ratio and at 34 °C.

population is in a more extended conformation in this part of αM1. In contrast, the intensity distribution between 56 and 52 ppm indicates that at least 90% of the peptides are helical near Leu07, and the same can be concluded from the ^{15}N MAS NMR spectrum (not shown) for the Ile14 position.

Upon hydration of the αM1/DTPC system, however, the intensity of the ^{13}C resonances at 172 and 52 ppm increased (see Fig. 5C), indicating that a larger proportion (~40–50%) of the peptides is non-helical at Leu18 and that also an estimated 30% of the peptide population is now in an extended conformation at Leu07. The corresponding ^{15}N MAS NMR spectrum (not shown) indicated that the α-helical component of the ^{15}N-Ile14 label is still dominant, but quantification of signal intensity was not possible due to the low signal-to-noise ratio of the spectrum. The corresponding ^{31}P NMR spectrum (not shown) with a chemical shift anisotropy of 36 ppm was typical of fully hydrated multilamellar vesicle dispersions in the biologically relevant liquid-crystalline phase [34,35]. In fact, given that the relative intensities of the Leu18 and Leu07 resonances in the ^{13}C MAS NMR spectra of αM1/DTPC (Fig. 5B) and αM1/POPC (Fig. 4C) are very similar, whereas the ^{31}P NMR chemical shift anisotropies of the two samples differ by a factor of two (spectra not shown), the conformation of αM1 does not seem to be modulated by the vesicle morphology.

For the hydrated αM1/DTPC bilayers, we investigated the effect of adding an anaesthetic on the secondary structure of αM1 at the Leu18 and Leu07 positions. The volatile anaesthetic halothane was injected into the hydrated αM1/DTPC sample, with a nominal halothane concentration of 0.1 M, which corresponds to a halothane/lipid molar ratio of 1:10 and a halothane/peptide ratio of 3:1, although some halothane would have evaporated during sample mixing. The ^{13}C MAS NMR spectrum of hydrated αM1/DTPC with halothane (not shown) exhibited a small increase in signal intensity at 172 and 52 ppm, suggesting that the anaesthetic had little effect on the proportion of αM1 peptides that are in a non-helical conformation. As with increasing hydration, any increase in extended structure could be attributed to changes in the physical properties of the bilayers such as a decrease in membrane order as has been previously observed for general anaesthetics [37].

4. Discussion

The αM1 analog employed in this study not only covers the hydrophobic residues predicted to be in contact with the membrane core (Fig. 1), but also the more polar flanking residues which typically interact with the lipid headgroups [4,38]. This extended αM1 analog was shown by CD to have an overall helical content of about 65% in unilamellar PC vesicles in excess water, in agreement with the ~60% overall helicity estimated for the vesicle-reconstituted proteolytic digest α210–242 [15]. Oriented CD measurements suggested that the αM1 segment traverses the hydrophobic membrane core as an α-helix, most likely with disordered ends on both sides of the bilayer. Our MAS NMR data indicated that, as a lyophilized powder, the entire αM1 peptide is not well structured, but that upon incorporation in a range of PC bilayers, the peptide attained a predominantly α-helical conformation at the labelled sites in the transmembrane stretch. However, we observed that the extent of helicity depends on the water content, most likely because the bilayer becomes more fluid and less ordered with increasing hydration.

Notably, at the more biologically relevant full hydration, populations of the αM1 analog with different conformations coexist. Although in multilamellar vesicles of DTPC the majority of the peptides were found to have an α-helical conformation at the labelled sites Leu07, Ile14 and Leu18, which correspond to residues αLeu212, αIle219 and αLeu223 of the nAChR (Fig. 1), a significant peptide fraction appeared to be non-helical at these positions. The largest percentage of non-helicity, an estimated 40–50%, was observed for residue Leu18. We construe that the large fraction of αM1 peptides with non-helical structural elements still exhibits a transmembrane topology. This is based on the observation that the relative resonance intensities are consistently different for the membrane samples than for the aggregated peptide powder (Figs. 3 and 4), and that αM1 significantly orders the lipid acyl chains of fully hydrated DMPC bilayers [20], whereas partially inserted surface-associated peptides generally cause a disordering of the acyl chains [39].

In contrast to our observations for αM1, deviations from helicity have not been reported for synthetic M2, M3 and M4 analogs in membranes or membrane mimicking environments [16–19]. However, the results of our model system study are in agreement with experiments on the entire nAChR receptor, which show that (only) for M1 the pattern of photolabelling is not consistent with a well-defined α-helical structure [10–12]. Because labelling patterns ruled out a β-sheet conformation for αM1 near Leu223 [10,12], the non-helical signal observed by MAS NMR for the Leu18 position in our synthetic analog probably represents a local distortion within an overall helical structure. It is interesting in this respect that a minor disruption of the α-helical conformation, in the form of a short stretch of 3_{10}-helix, has recently been reported for the αM3 segment [40]. For the αM1 peptide, the non-helicity near Leu18 could well be induced by the proline residue at position 16. A proline and about four surrounding residues typically form a kink in the transmembrane stretch with an angle that can vary between 5° and 60°, and these hinge regions are thought to play a key role in membrane proteins because of their expected inherent flexibility [41,42]. Preliminary two-dimensional ^{1}H NMR spectra of αM1 in perdeuterated SDS and DPC micelles were characterized by broad and low-intensity resonances (spectra not shown), which could indeed be consistent with a flexible peptide undergoing conformational exchange.

In the entire receptor, the presence of the other M-segments will impart a different, most likely more restricted, conformation on αM1 than in the case of an isolated peptide. However, in the most recent structural model of the nAChR transmembrane region, the helical M-segments are rather loosely packed, an arrangement that facilitates the segment rotations involved in the gating mechanism. The outer ring of M1, M3 and M4 helices appears to be in extensive contact with the lipid matrix, as well as these M-segments being largely separated by water-filled cavities from the inner ring of M2 helices [5]. Conformational equilibria of the nAChR are not only controlled by the binding of acetylcholine, but are also modulated by the lipid composition of the membrane [21,43], and many anaesthetics inhibit channel activity by targeting the lipid-exposed M-segments [44,45]. The mechanism underlying these interactions is unknown, but is expected to involve a conformational change in the lipid-exposed M1, M3 or M4 segments, which is subsequently transmitted to the channel-lining M2 helices. Given that the M1-segments are the only M-segments of the nAChR with a conserved proline in the hydrophobic part of the transmembrane sequence (Fig. 1), that an isolated αM1 peptide has distinct non-helical tendencies and may exhibit flexibility near this residue, and that the M1 and M2 segments are in close proximity at this site in the receptor [5], we propose that the M1 segments are the most likely candidates to transmit events at the protein–lipid interface to the channel lumen through transient deviations from full helicity.

Acknowledgements

This work was supported by fellowship S81-683 of The Netherlands Organization for Scientific Research (NWO) and by the Melbourne Research Development Grants Scheme (MRDGS).

References

[1] A. Karlin, Emerging structure of the nicotinic acetylcholine receptors, Nat. Rev., Neurosci. 3 (2002) 102–114.
[2] J.P. Changeux, S.J. Edelstein, Allosteric receptors after 30 years, Neuron 21 (1998) 959–980.
[3] N. Unwin, Acetylcholine receptor channel imaged in the open state, Nature 373 (1995) 37–43.
[4] M. Noda, H. Takahashi, T. Tanabe, M. Toyosato, S. Kikyotani, Y. Furutani, T. Hirose, H. Takashima, S. Inayama, T. Miyata, S. Numa, Structural homology of *Torpedo californica* acetylcholine receptor subunits, Nature 302 (1983) 528–532.
[5] A. Miyazawa, Y. Fujiyoshi, N. Unwin, Structure and gating mechanisms of the acetylcholine receptor pore, Nature 423 (2003) 949–955.
[6] F.J. Barrantes, Lipid matters: nicotinic acetylcholine receptor–lipid interactions (Review), Mol. Membr. Biol. 19 (2002) 277–284.
[7] M. Noda, H. Takahashi, T. Tanabe, M. Toyosato, Y. Furutani, T. Hirose, M. Asai, S. Inayama, T. Miyata, S. Numa, Primary structure of alpha-subunit precursor of *Torpedo californica* acetylcholine receptor deduced from cDNA sequence, Nature 299 (1982) 793–797.
[8] J.E. Baenziger, N. Méthot, Fourier transform infrared and hydrogen/deuterium exchange reveal an exchange-resistant core of α-helical peptide hydrogens in the nicotinic acetylcholine receptor, J. Biol. Chem. 270 (1995) 29129–29137.
[9] N. Méthot, J.E. Baenziger, Secondary structure of the exchange-resistant core from the nicotinic acetylcholine receptor probed directly by infrared spectroscopy and hydrogen/deuterium exchange, Biochemistry 37 (1998) 14815–14822.
[10] M.P. Blanton, J.B. Cohen, Identifying the lipid–protein interface of the Torpedo nicotinic acetylcholine receptor: secondary structure implications, Biochemistry 33 (1994) 2859–2872.
[11] M.P. Blanton, L.J. Dangott, S.K. Raja, A.K. Lala, J.B. Cohen, Probing the structure of the nicotinic acetylcholine receptor ion channel with the uncharged photoactivable compound [^{3}H]diazofluorene, J. Biol. Chem. 273 (1998) 8659–8668.
[12] M.H. Akabas, A. Karlin, Identification of acetylcholine receptor channel-lining residues in the M1 segment of the α-subunit, Biochemistry 34 (1995) 12496–12500.
[13] S. Tamamizu, G.R. Guzmán, J. Santiago, L.V. Rojas, M.G. McNamee, J.A. Lasalde-Dominicci, Functional effects of periodic tryptophan substitutions in the α M4 transmembrane domain of the *Torpedo californica* nicotinic acetylcholine receptor, Biochemistry 39 (2000) 4666–4673.
[14] F.J. Barrantes, S.S. Antollini, M.P. Blanton, M. Prieto, Topography of nicotinic acetylcholine receptor membrane-embedded domains, J. Biol. Chem. 275 (2000) 37333–37339.
[15] J. Corbin, N. Méthot, H.H. Wang, J.E. Baenziger, M.P. Blanton, Secondary structure analysis of individual transmembrane segments of the nicotinic acetylcholine receptor by circular dichroism and Fourier transform infared spectroscopy, J. Biol. Chem. 273 (1998) 771–777.
[16] S.J. Opella, F.M. Marassi, J.J. Gesell, A.P. Valente, Y. Kim, M. Oblatt-Montal, M. Montal, Structures of the M2 channel-lining segments from nicotinic acetylcholine and NMDA receptors by NMR spectroscopy, Nat. Struct. Biol. 6 (1999) 374–379.
[17] P.T.F. Williamson, B.B. Bonev, F.J. Barrantes, A. Watts, Structural characterization of the M4 transmembrane domain of the acetylcholine receptor: An NMR study, Biophys. J. 78 (2000) 147A.
[18] V.S. Pashkov, I.V. Maslennikov, L.D. Tchikin, R.G. Efremov, V.T. Ivanov, A.S. Arseniev, Spatial structure of the M2 transmembrane segment of the nicotinic acetylcholine receptor alpha-subunit, FEBS Lett. 457 (1999) 117–121.
[19] A.A. Lugovskoy, I.V. Maslennikov, Y.N. Utkin, V.I. Tsetlin, J.B. Cohen, A.S. Arseniev, Spatial structure of the M3 transmembrane segment of the nicotinic acetylcholine receptor α subunit, Eur. J. Biochem. 255 (1998) 455–461.
[20] M.R.R. de Planque, D.T.S. Rijkers, R.M.J. Liskamp, F. Separovic, The αM1 transmembrane segment of the nicotinic acetylcholine receptor interacts strongly with model membranes, Magn. Reson. Chem. 42 (2004) 148–154.
[21] C.J.B. daCosta, A.A. Ogrel, E.A. McCardy, M.P. Blanton, J.E. Baenziger, Lipid–protein interactions at the nicotinic acetylcholine receptor, J. Biol. Chem. 277 (2002) 201–208.
[22] L.A. Carpino, 1-Hydroxy-7-azabenzotriazole. An efficient peptide coupling additive, J. Am. Chem. Soc. 115 (1993) 4397–4398.
[23] L.A. Carpino, A. El-Faham, C.A. Minor, F. Albericio, Advantageous applications to azabenzotriazole-based coupling reagents to solid-phase peptide synthesis, J. Chem. Soc., Chem. Commun. (1994) 201–203.
[24] E. Kaiser, R.L. Colescott, C.D. Bossinger, P.I. Cook, Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides, Anal. Biochem. 34 (1970) 595–598.
[25] J.A. Killian, I. Salemink, M.R.R. de Planque, G. Lindblom, R.E. Koeppe II, D.V. Greathouse, Induction of nonbilayer structures in diacylphosphatidylcholine model membranes by transmembrane α-helical peptides: importance of hydrophobic mismatch and proposed role of tryptophans, Biochemistry 35 (1996) 1037–1045.

[26] M.R.R. de Planque, J.A.W. Kruijtzer, R.M.J. Liskamp, D. Marsh, D.V. Greathouse, R.E. Koeppe II, B. de Kruijff, J.A. Killian, Different membrane anchoring positions of tryptophan and lysine in synthetic transmembrane α-helical peptides, J. Biol. Chem. 274 (1999) 20839–20846.
[27] N. Greenfield, G.D. Fasman, Computed circular dichroism spectra for the evaluation of protein conformation, Biochemistry 8 (1969) 4108–4116.
[28] V.E. Yushmanov, Y. Xu, P. Tang, NMR structure and dynamics of the second transmembrane domain of the neuronal acetylcholine receptor β_2 subunit, Biochemistry 42 (2003) 13058–13065.
[29] H.H.J. de Jongh, E. Goormaghtigh, J.A. Killian, Analysis of circular dichroism spectra of oriented protein–lipid complexes: toward a general application, Biochemistry 33 (1994) 14521–14528.
[30] D.E. Warschawski, P.F. Devaux, Polarization transfer in lipid membranes, J. Magn. Reson. 145 (2000) 367–372.
[31] A. Drechsler, F. Separovic, Solid-state NMR structure determination, IUBMB Life 55 (2003) 515–523.
[32] A. Shoji, T. Ozaki, T. Fujito, K. Deguchi, I. Ando, High-resolution ^{15}N NMR study of solid homopolypeptides by the cross-polarization-magic angle spinning method: conformation-dependent ^{15}N chemical shifts characteristic of the α-helix and β-sheet forms, Macromolecules 20 (1987) 2441–2445.
[33] H. Saitô, Conformation-dependent ^{13}C chemical shifts: a new means of conformational characterization as obtained by high-resolution solid-state NMR, Magn. Reson. Chem. 24 (1986) 835–852.
[34] J. Seelig, ^{31}P nuclear magnetic resonance and the head group structure of phospholipids in membranes, Biochim. Biophys. Acta 515 (1978) 105–140.
[35] I.C.P. Smith, I.H. Ekiel, Phosphorus-31 NMR of phospholipids in membranes, in: D.G. Gorenstein (Ed.), Phosphorus-31 NMR: Principles and Applications, Academic Press Inc., Orlando, 1984, pp. 447–475.
[36] R. Smith, F. Separovic, F.C. Bennett, B.A. Cornell, Mellitin-induced changes in lipid multilayers, Biophys. J. 63 (1992) 469–474.
[37] J. Baber, J.F. Ellena, D.S. Cafiso, Distribution of general anesthetics in phospholipid bilayers determined using ^{2}H-NMR and ^{1}H–^{1}H NOE spectroscopy, Biochemistry 34 (1995) 6533–6539.
[38] M.R.R. de Planque, J.A. Killian, Protein–lipid interactions studied with designed transmembrane peptides: role of hydrophobic matching and interfacial anchoring (Review), Mol. Membr. Biol. 20 (2003) 271–284.
[39] A. Zemel, A. Ben-Shaul, S. May, Membrane perturbation induced by interfacially adsorbed peptides, Biophys. J. 86 (2004) 3607–3619.
[40] G.R. Guzmán, J. Santiago, A. Ricardo, R. Martí-Arbona, L.V. Rojas, J.A. Lasalde-Dominicci, Tryptophan scanning mutagenesis in the αM3 transmembrane domain of the *Torpedo californica* acetylcholine receptor: functional and structural implications, Biochemistry 42 (2003) 12243–12250.
[41] F.S. Cordes, J.N. Bright, M.S.P. Samson, Proline-induced distortions of transmembrane helices, J. Mol. Biol. 323 (2002) 951–960.
[42] B. Arshava, I. Taran, H. Xie, J.M. Becker, F. Naider, High resolution NMR analysis of the seven transmembrane domains of a heptahelical receptor in organic-aqueous medium, Biopolymers 64 (2002) 161–176.
[43] T.M. Fong, M.G. McNamee, Correlation between acetylcholine receptor function and structural properties of membranes, Biochemistry 25 (1986) 830–840.
[44] H.R. Arias, Binding sites for exogenous and endogenous non-competitive inhibitors of the nicotinic acetylcholine receptor, Biochim. Biophys. Acta 1376 (1998) 173–220.
[45] H.R. Arias, W.R. Kem, J.R. Trudell, M.P. Blanton, Unique general anesthetic binding sites within distinct conformational states of the nicotinic acetylcholine receptor, Int. Rev. Neurobiol. 54 (2003) 1–50.