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A spectroscopic study of the membrane interaction of tuberoinfundibular peptide of 39 residues (TIP39)

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Abstract

The membrane interaction of tuberoinfundibular peptide of 39 residues (TIP39), which selectively activates the parathyroid hormone 2 (PTH2) receptor (PTH2-R), has been studied by fluorescence and NMR spectroscopic techniques. Membrane binding would be the first step of a potential membrane-bound activation pathway which has been discussed for a number of neuropeptides and G-protein coupled receptors (GPCRs). Here, the orientation of TIP39 on the surface of membrane mimicking dodecyl-phosphocholine (DPC) micelles was monitored by Photo-CIDNP (chemically-induced dynamic nuclear polarization) NMR which indicates that both Trp25 and Tyr29 face the membrane surface. However, the PTH2 receptor is located in the hypothalamus membrane, for which a more realistic model is required. Therefore, liposomes containing different mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine (POPS) and cholesterol were used for fluorescence and solid-state NMR spectroscopy. Fluorescence spectroscopy showed that a large proportion of TIP39 added to these liposomes binds to the membrane surface. Proton-decoupled ^{31}P-MAS NMR is used to investigate the potential role of individual lipid headgroups in peptide binding. Significant line-broadening in POPC/cholesterol and POPC/POPS liposomes upon TIP39 association supports a surface binding model and indicates an interaction which is slightly mediated by the presence of POPS and cholesterol. Furthermore, smoothed order parameter profiles obtained from ^{2}H powder spectra of liposomes containing POPC-d31 as bulk lipid in addition to POPS and cholesterol show that TIP39 does not penetrate beyond the headgroup region. Spectra of similar bilayers with POPS-d31 show a small increase in segmental chain order parameters which is interpreted as a small but specific interaction between the peptide and POPS. Our data demonstrate that TIP39 belongs to a class of signaling peptides that associate weakly with the membrane surface but do not proceed to insert into the membrane hydrophobic compartment.

Keywords: GPCR; PTH-2 receptor; TIP39; Solid-State NMR; Peptide–lipid interaction; Photo-CIDNP

1. Introduction

Tuberoinfundibular peptide of 39 residues (TIP39) is a neuropeptide agonist for the parathyroid hormone 2 receptor (PTH2-R) which belongs to class B of the G-protein coupled receptor (GPCR) family [1]. It is suggested that TIP39 mediates both endocrine and nociceptive effects [2] and might be involved in regulating auditory information processing based on studies on expression and distribution of TIP39 in the rat central nervous system [3]. TIP39 is a member of a small peptide family that includes parathyroid hormone (PTH) and parathyroid-related peptide (PTHrP). These peptides match the sequence of TIP39 at only four to six positions yet the three-dimensional structure of TIP39 is very similar to that of PTH [4]. Both PTH and TIP39 are potent activators of human PTH2-R [1]. The potency of PTH, however, in activating the same receptor from rats is much reduced and is only 40% of that of TIP39, whilst PTHrP activates neither rat nor human PTH2-R [5]. The relationship between the peptide structure and how it activates its specific receptor are hence of some interest as

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the peptide and/or receptor may in future become important therapeutic targets.

Until recently, the nature of ligand binding interactions in class B GPCRs was poorly understood, with very little mutation and ligand binding data available [6]. However, amongst a body of work on the PTH receptor [7], two contact sites for PTH analogs in PTH2-R have been found using photoaffinity cross-linking [8]. One contact site was found in the sixth transmembrane domain and part of the third extracellular loop of this seven transmembrane helix protein whilst the second was found in the N-terminal extracellular tail thus indicating that PTH is likely to bind at the extracellular surface of the receptor. The structure of TIP39, which is poorly soluble in either aqueous solution or chloroform, was determined in the presence of dodecylphosphocholine (DPC) [4] to mimic the membrane environment of the PTH2 receptor. Relaxation studies in the presence of paramagnetic spin label (5-doxylstearic acid) in combination with MD simulations in a water/decane cell revealed a preferred topological orientation at the water/decane interface with the C-terminal helix partially entering the hydrophobic decane phase [4]. These experiments have been interpreted as involvement of TIP39 in a membrane-bound activation pathway which can be seen in the light of a more general 'lock-and-key' concept as previously discussed for class A GPCR–peptide interaction [9]. It has been postulated that the membrane surface of the target cell influences regulatory peptides in their receptor selection by accumulating these ligands in the membrane and by imposing restriction onto their conformation and orientation [9].

In this paper, we present data acquired using a variety of non-perturbing biophysical techniques that describe the interaction of TIP39 with detergent micelles and lipid vesicles in the absence of PTH2-R. The results give direct evidence that TIP39 does not penetrate deep into the membrane but remains associated with the surface of lipid vesicles. The data support a model where TIP39 associates with the membrane surface prior to binding and activation of the receptor at its extracellular surface.

2. Materials and methods

2.1. Peptide and lipids

The peptide, bovine TIP39, SLALA DDAAF RERAR LLAAL ERRHW LNSYM HKLLV LDAP was synthesized using standard FMOC solid-state chemistry. Peptide purity was higher than 95% as determined by HPLC and electrospray mass spectroscopy. The lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), 1-palmitoyld31-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC-d31), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine (POPS), 1-palmitoyld31-2-oleoyl-*sn*-glycero-3-phosphatidylserine (POPS-d31) and cholesterol were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. All other reagents were analytical grade or better.

2.2. Sample preparation for liquid-state CIDNP NMR

For CIDNP measurements, two samples of 0.5 mM TIP39 in 100% D_2O and 0.2 mM FMN were prepared at a pH of 7.2. The pH was not adjusted for the isotope effect. One sample also contained 44 mM DPC micelles to mimic an amphiphilic membrane environment. DPC was purchased from Avanti Phospholipids and used without further purification. FMN was purchased from SIGMA and used without further purification.

2.3. Sample preparation for solid-state NMR

For solid-state NMR, samples with different lipid compositions were prepared: POPC, POPC/cholesterol, POPC/POPS, POPC-d31, POPC-d31/POPS, POPC/POPS-d31, POPC-d31/POPS/cholesterol and POPC/POPS-d31/cholesterol. The ratios of POPC/POPS, POPC/cholesterol and POPC/POPS/cholesterol were 85:15, 85:15 and 70:15:15, respectively. A total of 10 mg lipids per sample were dissolved and mixed in chloroform and dried down under rotor-evaporation at room temperature. The lipid films were further dried under vacuum overnight and then rehydrated with 5 ml of a suspension of TIP39 in 0.1 M Tris, 0.1 M KCl buffer at pH 7 at room temperature. The total lipid/peptide ratio was 40:1. The samples were briefly sonicated in a bath sonicator to improve exposure of all lipids to the peptide. Samples were subjected to five rapid freeze–thaw cycles for further sample homogenization and then centrifuged at 21,000×*g* for 20 min at 4 °C. The pellets, containing lipid vesicles and associated TIP39 were transferred to Bruker 4 mm MAS rotors for NMR measurements.

2.4. High resolution NMR and photo-CIDNP

All solution state ^{1}H-NMR and ^{1}H-CIDNP spectra were recorded on a home-built NMR spectrometer (OCMS laboratories, Oxford University) operating at 600 MHz. Suppression of a residual water peak was achieved using triple-axis pulsed field gradients which were built in to the probehead. A Peltier semiconductor device was used to maintain the sample temperature at 25 °C. The CIDNP experiments were performed using a continuous wave Spectra-Physics 2016-05 argon ion laser as light source, operating at 4 W in multiline mode, with the principal power output at 488 and 514 nm. The beam itself was gated into 100 ms pulses using a mechanical shutter controlled from the spectrometer. The beam was coupled into a 1-mm diameter optical fibre, the other end of which was mounted in a coaxial capillary insert (Wilmad WGS 5BL) inside the 5-mm NMR sample tube [10].

A 'light' acquisition sequence consisted schematically of '*solvent suppression*'–'*spectral irradiation*'–*laserflash–10 ms delay–acquisition*. A 'dark' acquisition has an identical sequence, but without sample irradiation. Solvent suppression was carried out with the help of 'excitation sculpting', in which strong pulsed field gradients are combined with r.f. pulses to achieve a solvent attenuation by a factor of up to 30,000 [11]. To 'crush' Boltzman polarization (spectral irradiation), a 90° pulse is used to transfer magnetization into the *xy* plane, and a subsequent magnetic field gradient along the *z*-axis of the NMR sample is then applied. 'Dark' spectra are subtracted from 'light' spectra in order to eliminate any remaining signals due to Boltzman polarization which builds up during the laser flash.

The shown CIDNP spectra (Fig. 1) are the results of subtracting 16 acquisitions without irradiation (dark) from the same number with irradiation (light). The laser flash (100 ms) and the acquisition pulse were separated by 10 ms to ensure that no radicals remained at the start of the data acquisition.

2.5. Fluorescence spectroscopy

Emission spectra of the intrinsic fluorescence of Trp25 were acquired using a Jasco FP-6300 spectrometer at room temperature. 100 μl of the vesicle suspensions prepared for solid-state NMR experiments above, containing TIP39 at a concentration of 0.4 mg/ml, was added to 2 ml of 0.1 M Tris, 0.1 M KCl buffer at pH 7 to produce a final peptide concentration of about 0.02 mg/ml. Following centrifugation (21000×*g*; 20 min; 4 °C) 100 μl of the supernatant was added to 2 ml of the buffer in the same way to detect TIP39 remaining in solution. Vesicle suspensions were also prepared as for solid-state NMR experiments above but omitting the freeze–thaw cycles to avoid light scattering. 150 μl of these suspensions containing TIP39 at a concentration of 0.1 mg/ml, was added to 0.85 ml of 0.1 M Tris, 0.1 M KCl buffer at pH 7 to produce a final peptide concentration of about 0.01 mg/ml. Emission spectra of lipid–peptide suspension before and after centrifugation were acquired by scanning from 310 to 450 nm using an excitation wavelength of 295 nm with a spectral bandwidth of 5 nm for both excitation and emission. For fluorescence quenching experiments, 40% acrylamide solution was added stepwise to a final concentration of 0.24 M.

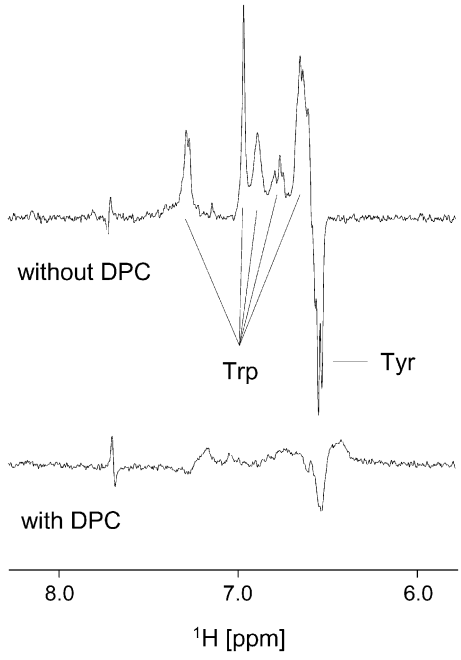

Fig. 1. The CIDNP spectra are a result of the subtraction of 16 acquisitions taken without a laser-flash prior to acquisition, from 16 'light' spectra. (Top) Aromatic region of the CIDNP spectrum of TIP39 in D_2O. CIDNP signal assignments to tryptophan and tyrosine are indicated. (Bottom) Aromatic region of the CIDNP spectrum of TIP39 and DPC in D_2O. The CIDNP signal attributable to tryptophan is clearly attenuated.

2.6. Solid-state NMR

^{31}P and ^{2}H NMR spectra were recorded on a Bruker Avance 600 NMR spectrometer equipped with a 4 mm MAS DVT probe. ^{2}H quadrupole-echo experiments [12] were performed at 92.12 MHz with a spectral width of 500 kHz and with recycle delay, echo delay, acquisition time and 90° pulse lengths of 0.5 s, 42 μs, 2 ms and 5 μs, respectively. The temperature was maintained at 298 K to keep the bilayers in their liquid-crystalline phase. During processing the first 22 points were removed in order to start Fourier-transformation at the top of the echo. Spectra were zerofilled to 8 k points and 50 Hz exponential line-broadening was applied. Smoothed deuterium order parameter profiles were obtained from symmetrised and dePaked ^{2}H-NMR powder spectra of POPC-d31 and POPS-d31 using published procedures [13–15].

Proton-decoupled ^{31}P MAS spectra were acquired at 242.94 MHz using a 6-μs 90° pulse followed by two pulse phase modulated (TPPM15) heteronuclear ^{1}H decoupling at a field strength of 62.5 kHz. The spectral width was 25 kHz and the recycle delay and acquisition time were 3 s and 49 ms, respectively. MAS spinning frequencies were 4 and 14 kHz. The temperature was maintained at 298 K. Spectra were zero filled to 16 k points and no line broadening was applied during processing. Processed spectra were deconvoluted using PeakFit (SeaSolve, USA) to obtain the full width at half height (FWHH) of the overlapping POPC and POPS resonances.

3. Results

3.1. High resolution NMR and photo-CIDNP

Changes in the surface accessibility of aromatic residues (tryptophan, tyrosine, histidine) may be monitored by initiating photochemical reactions between the amino acids

and a chosen dye molecule, which result in what is known as photo-chemically induced dynamic nuclear polarization (photo-CIDNP) [16–18]. Photo-CIDNP manifests itself in the NMR signals of the reaction products by signal intensities which deviate from those which are attributable to the usual Boltzmann polarization. In the past, photo-CIDNP has been useful for gauging the interactions between proteins and other macromolecules, by using the changes of the CIDNP signal as an indication of the changes in accessibility between the dye molecule, usually a flavin derivative and the amino acid residue.

CIDNP measurements on TIP39 were carried out in neat water, and in aqueous solutions of dodecylphosphocholine (Fig. 1). In Fig. 1 (top), the CIDNP signals of the single tryptophan (Trp25) and tyrosine (Tyr29) residues are clearly discernible and readily assigned due to their typical appearance [16]. The signals of the H3 and H5 protons of tyrosine are in emissive phase, whilst those peaks in absorptive mode may be ascribed to tryptophan. TIP39 has no histidine or other tyrosines or tryptophans and so the signals are attributable solely to these two residues. On addition of DPC, the CIDNP signal of tryptophan is clearly attenuated (see Fig. 1 bottom), whilst a small residual signal of the tyrosine is still detectable, which indicates that the tryptophan residue is inaccessible to the flavin dye, and may thus be interpreted as being oriented towards the micelle surface.

3.2. Fluorescence spectroscopy

As described above, CIDNP-NMR gives an indication of aromatic amino-acid specific TIP39 interaction with DPC micelles. However, a natural membrane consists of a number of lipids such as PC, PS and cholesterol which form a rather different electrostatic and hydrophobic environment which may influence binding and interaction of TIP39 with membranes. Therefore, fluorescence spectroscopy has been used to assess the binding and interaction of TIP39 with a number of mixed liposomes to determine the qualitative and quantitative effects on binding of POPS and cholesterol. Spectra were obtained for TIP39 alone and in the presence of liposomes before, and after, centrifugation. The spectral intensity was reduced by about 57% after centrifugation of the liposomal suspensions (Fig. 2A) indicating that about 2/3 of added peptide is bound to liposomes and resulting in an effective peptide to lipid ratio of c 1:70 compared with a starting ratio of 1:40. The same reduction was observed for liposomes containing POPC alone or with POPS, cholesterol or POPS and cholesterol added indicating that the membrane composition did not significantly affect the amount of TIP39 associated with the liposomes. Fluorescence quenching experiments [19,20] (Fig. 2B), performed by sequentially adding small volumes (15 μl) of 40% acrylamide, an efficient neutral quencher, caused signal reduction for both TIP39 in solution as well as bound to liposomes. However, the Stern Volmer plot reveals

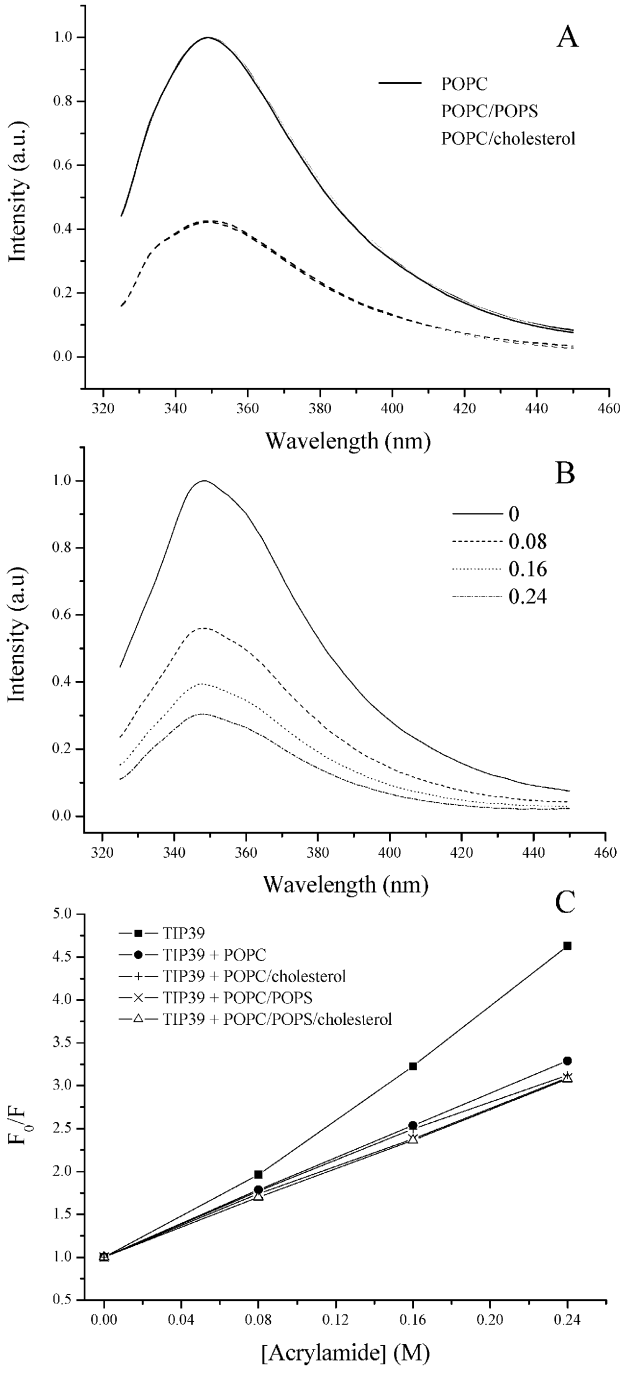

Fig. 2. (A) Intrinsic fluorescence spectrum of TIP39, arising from Trp25, in the presence of POPC, POPC/POPS (molar ratio 85:15) and POPC/cholesterol (85:15) vesicles (solid line) and after removal of vesicles from suspension by centrifugation (dashed line). A contribution at 332 nm from the buffer has been subtracted. (B) Intrinsic fluorescence spectra of TIP39 in the presence of POPC vesicles following the addition of 40% acrylamide to final concentrations of 0, 0.08, 0.16 and 0.24 M. Spectra were acquired at room temperature in 0.1 M Tris/KCl buffer at pH 7 using an excitation wavelength of 295 nm. (C) Stern Volmer plot of initial fluorescence intensity / intensity after addition of acrylamide for TIP39 in solution and in the presence of a variety of mixed lipid vesicles. The presence of lipid vesicles significantly reduces the quenching effect of aqueous acrylamide on TIP39 whilst the presence of PS in the lipid vesicles causes a further small but noticeable reduction of acrylamide induced quenching.

that the presence of liposomes significantly reduces the quenching effect (Fig. 2C). In addition, a small but noticeable reduction in the quenching ability is observable when PS in included in the lipid vesicles (Fig. 2C). Interestingly, the Trp fluorescence emission maximum shows a small blue shift upon increasing the amount of POPS in POPC/cholesterol vesicles; from 349±1 nm (0% POPS) to 346±1 nm (25% POPS). For all other lipid mixtures, the emission was found at 348±1 nm. A blue shift of the emission maximum in the presence of liposomes of any description would be expected if a tryptophan were to move into a more hydrophobic environment. The small size of the observed effect and the accessibility of the tryptophan to an aqueous quencher indicate, that Trp25 of TIP39 cannot be buried deeply within the hydrophobic core of the membrane but that instead it is weakly associated with the membrane surface with significant amounts remaining in solution.

The fluorescence data, described here for binary lipid mixtures, can be compared directly with the ^{31}P MAS spectra described below. Fluorescence spectra of TIP39 in the presence of vesicles containing deuterated lipids were also acquired and demonstrated no significant changes in TIP39 binding indicating that TIP39 binds at the membrane surface in similar quantities for all lipid mixes studied. In addition, to enable a comparison of TIP39 interactions with DPC micelles and lipid vesicles, fluorescence emission spectra were obtained for TIP39 dissolved in water, in 0.1 M Tris, 0.1 M KCl buffer at pH 7 and in the presence of DPC micelles in both water and buffer. Again, an average emission maximum of 348±1 nm was observed in all spectra and, in the presence of DPC micelles, the intrinsic tryptophan fluorescence responded to the addition of acrylamide in the same fashion as described for TIP39 in the presence of vesicles.

3.3. Proton-decoupled-^{31}P MAS NMR

A more specific view of the potential role of individual lipids in peptide binding can be obtained by solid-state NMR, either by observing headgroup deuterated lipids or by monitoring the properties of ^{31}P nuclei located in each phospholipid headgroup. Peptide binding to membrane surfaces could alter the dynamic properties and/or the electronic environment of the phosphorous nuclei in lipid headgroups. Changes in the electronic environment cause variations of the ^{31}P chemical shift anisotropy (CSA), which would be observed by MAS-NMR spectroscopy as variations of isotropic chemical shift and spinning sideband intensities [21]. Differing dynamic properties could also affect the size of the ^{31}P-CSA as well as the lineshape of ^{31}P-MAS resonance lines. The ^{31}P CSA in lipid headgroups is significantly scaled down and axial symmetric because of fast rotational diffusion of lipids in a fluid bilayer. If restrictions on these fast motions occur, for example, in their gel-phase, changes in the CSA can be expected. Furthermore, the line-width of MAS resonances may also respond to peptide binding. Line-broadening can be caused either by changes in the transverse relaxation time T2 and by insufficient proton decoupling due to reduced lipid mobility. Contributions from slow, intermediate or fast exchange of lipids between peptide-free and peptide associated parts in the bilayer can be expected as well [21].

^{31}P spectra of the centrifuged lipid mixtures which were also used for fluorescence spectroscopy were acquired at spinning frequencies of 4 and 14 kHz. The higher spinning frequency was chosen to obtain optimum resolution of differing phosphorus populations in the isotropic region whilst the lower spinning frequency was chosen to allow analysis of the chemical shift anisotropy which is refocused into the spinning sidebands. None of the samples showed any significant changes in spinning sideband intensities upon peptide binding. Proton-decoupled ^{31}P MAS spectra at 14 kHz spin rate obtained for POPC, POPC/Cholesterol and POPC/POPS are shown in Fig. 3. The addition of TIP39 causes almost no broadening of pure POPC bilayers but a broadening of the POPC resonance in the presence of cholesterol from 23 Hz to 34 Hz is observed. Deconvolution of the spectra obtained with mixed POPC/POPS liposomes reveals two sharp components comprising the POPC and POPS resonances, which are broadened from 35 to 46 Hz and from 45 to 73 Hz, respectively when TIP39 is added, and a broad component. An optimum fit is not obtained without the addition of a broad component, in contrast to the other samples lacking an anionic lipid fraction, and hence the presence of this broad population is attributed to being due to interactions between anionic and zwitterionic lipid. Supporting this analysis, the broad component is much reduced on addition of TIP39 where peptide–lipid interactions may become more important. A similar broadening of the POPC and POPS resonances is observed in the presence of TIP39 when cholesterol is included in the mixed membranes and a very small upfield shift of 0.016 ppm of the POPS resonance can be discerned. These spectra indicate that the membrane interaction of TIP39, as monitored by the linebroadening effect, is slightly more pronounced in the presence of either POPS or cholesterol.

3.4. ^{2}H-NMR spectroscopy

Whilst CIDNP- and ^{31}P-MAS-NMR spectroscopy provide an insight into TIP39 membrane interaction at the membrane–water interface, deuterium powder spectra of chain-deuterated lipids allow a more detailed view into the ability of TIP39 to penetrate into the hydrophobic core of the bilayer. ^{2}H-NMR spectra of both POPC-d31 as well as POPS-d31 in POPC/POPS and POPC/POPS/cholesterol mixtures, with and without TIP39 were recorded. No effect of TIP39 binding was observed in powder spectra of pure POPC-d31 of mixed bilayers POPC-d31/POPS, POPC-d31/cholesterol and of POPC-d31/POPS/cholesterol. Interestingly, however, the typical ^{2}H-powder spectrum of POPC-

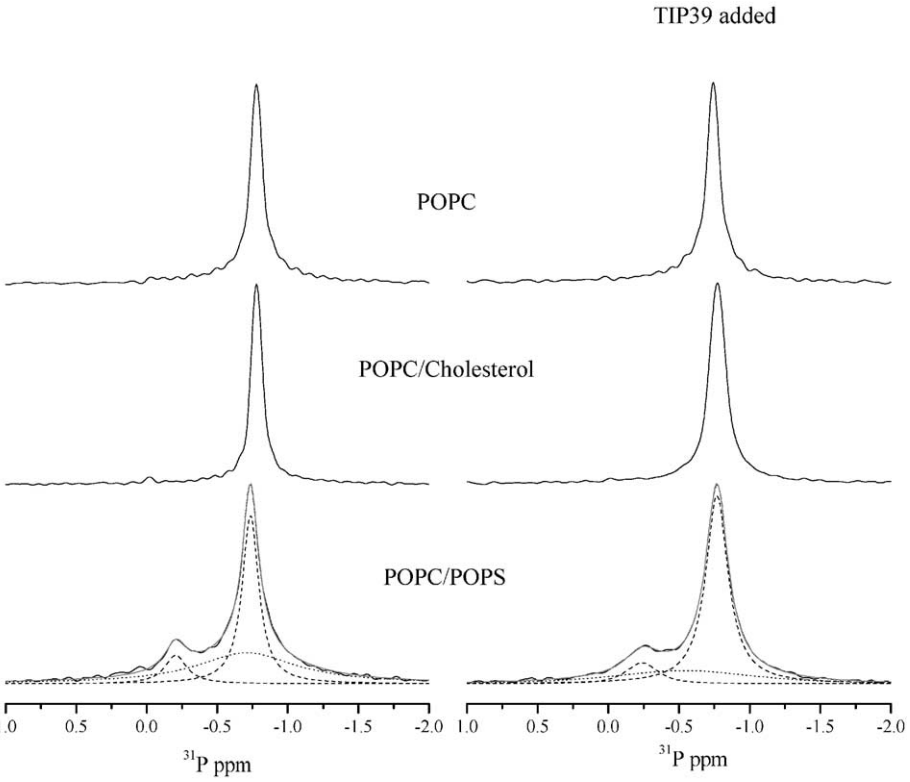

Fig. 3. Proton-decoupled ^{31}P MAS spectra acquired at 14 kHz MAS frequency and 298K show only a small effect of TIP39 binding for POPC a slightly larger effect for POPC/cholesterol (molar ratio, 85:15) and a significant broadening for POPC/POPS (85:15). De-convolution of the POPC/POPS spectra using three Lorentzian curves revealed a significant increase in line-width, from 45 to 73 Hz, for POPS and a smaller increase, of 35 to 46 Hz, for POPC. A broad component was also detected in peptide free liposomes with line-width of 257 Hz which is greatly reduced in intensity on addition of TIP39. Original spectra (solid lines) can be compared with the fitted spectra (dotted and dashed lines) and their sum (grey line). The peptide to lipid ratio prior to pelleting was 1:40 in each case.

d31 is reduced in width from 33 kHz to 26 kHz in POPC-d31/ POPS bilayers (irrespective of the presence of TIP39). The spectrum broadens again to 33 kHz when cholesterol is incorporated in the bilayer at the ratio described before. The decrease of chain order in mixed PC/PS bilayers is probably caused by increased headgroup distances due to the electrostatic effect of PS. Adding cholesterol reduces the available space in the hydrophobic core of the bilayer and therefore causes again an increase in chain order.

The same experiments were carried out using POPS-d31 instead of POPC-d31 as reporter. No effect of peptide binding to POPC/POPS-d31 could be observed (Figs. 4Aa and Ab), however, as seen for POPC-d31, adding cholesterol also causes an increase in the observed quadrupole splittings of POPS-d31 from 26 kHz to 33 kHz (Fig. 4Ac). In this sample, on addition of TIP39 to these mixed POPC/POPS-d31/cholesterol bilayers, a significant increase in some of the quadrupole splittings could be observed (Fig. 4Ad). A dePake-ing analysis (Fig. 4Ae) of these spectra (Figs. 4Ac and Ad) reveals that almost all CD2 chain segments show a slightly larger quadrupole coupling in the presence of TIP39. The corresponding order parameter profile (Fig. 4B) reveals that the effect of TIP39 addition was propagated throughout the length of the acyl chain in POPS-d31. However, the increase of the segmental order parameter upon peptide binding is largest at the beginning of the chain and becomes smaller with increasing chain position.

No perturbation of bulk lipid (POPC-d31) upon peptide binding was observed which would agree with our fluorescence data that the peptide is not penetrating deeply into the bilayer. However, the slightly increased order parameters of POPS-d31 in POPC/POPS-d31/cholesterol bilayers indicate a specific interaction of POPS with TIP39 which can only be observed in the presence of cholesterol.

4. Discussion

Experimental evidence points towards a hypothesis where TIP39 may be a peptide hormone that is involved in a membrane bound activation pathway: TIP39 is poorly soluble in aqueous solution or chloroform, whilst in the presence of DPC micelles, 5-doxylstearic acid nitroxide radical induced linebroadening of ^{1}H signals indicated that the peptide interacts with the micelles at the lipid–water interface [4]. Furthermore, the peptide binding sites are located close to the membrane surface of the receptor [8] making them more easily accessible from the lipid environment.

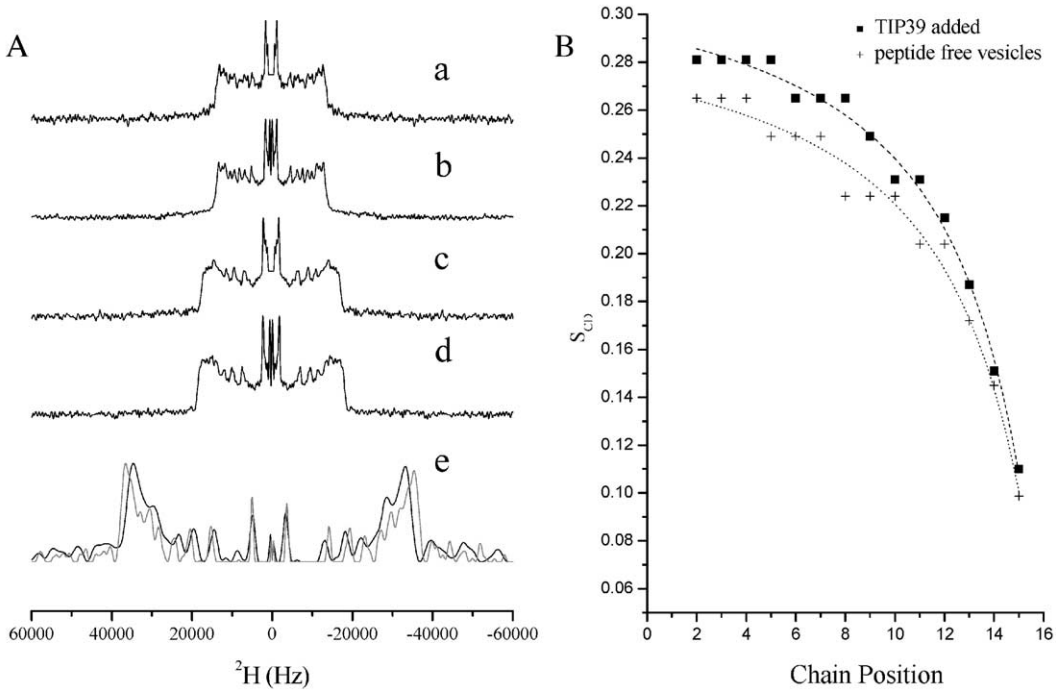

Fig. 4. Influence of TIP39 on ^{2}H NMR powder pattern of POPS-d31 (A) The spectrum of POPC/POPS-d31 (a) does not change upon adding TIP39 (b) whilst POPC/POPS-d31/cholesterol (c) shows a small broadening upon adding TIP39 (d). DePakeing of spectra (c) and (d) reveals largest changes at the beginning of the chains close to the bilayer interface region (e: black line, peptide free vesicles; grey line, with Tip39 added) which can be further analysed in terms of a smoothed order parameter profile for POPS-d31 (B).

Our CIDNP data agree with the topology derived from relaxation studies [4]. However, we have sought to clarify the nature of the peptide interaction with artificial lipid membranes that mimic more closely the physiological situation compared to DPC micelles. The polar aromatic residues tryptophan and tyrosine have a known specific affinity for a region near the lipid carbonyls [22] and our results support a surface association model for TIP39 with residues Trp25 and Tyr29 located in the membrane headgroup region. Our fluorescence data clearly show a similar proportion of TIP39 is associated with liposomes independent of their composition, whilst only very small blue shifts in the emission maximum of Trp25 are observed upon membrane binding. Emission spectra of proteins change when the tryptophanyl environments shift toward lesser polarity [19]. Blue shifts as large as 24 nm have been observed, for example when β-glucosidase inserts into brain phosphatidylserine liposomes in the presence of saposin C [20]. Fluorescence quenching experiments, using acrylamide in solution, showed signal reduction for both TIP39 in solution and associated with liposomes. A comparison of Stern Volmer plots for the quenching ability of acrylamide revealed that the presence of liposomes causes a small but significant reduction in quenching. This confirms that the peptide is associated with the lipid vesicles but also indicates that Trp25 is accessible to quenching by aqueous acrylamide. Therefore, TIP39 is likely to be located in the membrane interface region.

The interaction of signaling peptides with membranes is an interplay between electrostatic [23] and hydrophobic forces [24]. In TIP39, negatively charged residues (Asp6, 7 and 37) are located close to C- and N-terminus as well as close to the centre of the peptide (Glu12 and 21), whilst six positively charged amino acids are distributed along the sequence (Arg11, 13, 15, 22, 23 and Lys32). This leaves a small positive net charge which may provide a source for an electrostatic interaction with negatively charged PS head-groups. Negatively charged phosphatidylserine as used in this study constitutes 18.5% of polar bovine brain extract [25] which also contains 15% cholesterol. Though not normally present in the outer leaflet of the hypothalamus membrane, addition of phosphatidylserine at a molar ratio of 15% of total lipid is used to artificially increase the negative surface charge of the membrane. Loss of PS asymmetry is observed under various pathological and physiological conditions. This increase in surface charge, however, does not lead to any significant increase in the level of TIP39 association as assessed by intrinsic tryptophan fluorescence. This suggests that, whilst electrostatic interaction between TIP39 and the lipid membrane are likely to exist, this is not what drives TIP39 association. This result is not so surprising when one considers that, despite the small net positive charge, there are also five negatively charged residues in the TIP39 sequence.

The membrane association of TIP39 is also supported by our ^{31}P data although the observed linebroadening is more significant in the presence of cholesterol and POPS. TIP39

seems to impose restrictions on the lipid mobility. Proton-decoupled ^{31}P-MAS has been used before to monitor electrostatic interactions between lipid headgroups and charged peptide residues by observing small changes (up to 0.3 ppm) in the isotropic chemical shift [21,26]. No such effect has been observed here. The small effect on the ^{31}P chemical shift depends on the orientation of the charged residues in the peptide with respect to the membrane surface and will be further reduced according to the Gouy–Chapman theory considering the physiological salt concentration used in this study (0.1 M KCl). Fluorescence control experiments with different salt concentrations to alter the shielding of membrane surface charges further demonstrated that peptide binding was not significantly influenced by surface charge. The effect of electrostatic charges on ^{31}P chemical shifts is here probably too small to be observed.

Besides the fact that many GPCRs require cholesterol for correct functioning, cholesterol may also affect hydrophobic peptide binding by occupying the lipid acyl chain region [23]. For TIP39, no indication supporting TIP39 penetration of the hydrophobic core was found. It is much more likely, considering our data in light of the location of the proposed peptide binding sites on PTH2-R, that in vivo the receptor activation pathway requires that TIP39 remains associated with the membrane surface and does not insert into the hydrophobic compartment. By extension, TIP39 should be expected to diffuse laterally and bind to the receptor at the lipid–water interface.

It may be conceivable that our ^{2}H-NMR techniques are not sensitive enough to small amounts of TIP39 being able to insert into the hydrophobic compartment. Therefore, attempts were made to force a greater association of peptide with lipid bilayers by co-reconstituting TIP39 and lipids by drying from chloroform/methanol solution followed by rehydration with aqueous buffer. However, no enhancement of the observed effects of TIP39 binding was observed.

The association of TIP39 with the membrane surface as derived from CIDNP-NMR, fluorescence and ^{31}P-MAS-NMR is also in agreement with the ^{2}H NMR spectra of POPC-d31 containing vesicles which showed no evidence for peptide insertion beyond the headgroup region into the hydrophobic compartment in any of the samples prepared.

Phosphatidylserine (PS) asymmetry, is maintained in mammalian cells by an ATP-dependent aminophospholipid translocase that catalyses the transport of aminophospholipids from the external leaflet to the internal leaflet of the plasma membrane [27]. Loss of PS asymmetry is observed under different pathological and physiological conditions including injury [28] and malignant transformation [29]. PS containing membranes have also recently been shown to trigger the formation of amyloid fibres [30]. In order to investigate whether a specific lipid–peptide interaction takes place between TIP39 and POPS, ^{2}H spectra of POPS-d31 were recorded which did indeed show a significant change in segmental chain order parameters upon peptide binding which could only be observed in the presence of cholesterol. The observed small increase in POPS-d31 chain order becomes larger close to the membrane interface region and smaller towards the methyl end of the chain (Fig. 4B) which would also agree with our previous findings about a membrane surface association of TIP39.

It is well established that cholesterol in the membrane has an ordering effect on lipid acyl chains [31]. This property is illustrated in Fig. 4A. The breadth the POPS-d31 spectrum obtained from mixed POPC/POPS liposomes (Fig. 4Aa) increases significantly when cholesterol is present (Fig. 4Ac) which has also been detected for bulk POPC-d31. This ordering effect is associated with an increase of average lipid chain lengths [32] and an altered packing and optimized spacing between lipid headgroups within the membrane. This could allow a better accessibility of negatively charged phosphatidylserine to peptide sidechains and could enable a slightly stronger electrostatic interaction with TIP39, explaining a further increase in POPS-d31 chain order upon peptide binding. Lipids with phosphatidylserine headgroups have been shown before to be involved in both non-specific [33] and specific electrostatic interactions as in the case of PMP1, a yeast plasma membrane protein [34]. The study of PMP1 18–38 is the only other study that we have found in the literature where POPS-d31 is used as a reporter in mixed POPC/POPS membranes. Interestingly, the maximum splitting for chain deuterated POPS was also around 26 kHz in mixed POPC/POPS membranes and a peptide–lipid interaction was observed when the fragment of PMP1 also caused significant ordering of the POPS lipid chains [34]. Our data therefore suggest that TIP39 can take part in an interaction with the negatively charged POPS headgroup when cholesterol is present. However, comparing the Stern Volmer plot for POPC/POPS/cholesterol with all other lipid mixtures shows a very small effect only (Fig. 2C). In addition, the Trp emission maximum shows only a very small blue shift of 3 nm upon adding 15% cholesterol to POPC/POPS. Together with our deuterium data, we conclude that the peptide–POPS interaction must be relatively weak and does not increase the level of TIP39 membrane association.

Studies of class A GPCR activators, such as opioid and neurokinin peptides, have allowed a general classification of some signaling peptides, according to their interactions with biological membranes [35]. Either the peptides associate with the anionic membrane surface or insert an amphiphilic helix into the hydrophobic compartment as well. Examples where peptides exhibit no discernable interaction with lipid membranes are known as well. The relative degree of insertion of the peptide has been shown to influence the selectivity of closely related receptors for the message containing peptides. Selection between peptides that associate close to the membrane surface may be based on electrostatic gradients [24,35]. For example, neurokinins

can be shown to bind to three principal mammalian NK receptor sites which correlate to their type of membrane interaction: The NK-1 binding site is selected by substance P which has one α-helical domain parallel to the membrane normal caused by a strong hydrophobic interaction of its C-terminal message segment. The NK-2 site is selected by neurokinin A on the basis of an electrostatic accumulation of the peptide at an anionic membrane surface [24] whilst electrostatic repulsion of neurokinin B by anionic membranes is correlated with the selection of the NK-3 binding site.

Studies of substance P binding to artificial membranes [36] demonstrated that electrostatic accumulation of peptide in anionic membranes preceded insertion of the peptide into the hydrophobic compartment, as was also observed for neutral membranes, demonstrating that both the hydrophobic and electrostatic properties of the membrane were involved in a membrane association pathway. Neutron diffraction studies confirmed the existence of two populations of substance P associated with lipid bilayers [25], this observation being consistent with a mechanism that involves membrane insertion of the C-terminal region followed by lateral diffusion of the peptide to reach the ligand binding site of the receptor [9].

Signaling peptides from other GPCR classes may also behave in a similar manner. Cholecystokinin (CCK) for example, has been shown to interact only transiently with small unilamellar vesicles. However, evidence that CCK is capable of lateral membrane penetration and activates its receptor at the lipid–water interface has been provided by showing that lipo-derivatized CCK inserted into bilayers maintained comparable binding affinity to unmodified CCK [37].

5. Conclusions

Our studies confirm earlier indications [4] that TIP39 belongs to a class of signaling peptides that associates with the anionic membrane surface but do not proceed to insert into the membrane hydrophobic compartment. Furthermore, our data show that positively charged TIP39 interacts specifically with anionic POPS. However, peptide accumulation at the membrane surface is not significantly increased by the presence of POPS.

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