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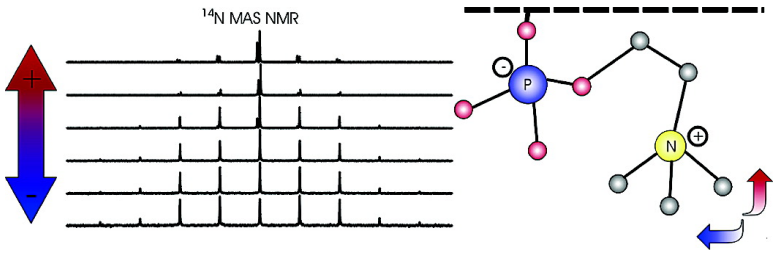

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Molecular Insight into the Electrostatic Membrane Surface Potential by $^{14}N/^{31}P$ MAS NMR Spectroscopy: Nociceptin−Lipid Association

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Abstract: Exploiting naturally abundant ^{14}N and ^{31}P nuclei by high-resolution MAS NMR (magic angle spinning nuclear magnetic resonance) provides a molecular view of the electrostatic potential present at the surface of biological model membranes, the electrostatic charge distribution across the membrane interface, and changes that occur upon peptide association. The spectral resolution in ^{31}P and ^{14}N MAS NMR spectra is sufficient to probe directly the negatively charged phosphate and positively charged choline segment of the electrostatic $P^--O-CH_2-CH_2-N^+(CH_3)_3$ headgroup dipole of zwitterionic DMPC (dimyristoylphosphatidylcholine) in mixed-lipid systems. The isotropic shifts report on the size of the potential existing at the phosphate and ammonium group within the lipid headgroup while the chemical shielding anisotropy (^{31}P) and anisotropic quadrupolar interaction (^{14}N) characterize changes in headgroup orientation in response to surface potential. The $^{31}P/^{14}N$ isotropic chemical shifts for DMPC show opposing systematic changes in response to changing membrane potential, reflecting the size of the electrostatic potential at opposing ends of the $P^-−N^+$ dipole. The orientational response of the DMPC lipid headgroup to electrostatic surface variations is visible in the anisotropic features of ^{14}N and ^{31}P NMR spectra. These features are analyzed in terms of a modified "molecular voltmeter" model, with changes in dynamic averaging reflecting the tilt of the $C_\beta-N^+(CH)_3$ choline and PO_4^- segment. These properties have been exploited to characterize the changes in surface potential upon the binding of nociceptin to negatively charged membranes, a process assumed to proceed its agonistic binding to its opoid G-protein coupled receptor.

Introduction

The interaction of peripheral proteins with biological lipid membranes plays a key role in many cellular processes and is typically governed by electrostatic interactions between positively charged protein residues and negatively charged membrane surfaces.[1−18] Examples of electrostatically driven processes include the adsorption of antimicrobial peptides and proteins such as Src and MARCKS that display clusters of basic residues triggering electrostatic anchoring to a membrane surface.[1,3,5,12] Even amyloidogenic systems such as the amyloid-β (Aβ) protein, can associate electrostatically, where the dramatic increase of its local membrane surface concentration triggers accelerated aggregation into toxic protofibrils, a process currently debated as a major neurotoxic pathway in Alzheimer's disease.[7,13,14] Initial electrostatic binding of proteins to their target membranes is not only controlled by the presence of charges at the protein surface, but crucially by the lipid composition.[8,12] As the huge variety of anionic lipids and their distribution pattern suggests, essential electrostatic interactions occur not only on the surface but also across the whole membrane interface. Therefore, not only is the net charge of the lipid headgroup important but also the residual charge distribution within a lipid headgroup structure, indicating the physiological significance of the internal charge location.[8,11,12]

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While the structural understanding of proteins at a molecular level has enhanced our understanding of how they achieve their membrane-bound state, detailed information about the significance of the charge distribution inside the membrane interface (as controlled by various specific lipids), is still quite restricted.[2,3,7,8,15,16]

Traditionally, effects of surface-active molecules and ions on membranes have been investigated by a range of biophysical methods including mobility,[1] fluorescence,[9] infrared,[10] EPR[11] and NMR[8,15,17−21] in addition to computational approaches.[1,6] In particular, wide-line ^{2}H NMR of lipids specifically deuterated in their headgroup region has led to a wealth of information about the response of the $P^-{-}O{-}CH_2{-}CH_2{-}N^+(CH_3)_3$ dipole in the headgroup of the zwitterionic PC lipids to variations in the membrane surface potential, summarized in a concept called "molecular voltmeter" pioneered by various groups.[20,22−25] Drawbacks of this approach included the synthesis of specifically deuterated lipids and severe limitations in studies of native membranes. Additionally, ^{2}H NMR reports only indirectly on the charged sites in the headgroup region by relying on changes in the internal headgroup conformation, a mechanism only recently studied in more detail by complimentary carbon-13 NMR studies.[16]

Here, we exploit the full potential of the naturally abundant spins nitrogen-14 and phosphorus-31 in the DMPC headgroup by high-resolution MAS NMR to obtain a molecular view of the electrostatic interface properties of mixed-lipid membranes. In our approach, the ^{14}N nucleus senses the electrostatic potential near the positively charged end of the DMPC headgroup, and the ^{31}P nucleus senses at the negatively charge phosphate segment. By applying MAS NMR techniques, the NMR features for each lipid component in mixed-lipid bilayers are resolved in the NMR spectra by their differing isotropic ^{14}N and ^{31}P chemical shifts. Analysis of the opposite charges on the phosphate and choline groups in the DMPC headgroup dipole permits a direct description of the electrostatic potential present at the membrane surface. This approach enables the study of charge distribution across the bilayer interface, the overall response of the DMPC headgroups to the modulations induced by cationic or anionic lipids (see Figure 1a), or the surface association of charged molecules. Here we have exploited this to study how the peptidic GPCR ligand nociceptin(1−13), a potent agonist of the opioid-like ORL1 GPCR involved in pain modulation and stress,[26,27] perturbs the charge distribution at the membrane surface upon binding. In common with many larger peptidic hormones that modulate their effects through GPCR-dependent pathways, the mechanism of the binding of nociceptin to the receptor is unclear. It is proposed that a nonspecific surface association of these hormones with the membrane surface is a necessary prerequisite to their lateral diffusion into the receptor binding site.[28] Here, the observed changes in ^{31}P isotropic chemical shift and ^{14}N quadrupole splittings indicate an electrostatically driven membrane association of nociceptin.

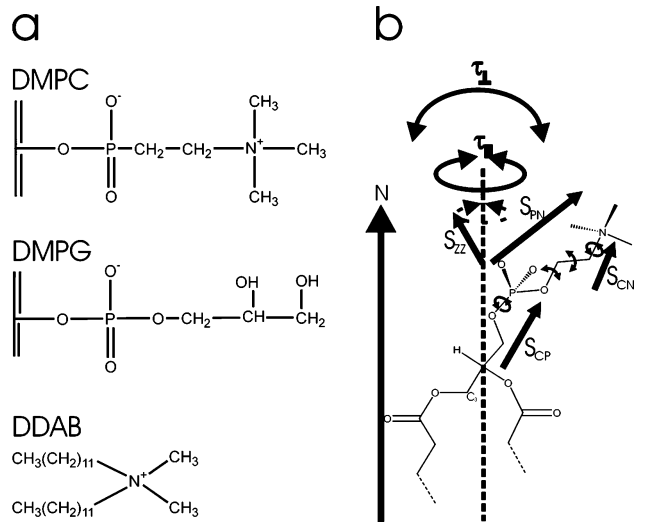

Figure 1. (a) Representation of the phospholipid headgroup of the membrane constituents DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine, and the negatively charged DMPG, 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol. In addition, a single lipid molecule of the positively charged membrane component DDAB, dimethyldidodecylammonium bromide, is shown. (b) Schematic presentation of the headgroup geometry and occurring motional modes in DMPC phospholipid membranes; S_{ZZ} is the relevant orientational order parameter of the lipid molecule in liquid-crystalline membrane; S_{PN} the dipole order parameter; and S_{CN} and S_{CP} the relevant segmental order parameters.

Materials and Methods

Materials. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), and didodecyldimethylammonium bromide (DDAB) were obtained from Sigma (UK). Nociceptin(1−13)-NH_2 was synthesized on an Applied Biosystems 433A peptide synthesizer according to Guerrini et al.[29,30] The molecular mass of the final compound was confirmed by electrospray mass spectroscopy (MH^+ 1383 Da), and the purity was determined to be over 90% by reversed phase HPLC.

Sample Preparation. Multilamellar vesicles were prepared by co-solubilizating the lipids in chloroform/methanol (3:1 v/v). Samples were dried under high vacuum, and subsequently the lipid film was resuspended in buffer (10 mM Tris, 10 mM KCl, 0.5 mM EDTA, pH 7.8) and subjected to five freeze−thaw cycles. Nociceptin was dissolved in 500 μL of trifluoracetic acid (TFA) to break up any aggregates. TFA was removed under a stream of nitrogen gas. The peptide film was resuspended twice in 5 mL of TFE (trifluoroethanol), followed by evaporation under high vacuum. Upon dissolving in buffer, nociceptin was added to lipids to a final lipid-to-peptide ratio of 15:1 and lipid-to-water ratio of 30:1.

Solid-State NMR Measurements. ^{14}N measurements were carried out on a 400 MHz Infinity spectrometer (Chemagnetics, U.S.A.) with a 4-mm double resonance MAS probe. ^{14}N MAS NMR spectra were acquired using a rotor-synchronized quadrupole echo sequence with a $\pi/2$ pulse duration of 7.3 μs and a repetition rate of 300 ms. For static ^{14}N NMR spectra a 200-μs interpulse delay was utilized. The isotropic shift of DMPC vesicles was used as an external reference (0.0 ppm).

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The number of scans collected for static ^{14}N NMR never exceeded 250 000. ^{31}P MAS NMR experiments were acquired under proton decoupling using a single $\pi/2$-pulse with 6.0 μs duration. A Hahn echo sequence was applied for ^{31}P static measurements with an interpulse delay of 50 μs. Some ^{31}P experiments were carried out on a 100 MHz Infinity spectrometer (Chemagnetics, U.S.A.) and a 7-mm double resonance probe (Bruker, Germany). Spectra were collected using a single pulse with proton decoupling (40 kHz) and a pulse width of 7.5 μs. All ^{31}P spectra were referenced externally to −0.9 ppm, using DMPC vesicles at 308 K; 100 to 15 000 transients were studied with a repetition time of 4 s.

Spectral Analysis. The spin interactions that dominate the ^{14}N and ^{31}P NMR spectra of DMPC are the quadrupolar interaction between the electric field gradient and the quadrupole moment of the nitrogen nucleus, and the ^{31}P chemical shielding anisotropy (CSA) arising from the interaction of the phosphorus nucleus with its local electronic environment, respectively. These interactions are anisotropic in nature, and the observed resonance postions depend on both the dynamics present within the sample and the orientation of the anisotropic interaction with respect to the magnetic field. For the quadrupolar interaction, this leads to the Hamiltonian

$$H_Q = \frac{\delta_Q(3\hat{I}_z - 2)}{2}\left(\frac{1}{2}(3\cos^2\beta - 1) - \frac{\eta}{2}\sin^2\beta\cos 2\alpha\right) \quad (1.1)$$

in the absence of any averaging where δ_Q is the anisotropy of the electric field gradient, η its asymmetry, and α and β the Euler angles characterizing the orientation of the PAS to the magnetic field. Similarly, the chemical shielding Hamiltonian in the lab frame is:

$$H_\sigma = \omega_0\left(\bar{\sigma} + \frac{\delta_\sigma}{2}(3\cos^2\beta - 1) - \eta_\sigma\sin^2\beta\cos 2\alpha\right)\hat{I}_z \quad (1.2)$$

Thus, for a static sample of lipid vesicles the observed line shape is composed of the sum of all orientations present in a powder dispersion. In the case of the ^{14}N spectra, the trace of the field gradient is zero, thus $\bar{\sigma} = 0$. The summation of the line shape distribution of both transitions leads to the characteristic Pake pattern.

Under MAS conditions the resonance frequency for a given molecular orientation becomes periodically time dependent. This modulation gives rise to an isotropic resonance condition at $\bar{\sigma}$ with sidebands spaced at intervals of ω_r. The intensity of these sidebands was used to analyze both the anisotropy and asymmetry of the anisotropic interaction as previously described by Herzfeld and Berger.[31] The powder averaging was performed according to Cheng with 1154 powder orientations per spectrum.[32] Fitting of the sideband intensities was performed using MINUIT.[33] The errors given are those obtained from MINUIT and represent one standard deviation.

Results

Natural Abundance ^{14}N and ^{31}P MAS NMR at Membrane Interfaces. Representative ^{14}N and ^{31}P NMR spectra of DMPC/DMPG/DDAB lipid vesicles at a molar ratio of 3:1:1 are displayed in Figure 2. The static NMR spectra (Figure 2a,d) are typical for lipid bilayers in their natural liquid crystalline phase where fast dynamic processes occur.[34,35] In the case of the ^{14}N NMR spectrum the line shape is composed of two subspectra originating from two quaternary nitrogen nuclei in the DMPC and DDAB molecules, while the phosphate moieties of DMPC and DMPG give rise to a superimposed ^{31}P NMR line shape. The ^{14}N NMR spectrum is dominated by the orientation-dependent quadrupole interaction between the electric quadrupole moment of the nitrogen nucleus with its spin $I = 1$, and the electric field gradient, present in the quaternary ammonium groups. The quadrupole interaction is partially averaged due to the fast axial rotation of the whole lipid molecule and the fast rotational dynamics of the rotation of the whole quaternary ammonium group around the C_β−N bond in the DMPC headgroup region (see Figure 1b).[16,36] In the case of ^{31}P ($I = {}^1/_2$), the MAS NMR line shapes reflect the partially averaged chemical shift anisotropies of the phosphate moieties.

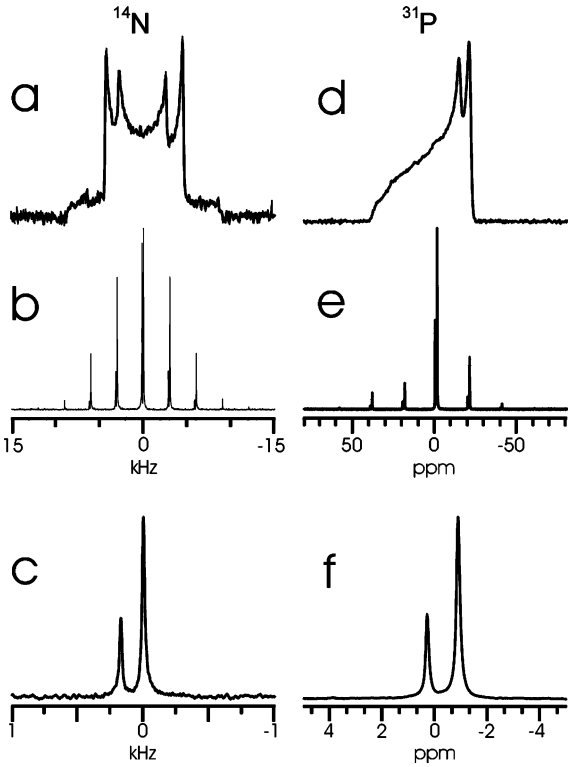

Figure 2. ^{14}N (left column) and ^{31}P NMR spectra at 400 MHz proton frequency of multilamellar DMPC/DMPG/DDAB vesicles (3/1/1 molar ratio) at 308 K; (a,d) static spectra; (b,e) MAS spectra at 3 kHz spinning speed; (c) expanded ^{14}N MAS spectrum at 10 kHz; (f) expanded ^{31}P MAS spectrum at 8 kHz spinning speed.

To permit a quantitative analysis of the spectral properties of each lipid in mixed bilayers, NMR experiments were performed under MAS spinning conditions, where the NMR resonances from the individual lipids are well resolved according to their isotropic chemical shift values, seen in Figure 2b,c (^{14}N) and Figure 2e,f (^{31}P). At 10 kHz spinning speed, the ^{14}N MAS NMR spectrum reveals the isotropic components at 6 and 0 ppm for DDAB and DMPC while the ^{31}P MAS NMR spectrum reveals resonances at 0.28 ppm and −0.89 ppm for DMPG and DMPC, respectively. MAS NMR experiments at intermediate speed (3 kHz), produce spectra where each isotropic central resonance is accompanied by its corresponding sideband family (Figure 2b,e), reflecting quantitatively the anisotropy of the quadrupolar and chemical shielding for each lipid component.[37]

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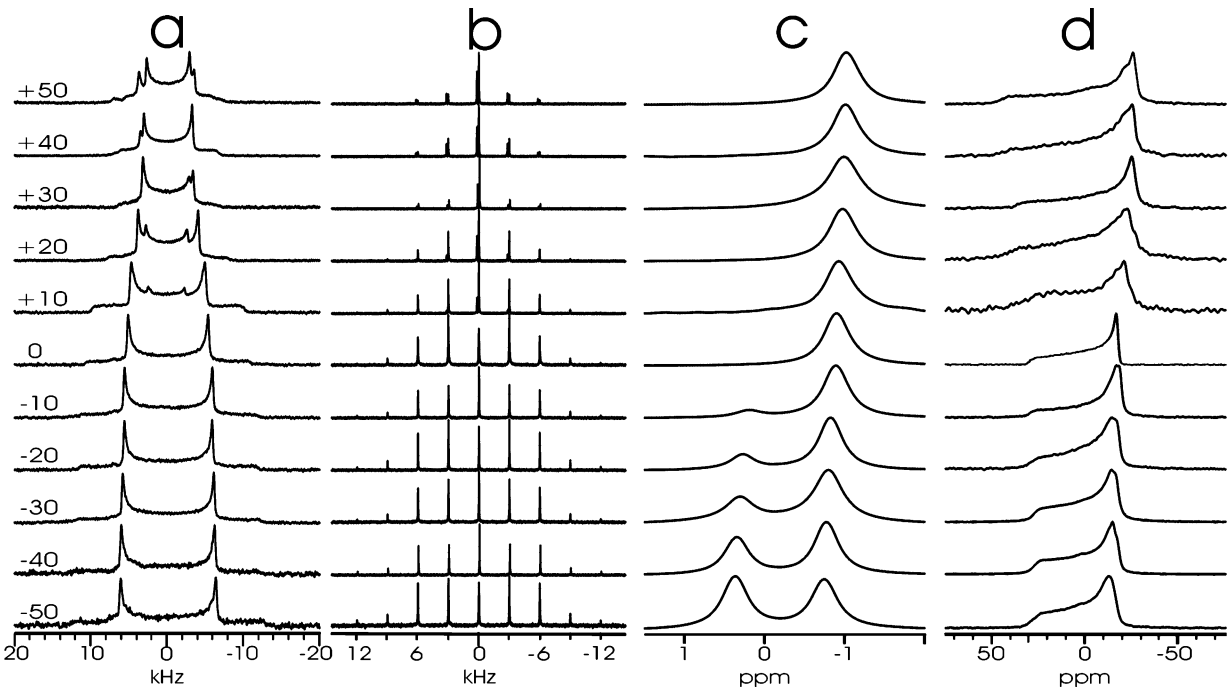

Figure 3. NMR spectra obtained at 400 MHz proton frequency of multilamellar DMPC vesicles containing either DMPG (−) or DDAB (+) at different molar fractions (mol %). All spectra were recorded at 308 K with the four columns representing (a) ^{14}N static spectra, (b) ^{14}N MAS spectra at 3 kHz spinning speed, (c) ^{31}P MAS spectra at 3 kHz spinning speed, and (d) ^{31}P static spectra.

Analysis of the sideband intensities for the neutral bilayers gives ^{14}N quadrupolar splittings of 6 and 10 kHz for DDAB and DMPC (asymmetry parameter η close to zero), and for the ^{31}P CSA anisotropies 53 and 47 ppm for DMPC and DMPG, respectively.

Variation in Membrane Surface Potential Monitored by ^{14}N/^{31}P MAS NMR. Typical ^{14}N NMR spectra of multilamellar DMPC lipid vesicles are shown in Figure 3, with a varying surface potential induced by increasing fractions of basic DDAB or acidic DMPG lipids. Already in the static ^{14}N NMR spectra as displayed in Figure 3a, the spectra show a pronounced dependency on the membrane potential. Although this has been reported previously for several systems[22,38−41] it has never been systematically exploited due to the lack of high-resolution MAS NMR techniques which provide the necessary sensitivity and spectral resolution for accurate analysis of the data.[36] To extract the quadrupole splitting of the DMPC choline group in mixed-lipid bilayers ^{14}N MAS NMR spectra at intermediate speed (3 kHz) were acquired (Figure 3b). Quantitative analysis of the ^{14}N sideband families shows a systematic increase in the quadrupolar splitting with increasing surface potential as summarized in Figure 4a. While for positively charged bilayers (50 mol % DDAB) a splitting of 4.68 ± 0.56 kHz is obtained, this value increased to over 12.49 ± 0.80 kHz when 50 mol % negatively charged DMPG lipids are present. The size of the quadrupole splitting reflects the response of the choline headgroup dipole to the existing membrane potential. The variation in surface potential as a function of membrane composition is also revealed in the isotropic chemical shift values of the central

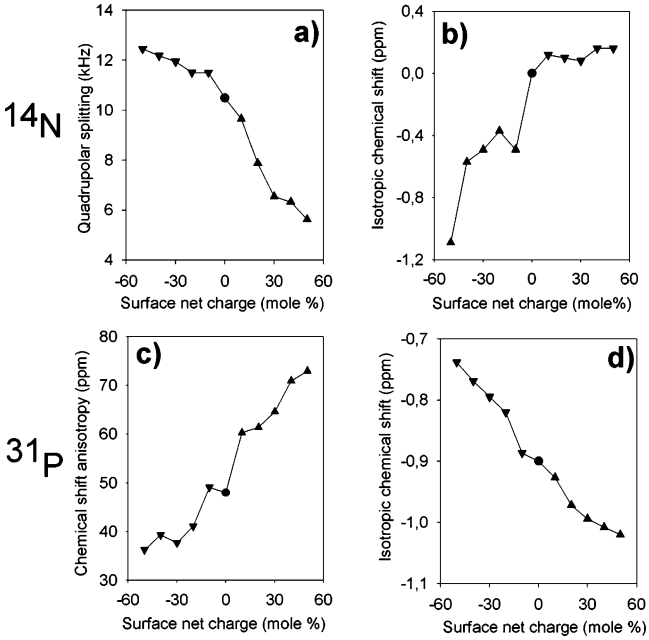

Figure 4. Variation in (a) ^{14}N quadrupolar splitting, (b) ^{14}N isotropic chemical shift, (c) ^{31}P chemical shift anisotropy, and (d) ^{31}P isotropic chemical shift at 308 K as a function of the molar percentage between DMPC and DMPG (−) or DDAB (+).

^{14}N resonance line of DMPC which undergoes an upfield shift as a function of an increasingly negative surface potential while remaining constant at an increasing positive potential, as seen in Figure 4b. Interestingly, the corresponding ^{31}P NMR spectra in Figure 3c,d, obtained for the negatively charge phosphate moiety of DMPC, show a counter-directional systematic behavior when compared to the ^{14}N NMR features. As summarized in Figure 4d, the isotropic ^{31}P chemical shift values vary systematically as functions of the surface net charge, moving downfield from −1.02 ppm for DMPC in positively charged

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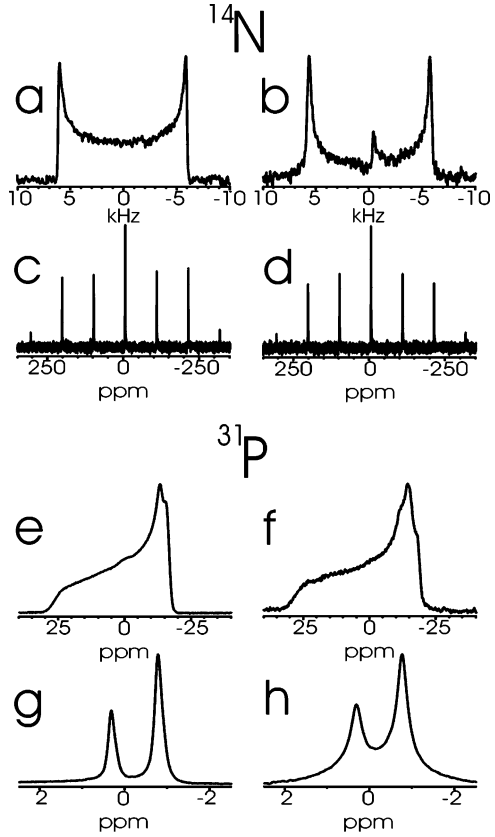

Figure 5. Left columns represent samples consisting of DMPC and DMPG at a 2:1 molar ratio before addition, and the right columns, after addition of nociceptin (NC) at a lipid-to-peptide molar ratio of 15:1. (a,b) ^{14}N NMR static spectra; (c,d) ^{14}N NMR MAS spectra at 3 kHz spinning speed; (e,f) ^{31}P NMR static spectra; (g,h) ^{31}P MAS NMR spectra at 3 kHz spinning speed. All spectra were acquired at 400 MHz proton frequency and at 308 K.

membranes to −0.74 ppm when 50 mol % acidic lipids are present. In addition, the dependency of the effective ^{31}P CSA (δ) on membrane composition is opposite to that observed for the ^{14}N quadrupole splitting, as seen in the static ^{31}P NMR spectra in Figure 3d. Through the analysis of ^{31}P MAS NMR spectra at intermediate speed (not shown), the dependency of the effective CSA ($\Delta\sigma$) upon surface charge can be determined (Figure 4c) where $\Delta\sigma$ ranges from 36 ppm for DMPC:DMPG at 1:1 molar ratio to 74 ppm for DMPC:DDAB at the same molar ratio.

Membrane Association of the GPCR Hormone Ligand Nociceptin. The effect of adding nociceptin (1−13) to negatively charged membranes (DMPC/DMPG at 2:1 molar ratio) is illustrated in Figure 5, with NMR spectra obtained before (left column) and upon addition (right column) of nociceptin. As nociceptin is added to the lipid system at a lipid-to-peptide ratio of 15:1, the resulting ^{14}N static spectrum in Figure 5b reveals a reduction in quadrupolar splitting from $\Delta\nu_Q$ 11.9 to 11.2 kHz, while in the relevant ^{31}P NMR spectrum (Figure 5f) an increase in $\Delta\sigma$ by 5 to 47 ppm is seen for DMPC (sideband analysis of MAS NMR spectra, not shown). This decrease in ^{14}N quadrupolar splitting is clearly reflected in the reduced intensity of the sidebands in Figure 5d and is consistent with a less negatively charged membrane surface as a result of interaction between the positively charged residues in nociceptin and the oppositely charged surface. This effect is also clearly visible in the ^{31}P MAS NMR spectra obtained at high spinning speed (Figure 5g,h). Here, the isotropic chemical shift values of DMPC and DMPG are shifted upfield from −0.77 to −0.79 and 0.33 to 0.28, respectively, indicating a reduced surface potential due to partial charge compensation, as previously observed in other systems including Aβ and pentalysine peptides.[7,37] The more pronounced effect seen for DMPG is most likely due to stronger repulsion of the negatively charged phophatidylglycerol headgroup. As seen in Figure 5h, the ^{31}P resonance lines for both lipids are broadened, indicating shorter spin−spin relaxation times (T_2) due to restricted motional dynamics in the headgroup region upon peptide association. This modulation of membrane fluidity is also reflected in a decreased intensity of the ^{14}N static spectrum (Figure 5b) and broadening of the isotropic ^{14}N resonance of DMPC in the MAS spectra from 14 to 20 Hz.

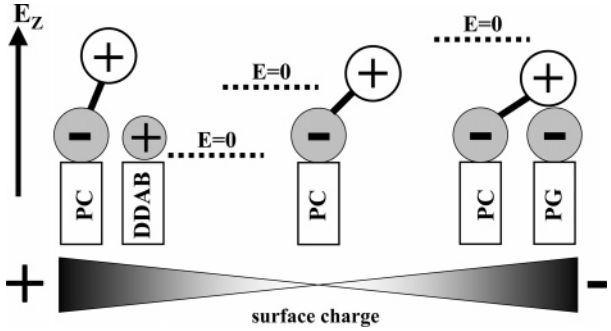

Figure 6. Schematic model of electrostatic situation at membrane interfaces as a function of lipid composition. The plane situated at the interface where the electric field component E_Z along the membrane normal changes sign ($E_Z = 0$ with $z = z_K$) is shown for three different situations: (left) positively charged membrane surface, (middle) neutral, (right) negatively charged.

Data Analysis

Isotropic NMR Chemical Shift: Size of Membrane Potential. The variation in membrane surface potential gives rise to systematic changes in the isotropic ^{14}N and ^{31}P chemical shift values of DMPC, which can be exploited as an indicator of the size of the electrical field originating at the membrane surface. The z component of the E vector changes as a function of its distance to the membrane surface and the existing potential and can therefore be described, as outlined earlier: [42]

$$E_Z = \frac{1}{2\epsilon_0}\sum_k \frac{\sigma_k(z - z_k)}{((A_0/\pi)^2 + (z - z_k)^2)^{1/2}} \qquad (1.3)$$

with A_0/π the radius of a lipid cylinder, σ_k the average charge density, z_k the position of a charge z in the membrane, and z describing the location along the membrane normal (parallel to E_Z).[42] For values such as $|z - z_k| \approx 0$ the sign and size of E_Z will vary strongly and can easily be correlated to the observed isotropic NMR chemical shift changes. Since the size and sign of E_Z will change differently at the phosphate and choline locations, the following three different scenarios can be assumed (Figure 6):

(i) In neutral DMPC bilayers, the average headgroup tilt is around 60° with respect to the membrane normal. With the PN vector nearly collinear with the dipole moment, and a PN length of 4.5 Å,[16] E_Z will be zero at 1.125 Å, reflecting a position

(42) Roux, M.; Neumann, J.-M.; Hodges, R. S.; Devaux, P. F.; Bloom, M. *Biochemistry* **1989**, *28*, 2313−2321.

right in the middle between both oppositely charged headgroup segments. Above this position, the field will have a positive value.

(ii) By increasing the negative surface potential via anionic DMPG, the plane with $E_Z = 0$ will move away from the surface. The phosphate region experiences a more negative field at the ^{31}P nucleus site, while the positive ^{14}N nucleus sees a reduced positive field. The chemical shift dependencies move in opposite directions for both nuclei, as seen in Figure 4. For ^{31}P, the values moves from −0.9 to −0.72 ppm and for ^{14}N from 0 to −1.1 ppm at 50 mol % DMPG.

(iii) Increasing the positive surface potential via cationic DDAB, the plane of zero field moves very close to the site of the phosphate segment with two consequences. First, at the choline position, the size of the positive E_Z component will not change significantly (as $|z - z_k| \ll A_o/\pi$), and therefore, charge-induced ^{14}N chemical shift changes are extremely weak (see Figure 4b, right part). This effect is even further enforced by a reduced tilt angle, which puts the choline group even further away from the surface. Second, the phosphate segment is very close to the position where the sign of the E_Z will change, and thus the observed linear upfield shift with increasing DDAB concentration.

Anisotropic ^{14}N/^{31}P NMR Line shapes: Headgroup Conformation versus Surface Potential. The variation of the membrane surface potential gives rise to systematic changes in the anisotropic features of the ^{14}N and ^{31}P NMR spectra, where the magnitude of the observed ^{14}N quadrupolar splitting $\Delta\nu_Q$ and ^{31}P chemical shift anisotropy $\Delta\sigma$ reflects the orientational response of the inherent P^{-}- - -N^{+} headgroup dipole to the surface potential a process previously described.[17,18,20,22,25] To analyze these changes in detail, a dynamic NMR model is applied (see Figure 1b), previously developed for liquid-crystalline systems.[16,34,35] Briefly, $\Delta\nu_Q$ and $\Delta\sigma$ do not provide direct structural, but time-averaged, orientational constraints. Using a molecular order parameter S_{mol} to describe this averaging of NMR interactions due to various, fast motional processes (see Figure 1b), the NMR observables can be expressed as $\Delta\nu_Q = (3e^2qQ/4h)S_{mol(N)}$ and $\Delta\sigma = S_{mol(P)}(\sigma_{||} - \sigma_{\perp})_{local}$. The order parameter can be dissected into a hierarchy of motional processes, with the intermolecular motions separated from intramolecular motions by deconvoluting S_{mol} into the orientational order parameter S_{ZZ} and conformational order parameters S_{CP} and S_{CN}. For pure DMPC bilayers at 308 K, S_{ZZ} is around 0.68,[34,35] a value fairly constant across the lipid mixtures with similar fatty acid composition.[22] Therefore, one can estimate changes in the average headgroup orientation by analyzing S_{CN} and S_{CP} which can be directly extracted from the NMR data according to:

$$\Delta\nu_Q = (3e^2qQ/4h)S_{ZZ}S_{CN} \quad \text{and} \quad \Delta\sigma = S_{ZZ}S_{CP}\,(\sigma_{||} - \sigma_{\perp})_{local} \tag{1.4}$$

Due to the small changes observed in isotropic ^{31}P chemical shifts, variations in observed $\Delta\sigma$ values (Figure 4) can be solely deduced to conformational reorientation of the phosphate group and its CSA tensor relative to the lipid long axis.[34] Using Dufourc's CSA phosphate tensor elements in the molecule fixed-coordinate system at 248 K ($\sigma_{xx} = -86$, $\sigma_{yy} = -24$, $\sigma_{zz} = 124$ ppm) and the concept of various axes of motional averaging (Figure 1b),[34] fast rotation around the O−P axis of the glycerol C_{glyc}−O−P hinge produces an axially symmetric tensor (appropriate Euler angles α,β (1°,85°)[34]) with a value of 124 ppm for $\Delta\sigma_{local}$. Including a further reduction by S_{ZZ}, $S_{CP} = \langle(3\cos^2\Theta - 1)/2\rangle$ reflects the time-averaged orientational offset of the O−P axis from the lipid axis. In the case of an extreme positively charged membrane surface, both axes are nearly parallel ($S_{CP} \approx 1$), with a maximal $\Delta\sigma$ value of 84 ppm. Therefore, the measured $\Delta\sigma$ can be used to determine Θ according to $\Delta\sigma/84\text{ ppm} = S_{CP}$. The measured $\Delta\sigma$ values of 74 ppm (+50% charge), 48 ppm (neutral), and 36 ppm, (−50% charge), correspond to Θ values of 16°, 32°, and 37°, respectively. Upon the binding of nociceptin to DMPC/DMPG bilayers, the change in $\Delta\sigma$ from 42 to 47 ppm, corresponds to a reduction in headgroup tilt from 35° to 33°.

In the case of ^{14}N, the static anisotropic ^{14}N quadrupole interaction (quadrupole constant e^2qQ/h around 135 kHz)[41] is averaged around the C_β−N bond in the choline C_β−$N(CH_3)_3$ and undergoes further additional averaging compared to the phosphate segment (Figure 1b). Using earlier ^{2}H NMR studies on DMPC deuterated at its choline methyl groups,[23] together with the known geometry in the choline headgroup for DMPC in neutral bilayers,[16] a value of 52 kHz for the preaveraged nitrogen quadrupole coupling constant around the C_β−N axis can be calculated. In addition to the motional processes the phosphate group experiences, the ^{14}N experiences additional rapid conformational changes in the choline segment, resulting in an observed splitting of 10.4 kHz in pure DMPC bilayers. Using the same approach as for the phosphate segment, the relative orientational changes of the choline segment defined by its C_β−N axis can be expressed as:

$$\Delta\nu_Q/\langle e^2qQ/h\rangle = S_{ZZ}S_{CP}S_{CN} \quad \text{with} \quad \langle e^2qQ/h\rangle = 52\text{ kHz}$$

and S_{CN} defining the fictive offset of the C_β−N rotation axis as effected by the intramolecular conformation changes. The corresponding angles as obtained from the splittings are therefore 47° (50 mol % DDAB), 36° (neutral DMPC), and 23° when 50 mol % DMPG is present. Upon the addition of nociceptin, a reduction in $\Delta\nu_Q$ value occurs from 11.9 to 11.2 kHz, corresponding to a change from 29° to 32°.

Discussion

As shown here, high-resolution, natural abundance ^{14}N and ^{31}P MAS NMR can be used as a noninvasive method to generate new insight at a molecular level into a biologically important key mechanism, namely the electrostatically driven association of proteins to their target membrane. By probing directly the negatively charged phosphate and positively charged choline part in the headgroup region of the zwitterionic DMPC lipid component in mixed bilayers, not only could the electrostatic potential present at a membrane surface be characterized but also the charge distribution across the membrane interface and, most significant, changes occurring upon protein binding or variation in the membrane constituents. The application of MAS NMR at high and intermediate rotation frequencies enabled us to use, in multicomponent systems, the isotropic chemical shift NMR parameters as an indicator of the size of the existing potential and the anisotropic NMR features to extract the

orientational response of lipid headgroups to changes in the surface potential.

The observed systematic modulations in the isotropic $^{14}N/^{31}P$ chemical shift values provided unique information about the size of the electrostatic surface potential, therefore enabling us for the first time to establish a molecular picture (Figure 6) of the charge distribution across a membrane interface for three different possible situations, with anionic and neutral membranes as found in vivo, and cationic membranes as used as DNA transfection agents.[12] All these different cases are well reflected in the nearly linear change of the isotropic ^{31}P resonance of DMPC across the whole variation in surface charge ranging from −50% to +50%. The ^{14}N resonance only shows variation when negatively charged lipids are present, a phenomenon easily explainable by our model in Figure 6. As shown here, the use of the isotropic NMR information, as extracted from anisotropic membrane systems, enables one in a elegant way to study not only the potential at a membrane surface but also the effect of a surface potential on each lipid component separately due to the superb resolution obtained using MAS NMR. As seen here, the obtained isotropic ^{31}P chemical shift values for PG change more dramatically upon addition of nociceptin than do the values for the PC lipid. This different behavior has been reported before for various proteins by us and others but without any insight into the underlying phenomena in detail.[5,37,43] By using a simple model of the electrostatic field present at a membrane surface,[23,42] we can easily correlate the observed changes in the isotropic NMR features to changes in the size and position of the electric field. Since isotropic isotropic chemical shifts values are invariant under rotation, any changes in their value must arise from change in the presence of an additional (or positionally shifted) electrostatic field component E_Z which distorts the local electron distribution around the phosphate group (induced atomic polarization).[44]

The observed variation in the anisotropic features of the $^{14}N/^{31}P$ NMR line shapes could be exploited here, to characterize directly the orientation of lipid headgroups - as defined by the P^--N^+ dipole - relative to the membrane surface as a function of the existing surface charge. To analyze the anisotropic NMR information, an approach based on an adapted "molecular voltmeter model",[17,18,22−25] was used together with the concept of dynamic molecular order parameters.[16,34,35] The combined tilt changes of the phosphate and choline region show a greater dependence on the presence of cationic charges (50 mol % DDAB: $\Delta(\Theta) = 12°(^{31}P) + 11°(^{14}N)$) compared to the presence of 50 mol % anionic DMPG ($\Delta(\Theta) = 5°(^{31}P) + 13°(^{14}N)$). There is clearly a major conformational flexibility inside the headgroup region with the phosphate segment more restricted than the choline group, as suggested by earlier ^2H NMR studies.[17,18,22−25] The values obtained here for the general change of the PN tilt angle are similar in size to those by MacDonald.[16] However our approach presented here is applicable to a wide variety of lipid and membrane-protein assemblies, not restricted at all as in studies using oriented lipid systems and/or specifically synthesized ^2H- and ^{13}C-labeled lipids.

Association of the peptide hormone nociceptin with negatively charged lipid vesicles produced characteristic changes in all ^{31}P and ^{14}N NMR spectra. Albeit small, these changes are all consistent with a reduction in membrane potential upon electrostatically driven peptide association, a process also observed in other membrane-bound systems such as Aβ-protein, melittin, and others.[7,36−41] The finding of electrostatically driven binding as a first step in its biological action is supported by isothermal titration calorimetry binding studies (not shown) determining $K_D \approx 50\ \mu M$. That the observed changes in the NMR spectra were not as pronounced as in the case of peripheral amphipathic proteins is mostly like due to the nature of the peptide as a highly soluble diffusive ligand involved in GPCR signal transduction. Nevertheless, binding of the peptide to the charged lipid surface supports the hypothesis that membrane association is an important prerequisite to the binding of nociceptin into its binding site on the ORL1 GPCR receptor.[18] Similar mechanisms have been proposed for a range of other peptidic hormones including substance P, PH2, and enkephalins, although a detailed understanding of this process is still limited.

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