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Membrane interaction of neuropeptide Y detected by EPR and NMR spectroscopy

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Abstract

Neuropeptide Y (NPY) is one of the most abundant peptides in the central nervous system of mammals. It belongs to the best-conserved peptides in nature, i.e., the amino acid sequences of even evolutionary widely separated species are very similar to each other. Using porcine NPY, which differs from human NPY only at position 17 (a leucine residue exchanged for a methionine), labeled with a TOAC spin probe at the 2nd, 32nd, or 34th positions of the peptide backbone, the membrane binding and penetration of NPY was determined using EPR and NMR spectroscopy. The vesicular membranes were composed of phosphatidylcholine and phosphatidylserine at varying mixing ratios. From the analysis of the EPR line shapes, the spectral contributions of free, dimerized, and membrane bound NPY could be separated. This analysis was further supported by quenching experiments, which selected the contributions of the bound NPY fraction. The results of this study give rise to a model where the α-helical part of NPY (amino acids 13–36) penetrates the membrane interface. The unstructured N-terminal part (amino acids 1–12) extends into the aqueous phase with occasional contacts with the lipid headgroup region. Besides the mixing ratio of zwitterionic and negatively charged phospholipid species, the electrostatic peptide membrane interactions are influenced by the pH value, which determines the net charge of the peptide resulting in a modified membrane binding affinity. The results of these variations indicate that NPY binding to phospholipid membranes depends strongly on the electrostatic interactions. An estimation of the transfer energy of the peptide from aqueous solution to the membrane interface ΔG supports the preferential interaction of NPY with negatively charged membranes.

Keywords: Liposome; TOAC; ^{1}H MAS NMR; Peptide lipid interaction; CROX; Membrane partitioning; Amphipathic helix

1. Introduction

Neuropeptide Y (NPY) is a 36-amino acid peptide that is widely distributed in the peripherally and the central nervous system [1]. The peptide is C-terminally amidated and was first isolated from porcine brain in 1982 [2]. NPY is found in all mammals as well as in a wide variety of animal species including birds, reptiles, amphibians, and fishes.

NPY acts both as a regulator of hormone secretion and a neurotransmitter [3]. The regulation of blood pressure and food uptake, especially that of carbohydrates, which can lead to fat deposition and weight gain, are also transmitted

Abbreviations: 5-doxyl PC, 1-palmitoyl-2-stearoyl-(5-doxyl)-*sn*-glycero-3-phosphocholine; AMPSO, 3-[(1,1-Dimethyl-2-hydroxy-ethyl)-amino]-2-hydroxy-propanesulfonic acid; CROX, potassium chromium oxalate ($K_3Cr(C_2O_4)_3^{\cdot}3H_2O$); DPC, dodecylphosphocholine; LUV, large unilamellar vesicles; MAS, magic-angle spinning; MES, 2-[N-morpholino]ethanesulfonic acid; MLV, multilamellar vesicles; NOE, nuclear Overhauser effect; NPY, neuropeptide Y; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; PS, phosphatidylserine; TOAC, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid

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by NPY [3]. Further, the control of the metabolism and the involvement in cardiovascular regulation are important functions of NPY. It potentiates the effects of other vasoactive neurotransmitters and vasoconstrictors, inhibits vasodilators in the sympathetic nervous system. The effects of NPY are initiated by five distinct receptors, which belong to the family of the heptahelical G-protein coupled receptors [4]. It has been suggested that binding of NPY to the phospholipid membrane is the important prerequisite for its interaction with the receptor [5,6]. When bound to the membrane, peptide diffusion is restricted to two dimensions and the likelihood of interacting with the receptor is drastically increased. Therefore, the characterization of the peptide in the membrane bound state represents a crucial step for the understanding of the NPY regulated processes. Consequently, the localization of NPY in the lipid membrane and the influence of the membrane surface potential on the binding properties of the molecules are important molecular features that determine the interaction of NPY with the receptors.

The solution structure and dynamics of NPY have been determined by NMR spectroscopy [7,8]. In aqueous solution at low pH, the nuclear Overhauser effects (NOE) of NPY indicate the presence of an amphipathic α-helix, which includes residues 13–36 [8]. The central part of this helix including residues 17–32 has been reported to be rigid [7]. The flanking helical regions and the rest of the peptide, however, have been found to be more flexible [8]. The N-terminal part of NPY (residues 1–12) does not show any ordered structure. Further, in aqueous solution NPY forms dimers, where the hydrophobic side of the amphipathic α-helix has been identified as the dimerization interface [7,8]. Both parallel and antiparallel orientations of the dimer exist in equilibrium with monomers. By reducing the concentration of NPY, the equilibrium shifts towards the monomers. Detergent micelles have widely been used as a model to study the interaction of peptides with membrane surfaces [9]. However, the weaknesses of micelles to mimic a planar phospholipid membrane are well known [10]. Liposomes, which are composed of true phospholipid bilayers and consequently a better membrane model, typically exceed the molecular weight limit of solution NMR and cannot be used for structure determination of membrane-associated peptides. The only alternative to use NMR techniques to study the structure and dynamics of membrane-associated peptides is solid-state NMR [11]. These techniques are very promising to obtain peptide structures and models of the peptide membrane interaction [12]. However, solid-state NMR techniques require either well-oriented membrane stacks or magic-angle spinning (MAS) of multilamellar vesicles typically investigated at low water concentration, which may complicate peptide binding studies. Therefore, at the moment, the best starting model to investigate NPY-membrane interaction is the NMR structure of the peptide bound to DPC micelles [7].

In the last years, electron paramagnetic resonance (EPR) techniques have been developed and successfully applied to obtain important structural and dynamical information on membrane-associated peptides [13–15]. These measurements are typically carried out on liposomes in excess buffer. Site-directed spin labeling involving nitroxide radicals as spin probes, which are often attached to the sulfhydryl group of a cysteine residue, has been the most frequently used EPR technique applied to biologically relevant systems [16,17]. These methods have been very successfully applied to build models of the interaction of peptide segments [18] and proteins [19] with the phospholipid membrane.

Alternatively, the non-proteinogenic amino acid TOAC, equipped with a nitroxide radical as the spin probe has been used [20]. The nitroxide is attached to the rigid piperidine ring, whose carbon atom at 4th position represents the Cα atom of the amino acid. This leads to a significant reduction of the flexibility of the TOAC moiety in comparison to cysteine bound spin probes and renders the TOAC probe a suitable tool for the investigation of the mobility of the peptide by EPR. A detailed conformational analysis on TOAC containing peptides has shown that TOAC is a strong stabilizer of β-turns and 3_{10}/α-helical structures [21].

The synthesis and application of TOAC labeled NPY mutants has first been described recently [5]. Three peptide mutants have been synthesized, where the TOAC label was introduced into the 2nd, 32nd, or 34th positions of the peptide backbone. In preliminary studies, EPR spectra with narrow and broad contributions from free and membrane-bound NPY have been reported allowing to determine rotational correlation times of the peptides [5].

In this paper, we investigate the binding of these TOAC labeled NPY mutants to negatively charged membranes composed of zwitterionic phosphatidylcholine (PC) and negatively charged phosphatidylserine (PS) membranes of varying composition using EPR and NMR methods. Membrane binding is strongly enhanced in negatively charged membranes and leads to an immobilization of NPY. We further determined the critical NPY concentration for self-association and the molar partition coefficients of NPY to negatively charged PS-LUVs at a concentration below the critical concentration for dimerization. Finally, the localization of the three TOAC labels in the membrane was determined by EPR and NMR spectroscopy.

2. Materials and methods

2.1. Materials

The TOAC labeled NPY analogs [$TOAC^{2}$]-NPY, [Ala^{31}, $TOAC^{32}$]-NPY, and [$TOAC^{34}$]-NPY were synthesized by solid-phase synthesis as reported in detail previously [5]. Human NPY was purchased from Bachem AG (Bubendorf, Switzerland). The phospholipids egg-phosphatidylcholine

(PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), brain-phosphatidylserine (PS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), 1-palmitoyl-2-stearoyl-(5-doxyl)-*sn*-glycero-3-phosphocholine (5-doxyl PC) were purchased from Avanti Polar-Lipids, Inc. (Alabaster, Al). The lipids and peptides were used without further purification. If not stated otherwise, the experiments were carried out in aqueous buffer solution containing 10 mM citric acid (pH 4 or pH 5), 10 mM MES (pH 6), 10 mM HEPES (pH 7.4), or 10 mM AMPSO (pH 9), respectively, and 100 mM NaCl.

2.2. Sample preparation

For EPR measurements, the peptides were dissolved in the respective buffer solution to yield stock solutions of 1 mM, which were further diluted to obtain the desired concentration. Large unilamellar vesicles (LUV) were prepared by extrusion according to standard procedures [22]. In brief, solutions of the lipids in chloroform in definite concentration were evaporated under high vacuum at room temperature with a rotary evaporator. The lipid film was resuspended in a defined buffer solution followed by vortexing. After 10 freeze–thaw cycles, the lipid buffer solution was extruded trough two stacked polycarbonate filters of 100 nm pore size in a LIPEX thermostated extruder (Biomembranes, Vancouver, BC, Canada). The final lipid concentration after extrusion was determined by a phosphate determination [23].

For ^{1}H NMR measurements, the NPY analogs were mixed in chloroform with POPC/POPS at a molar ratio of 1:80:20. After evaporating the chloroform under a stream of nitrogen, the samples were redissolved in cyclohexane and lyophilized to obtain a fluffy powder. These samples were hydrated with 70 wt.% D_2O and equilibrated by several freeze–thaw cycles and gentle centrifugation. Subsequently, the samples were transferred into 4-mm HR MAS rotors with spherical Kel-F inserts providing a volume of ~15 μl.

2.3. EPR measurements

The EPR spectra were acquired on a Magnettech Miniscope MS 100 (Magnettech, Berlin, Germany) using a microwave frequency of 9.45 GHz. Temperature control was performed with a Magnettech Temperature controller H01. The value of the center field was 3344 G for all measurements, the scan width was 120 G, and a microwave power of 50 mW was used. In all cases, the modulation amplitude was 1 G to avoid line shape distortion of the EPR signal. The accumulation and the gain were adapted to each particular measurement. LUVs, peptides, and buffer were mixed at the desired ratio, filled into glass capillaries (50 μl), and sealed for EPR measurements. The isotropic hyperfine coupling constant $a_0 = 1/3(A_{\parallel} + 2A_{\perp})$ was calculated from the EPR spectra according to [24]. $A_{\parallel}$ and $A_{\perp}$ can be easily determined from the A_{min} and A_{max}, which were derived from the EPR spectrum (see Fig. 4), where $2A_{\parallel} \approx 2A_{max}$ and $2A_{\perp} \approx A_{min} + 1.4(G)\{1 - (A_{max} - A_{min})/[A_{zz} - 0.5(A_{xx} + A_{yy})]\}$ [25]. The values of the hyperfine tensors A_{xx}, A_{yy}, and A_{zz} of nitroxides oriented in single crystals were taken from literature. For the TOAC analogs the values relative to tempone, $A_{xx} = A_{yy} = 5.2$ G and $A_{zz} = 31.0$ G [26] and for the *n*-doxyl stearic acids the values relative to 2-doxyl propane $A_{xx} = 5.9$ G, $A_{yy} = 5.4$ G, and $A_{zz} = 32.9$ G were used [27].

2.3.1. ^{1}H MAS NMR

^{1}H MAS NMR spectra were acquired on a DRX600 NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at a resonance frequency of 600.1 MHz for ^{1}H using a 4 mm HR MAS probe at a MAS frequency of 8 kHz and a spectrum width of 10 kHz. Typical π/2 pulse lengths were 8.8 μs. T_1 relaxation times were measured using the inversion recovery pulse sequence with 13 delays between 1 ms and 4 s and a relaxation delay of 4 s. All spectra were recorded at a temperature of 30 °C.

Due to the presence of the unpaired electron in the TOAC label, a fast paramagnetic relaxation mechanism is introduced and the total relaxation rate $R_1 = 1/T_1$ is the sum of paramagnetic and dipolar relaxation rates ($R_1 = R_{1,p} + R_{1,d}$). After measuring $R_{1,d}$ from a phospholipid sample in the absence of spin labeled NPY, $R_{1,p}$ can be easily determined from a relaxation measurement in the presence of NPY for each molecular segment of the lipids [28].

3. Results

3.1. EPR spectra of TOAC labeled NPY mutants

Using the three spin labeled NPY mutants, the binding and penetration of the TOAC labeled segments of the peptide into the phospholipid membranes was determined. According to the NMR structure of NPY in DPC micelles [7], the spin probe of [TOAC^2]-NPY is located at the N-terminus in the unstructured part of NPY. The C-terminal α-helical part of NPY is labeled at the 32nd and 34th positions with a TOAC spin probe in the mutants [Ala^{31}, TOAC^{32}]-NPY and [TOAC^{34}]-NPY, respectively.

The EPR spectra of [Ala^{31}, TOAC^{32}]-NPY, [TOAC^{34}]-NPY, and [TOAC^2]-NPY in aqueous buffer show the characteristics of isotropically tumbling peptides ($\tau < 10^{-11}$ s) with identical spectral features for all three mutants (spectra not shown). However, significant differences are observed for the EPR spectra of the NPY analogs in the presence of lipid membranes.

Fig. 1 shows the EPR spectra of [TOAC^{34}]-NPY bound to membranes of varying PS and PC content. The EPR spectra of [TOAC^{34}]-NPY in solution (Fig. 1A) and in the presence of purely zwitterionic PC-LUV (Fig. 1B) are nearly identical. Only the three sharp lines characteristic for a nitroxide radical that undergoes rapid rotation can be

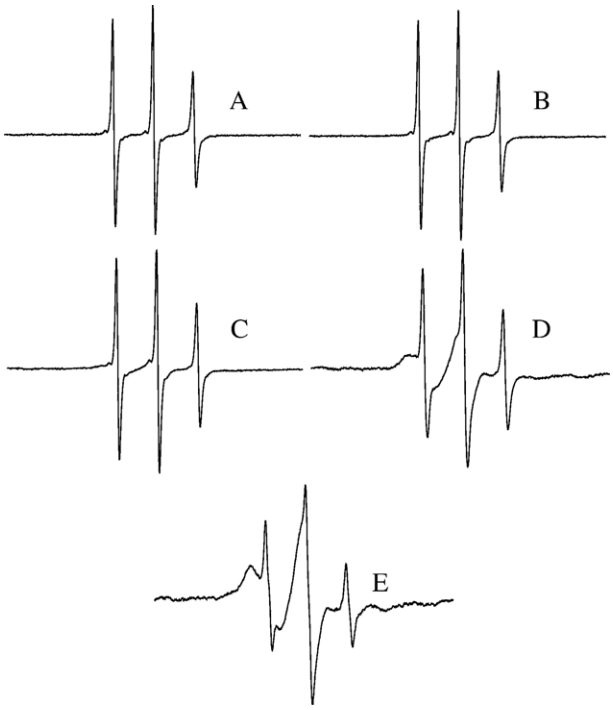

Fig. 1. Second integral normalized EPR spectra of [TOAC^{34}]-NPY (200 μM) in the presence of LUV (15 mM) composed of varying mixing ratios of PS and PC (A, no lipid; B, pure PC-LUV; C, PS/PC-LUV (1:5 mol/mol); D, PS/PC-LUV (1:2 mol/mol); E, pure PS-LUV) at pH 6 and at a temperature of 37 °C.

observed in both EPR spectra. Consequently, the binding of NPY to zwitterionic phospholipid membranes is negligible. Increasing the molar fraction of the negatively charged PS in the membrane results in the detection of the additional component of the EPR spectra, indicative of a slowly tumbling probe (Fig. 1C–E). These experiments suggest that NPY binds preferentially to negatively charged lipid surfaces. In order to investigate details of the peptide binding process, the EPR spectra of the three NPY analogs were investigated in detail in mixed PC/PS (2:1, mol/mol) membranes.

The EPR spectra of [Ala^{31}, TOAC^{32}]-NPY, [TOAC^{34}]-NPY, and [TOAC^{2}]-NPY in the presence of PC/PS-LUV (2:1, mol/mol) are shown in Fig. 2. The line width and the line spacing of the EPR spectra provide a measure of the mobility of the TOAC probe. The three sharp lines characteristic of the mobile nitroxide spin probe TOAC in aqueous solution are observable for all three TOAC labeled NPY analogs. In addition, the EPR spectra of the C-terminally labeled peptides ([Ala^{31}, TOAC^{32}]-NPY, [TOAC^{34}]-NPY) are composed of a superposition of two characteristic line shapes, one for highly mobile spin probes undergoing rapid rotation (as described above) and one for slowly tumbling immobilized TOAC probes. These contributions can be assigned to free and membrane bound peptides, respectively. The line width and line spacing of the slowly tumbling component of the EPR spectrum are increased in comparison to an EPR spectrum of a highly mobile nitroxide spin probe. The EPR spectrum of [TOAC^{2}]-NPY does not show this slowly tumbling component indicating that the N-terminal part of membrane bound NPY is very flexible while the C-terminal part of the molecule is immobilized when bound to negatively charged vesicles. However, the line width of the EPR spectrum of [TOAC^{2}]-NPY is slightly increased compared to the EPR spectra of highly mobile

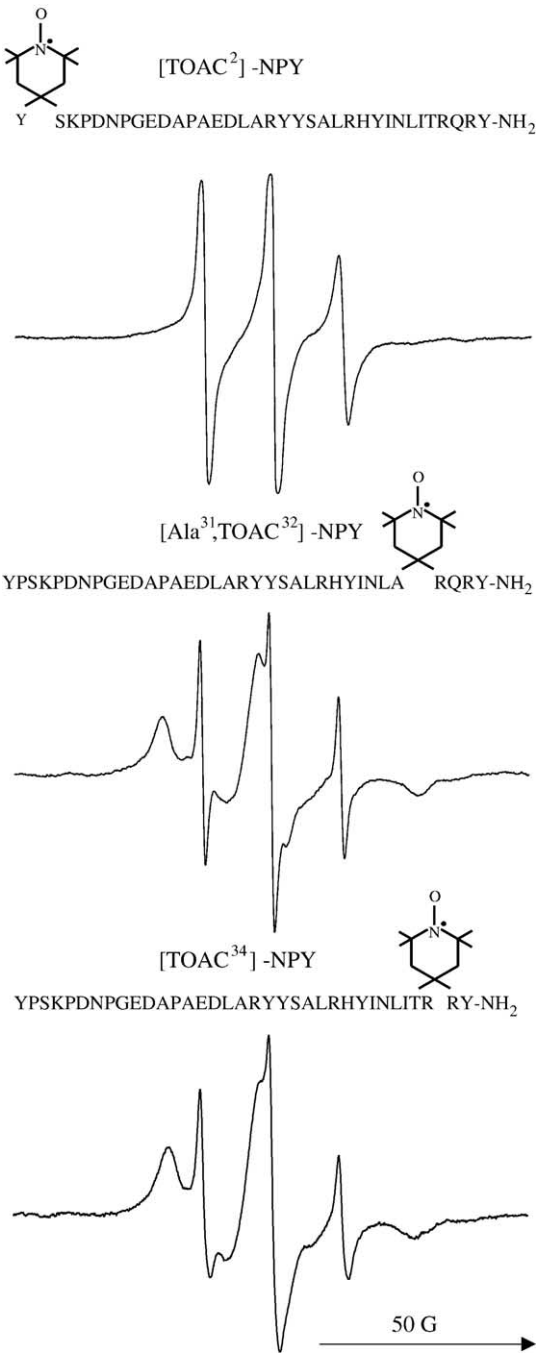

Fig. 2. EPR of the three TOAC labeled NPY analogs (200 μM) in presence of PS/PC-LUV (molar ratio 1:2) at pH 6 and a temperature of 37 °C. The total lipid concentration was 15 mM (buffer: 100 mM NaCl, 10 mM MES). The amino acid sequence of each NPY mutant is also given.

spin probes but still exhibits the characteristics of a highly mobile spin probe.

3.2. Determination of the EPR spectra of membrane bound NPY

The EPR signal of the TOAC spin label can be quenched by the hydrophilic molecule potassium chromium oxalate (CROX). This property is used to differentiate between peptides bound to the phospholipid vesicles and free peptides in solution [29]. Since the hydrophilic CROX ions do not penetrate the membrane, only the free peptide signal is quenched, while membrane inserted spin labeled segments should preserve their characteristic broad EPR spectra. Therefore, addition of CROX to the solution should quench the EPR signal of the free peptide but preserve the EPR signal of the membrane bound fraction of NPY. This should be particularly important for [TOAC^{2}]-NPY, where the spectra of free and membrane bound peptides are difficult to distinguish.

The EPR spectra of the three labeled peptides in the presence of 75 mM CROX are shown in Fig. 3. The EPR signals of TOAC labeled NPY in solution are almost entirely quenched by CROX (Fig. 3, left column). The residual broad component may arise from NPY dimers that are not accessed by CROX and show a slightly broader EPR spectrum due to their slower tumbling in solution.

In the presence of PS-LUV and 75 mM CROX (Fig. 3, right column), the EPR spectra of the three NPY analogs only show a broad component. For the [TOAC^{2}]-NPY spectrum in the presence of PS a line spacing of $2A_{\|}$=43.5 G is obtained, which is characteristic of a solution spectrum of the nitroxide spin probe. Therefore, the high mobility of the

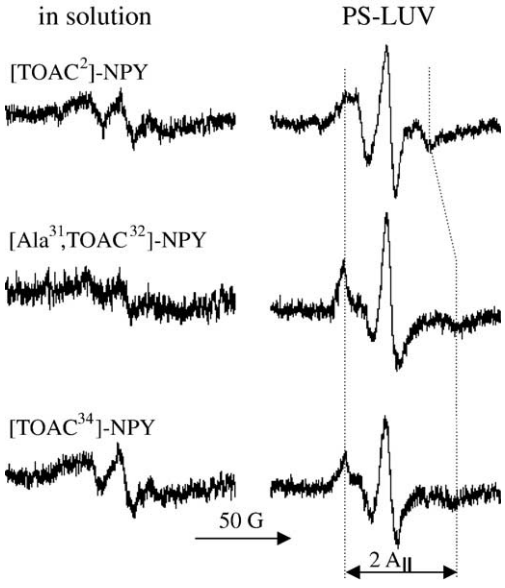

Fig. 3. EPR spectra of the three TOAC labeled NPY analogs (200 μM) in the absence (left column) and in the presence of PS-LUV (15 mM) (right column). The samples contained 75 mM CROX (buffer: 100 mM NaCl, 10 mM MES) and were recorded at pH 6 and a temperature of 32 °C.

membrane bound [TOAC^{2}]-NPY suggests that the N-terminal part of NPY is not inserted into the phospholipid membrane of the PS-LUV. Rather, the N-terminus is flexible undergoing fast motions with large amplitudes.

The at first sight surprising result that the EPR signal of [TOAC^{2}]-NPY is not quenched in the presence of CROX can be understood by considering electrostatic arguments. Due to the electrostatic repulsion between CROX and the membrane, the concentration of CROX molecules in the vicinity of the membrane surface is significantly reduced. Since the N-terminus of NPY is localized in this region, there is no full quenching of EPR signal of [TOAC^{2}]-NPY.

For the [Ala^{31}, TOAC^{32}] and [TOAC^{34}]-NPY analogs, the broad spectral components of the EPR spectra of Fig. 2 are selectively measured in the presence of CROX (Fig. 3). The line spacing of the EPR signal of membrane bound [Ala^{31},TOAC^{32}]-NPY is $2A_{\|}$=58 G and for [TOAC^{34}]-NPY $2A_{\|}$=56 G. These values are typical for EPR spectra of slowly tumbling spin probes. These results indicate again that the C-terminal part of NPY is clearly immobilized in comparison to the N-terminal part of the peptide.

The spectrum of [TOAC^{34}]-NPY in the presence of 75 mM CROX allows the determination of the isotropic hyperfine coupling constant a_0 (see Materials and methods). The value a_0 of [TOAC^{34}]-NPY was determined to (15.5±0.4) G, the a_0 value of TOAC in aqueous solution is 16.1 G [30]. The difference between these two values $\Delta a_0 = a_0(\text{aqueous}) - a_0(\text{measured})$ = (0.6±0.4) G provides a measure of the penetration depth of the TOAC labeled peptide into the lipid membrane as discussed below [20].

The comparison between the EPR spectra in the absence (Fig. 2) and in the presence of 75 mM CROX (Fig. 3) reveals a significant difference in the signal to noise ratio. This is at first sight surprising, since the anionic CROX should be repelled from the membrane surface and, therefore, only quench the soluble fraction of NPY. However, two facts may explain this apparent discrepancy: First, the binding affinity of NPY to negatively charged membranes is altered by CROX. The electrostatic attraction between NPY and the negatively charged membrane surface is screened by the addition of 75 mM CROX. This leads to a reduction of the Debye length from 9.7 Å in aqueous buffer with 100 mM NaCl to 5.4 Å (in the presence of 100 mM Na^{+} and 225 mM K^{+}), which weakens the electrostatic attraction between peptide and membrane and reduces the number of bound molecules. Second, depending on pH, NPY has 5 to 6 positively charged residues, which may attract the negatively charged CROX ions, which may also result in partial quenching of the EPR signal of bound NPY molecules.

To improve the signal to noise ratio of the EPR spectra of the three TOAC labeled NPY analogs in the presence of CROX, we decided to use pure PS-LUV instead of a more physiological mixture of PS and PC. The stronger electrostatic interaction between the positively charged amino acids and negatively charged PS increases the binding affinity of the peptide.

Because of the poor signal to noise ratio of the peptide EPR spectra in the presence of CROX, we decided to subtract the free component of the EPR signals in Fig. 2 using a deconvolution procedure to obtain the spectra of the membrane-associated peptide. To fit the spectral contribution of rapidly tumbling free NPY, we used two first derivatives of Gauss functions for each of the three EPR peaks. One Gaussian for the free monomeric component and one for the free dimerized component of the peptide. The difference between the original EPR spectrum and the fitted free components provides the EPR spectrum of the lipid bound fraction of the peptide (Fig. 4) and allows the determination of the isotropic hyperfine coupling constant a_0. The EPR signal to noise ratio in these spectra is much increased in comparison to the CROX spectra (Fig. 3) and allows to more accurately determine Δa_0 of the peptide.

The difference between the isotropic hyperfine coupling constant in solutions and in the membrane bound state provides a measure for the immersion depth of an EPR probe in the membrane [20]. The results of the penetration of the [Ala^{31}, TOAC^{32}]-NPY and [TOAC^{34}]-NPY are summarized in Table 1. Depending on the lipid membrane composition (pure PS or mixtures of PS and PC), values for Δa_0 between approximately 0.1 and 0.9 G are found. To translate these values into the depth of membrane penetration of the TOAC probe, the Δa_0 of 5-doxyl PC in a PC/PS and a pure PS matrix was considered for comparison. For 5-doxyl PC, Δa_0=(0.8±0.2) G, indicating that the TOAC labels of NPY at positions 32 or 34 penetrate the membrane at most to the upper chain region. In slight contrast, a previous study has shown that Δa_0=0.8 G denotes a position of the TOAC label around the 8th or 9th carbon atom in the chain. However, since EPR probes are not localized at a fixed position but rather broadly distributed [28] and our Δa_0 values for the TOAC label in the C-terminal α-helix of NPY scatter between 0.1 and 1 G, it is fair to say that the EPR probes of the α-helical part of NPY are located in the lipid–water interface region of the bilayer.

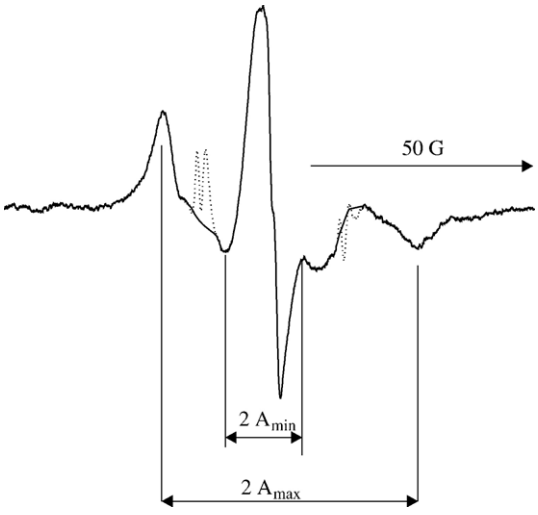

Fig. 4. The bound component of the EPR spectrum of [TOAC^{34}]-NPY (200 μM) in the presence of 15 mM PS/PC-LUV (molar ratio 1:2). The spectrum was derived after subtraction of the free and dimerized NPY components, obtained from a lineshape fit (deconvolution procedure described in the text). The dotted lines represent subtraction errors from the fit.

Table 1
EPR spectral parameters $2A_{max} \approx 2A_{||}$, $2A_{min}$, $2A_{\perp}$, a_0, and Δa_0 for membrane bound [Ala^{31}TOAC^{32}]-NPY and [TOAC^{34}]-NPY in the presence of phospholipid vesicles using spectral deconvolution methods as described in the text

	$2A_{max} \approx 2A_{||}$ [G]	$2A_{min}$ [G]	$2A_{\perp}$ [G]	a_0 [G]	Δa_0 [G]
[Ala^{31},TOAC^{32}]-pNPY[a]	59	16.5	17	15.5	0.6±0.4
[TOAC^{34}]-pNPY[a]	58	17	17.6	15.5	0.6±0.4
[Ala^{31},TOAC^{32}]-pNPY[b]	59.8	16.5	17	15.6	0.5±0.4
[TOAC^{34}]-pNPY[b]	57.2	17.6	18.3	15.6	0.5±0.4
[Ala^{31},TOAC^{32}]-pNPY[c]	60.0	16.5	16.9	15.6	0.5±0.4
[TOAC^{34}]-pNPY[c]	56.8	17.8	18.5	15.6	0.5±0.4
PC/PS 2:1,5 Doxyl[d]	49.1	19.0	20.2	14.9	0.8±0.2
PS, 5 Doxyl[d]	49.1	19.0	20.2	14.9	0.8±0.2

For comparison, spectral parameters for 5-doxyl PC are also given.
[a] Values were derived from the EPR spectra of the TOAC labeled NPY (200 μM) in the presence PS-LUV and 75 mM CROX at pH 6 and a temperature of 32 °C.
[b] Values were derived from EPR spectra of the TOAC labeled NPY (200 μM) in the presence of PS/PC-LUV(molar ratio 1:2, 15 mM) at pH 6 and at a temperature of 37 °C.
[c] Values were derived from EPR spectra of the TOAC labeled NPY (200 μM) in the presence of PS-LUV (15 mM) at pH 6 and at a temperature of 37 °C.
[d] Values were derived from EPR spectra of 5-doxyl PC in PC/PS LUV (molar ratio 2:1) and PS-LUV containing 1 mol% of 5 doxyl PC.

The comparison between the TOAC and the doxyl spin label to determine the penetration depth of the peptide has to be carried out very carefully. The problem is the different a_0 values of TOAC (a_0=16.1 G) and doxyl (a_0=15.7 G) [24] in aqueous solution. Therefore, the difference Δa_0 is used to determine the penetration depth. Further, the anisotropy caused by the rotational motion of the TOAC spin label, which is attached to the backbone of the peptide is different from that of the doxyl label, which is attached to the acyl chain of the PC molecule. The anisotropy of the motion of small membrane bound peptides is dominated by the mobility of the phospholipid matrix. This supports the assumption that rotational correlation times of the TOAC and doxyl labels are comparable for small peptides. Because of these problems related to the determination of the Δa_0 values, we decided to investigate the peptide penetration into membranes by an independent ^{1}H NMR method (*vide infra*).

3.3. Investigation of peptide aggregation

The EPR signal amplitude of the central (h_0) line as a function of the concentrations of [TOAC^{34}]-NPY in the absence of phospholipid vesicles is shown in Fig. 5. It is clearly seen that the signal amplitude does not change

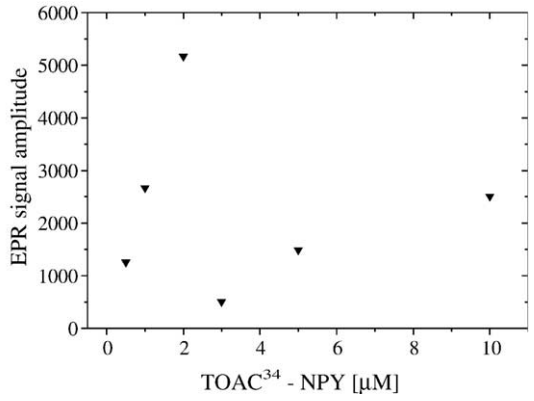

Fig. 5. A characteristic plot of the EPR signal amplitude of the h_0 line of [TOAC^{34}]-NPY as a function of the total peptide concentration. All experiments were carried out at a temperature of 37 °C at pH 7.4.

linearly as a function of the peptide concentration. This is a result of peptide dimerization, which increases the rotational correlation time of the TOAC labeled peptides and broadens the EPR signal. Taking into account that the second integral of the EPR intensity is proportional to spin density of the TOAC label and to the concentration of NPY, respectively, the amplitude of a slowly tumbling spin probe is decreased in comparison to a highly mobile spin probe.

Because of the low concentration of the peptide the slowly tumbling component is not observable at the EPR spectrum of [TOAC^{34}]-NPY. It needs to be added that the reproducibility of the EPR amplitudes as a function of NPY concentration is rather poor. However, all measurements indicated a discontinuity of the EPR amplitudes at around 2 μM. Therefore, the dramatic step of the EPR amplitude of the center line (h_0) in Fig. 5 is very likely caused by the dimerization of NPY. [TOAC^{34}]-NPY was used for dimerization studies because the TOAC label on the 34th position is located in the amphipathic α-helix of NPY, which is known as the dimerization interface of this peptide [31]. In agreement with other measurements, the critical dimerization concentration of NPY was determined to be ~2 μM [7].

3.4. NPY membrane partition coefficients

Dimerization of peptides always complicates the determination of partition coefficients in a peptide lipid system resulting in non reproducible (EPR) signal amplitudes. Using a concentration of the peptide below the critical dimerization concentration and increasing the signal to noise ratio by accumulation is the most simple way to avoid this problem. Pure PS-LUVs were used to determine the molar partition coefficients K of NPY (Fig. 6) to detect the interaction of the peptide at maximum binding. In particular at low pH, the interaction of NPY with mixed PC/PS membranes is too weak to obtain reproducible data. The amplitude of the central line (h_0) is linked to the concentration of free NPY in solution. By titrating liposomes to the [TOAC^{34}]-NPY buffer solution, the amplitude of the central line (h_0) is reduced because NPY binds to the lipid membrane. Because of reduced mobility of the bound peptide, the full width at half maximum of the h_0 peak is increased. This broadening causes a decrease of the intensity because the integral of the peak has to remain constant. The lipid bound component of NPY is not further detectable because the EPR signal amplitude of the bound (slowly tumbling) peptide falls below the noise level in this concentration range. Accordingly, the binding of the peptide to the vesicles is only linked to the reduction of the EPR signal amplitude. The fraction of the bound peptide f_b was calculated from $f_b=(I_0-I)/I_0$, where I_0 is the amplitude of the central line (h_0) of the EPR signal of 1 μM [TOAC^{34}] - NPY in the absence of lipid. The molar partition coefficient was calculated from these data using $f_b=(K\,[L])/(1+K[L])$ [32]. Here, $[L]$ is the accessible lipid concentration. For different pH values, the molar partition coefficients have the following values for $K=(7000\pm1000)$ M^{-1} (pH 5), $K=(2700\pm500)$ M^{-1} (pH 7.4), and $K=(800\pm100)$ M^{-1} (pH 9). The scatter in the data shown in Fig. 6 is a result of the low peptide concentration of 1 μM, which was used to avoid dimerization of NPY.

3.5. Determination of the peptide penetration depth by ^{1}H NMR MAS

To investigate the membrane interaction of the peptide attached TOAC labels, a complementary NMR method was used. In the presence of paramagnetic spin probes, the nuclear relaxation rates are increased. For instance, if the peptide attached paramagnetic TOAC spin label penetrates the lipid membrane, an increase in the phospholipid relaxation rates can be observed. Even for membranes with completely immobilized peptides well resolved ^{1}H NMR spectra of the phospholipids can be obtained under MAS. Fig. 7 shows representative 600 MHz ^{1}H MAS NMR spectra of POPC/POPS membranes (80/20, mol/mol) in the

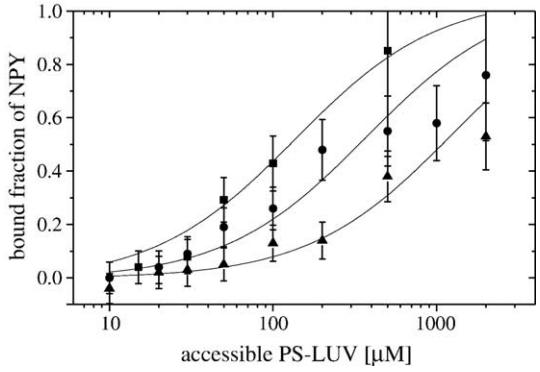

Fig. 6. Binding of 1 μM [TOAC^{34}]-NPY to PS LUV at varying pH values as a function of lipid concentration. The lines represent best fits to the model described in the text using the following molar partition coefficients: $K_p=(7000\pm1000)$ M^{-1} (■ pH 5), $K_p=(2700\pm500)$ M^{-1} (● pH 7.4), and $K_p=(800\pm100)$ M^{-1} (▲ pH 9). All molar partition coefficients were measured at the temperature of 37 °C.

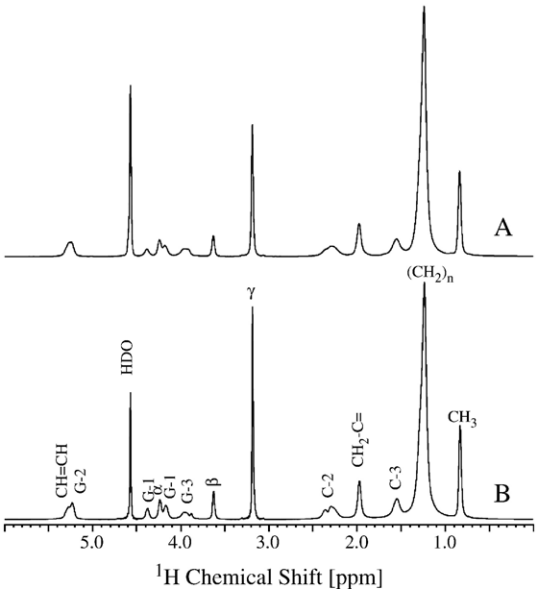

Fig. 7. 600.1 MHz ^{1}H MAS NMR spectra of POPC/POPS membranes (80/20, mol/mol) in the absence (A) and in the presence of [Ala^{31}, TOAC^{32}]-NPY (B). The NMR spectra were obtained at a water content of 70 wt.%, a temperature of 30 °C, and a MAS rotational frequency of 8 kHz. Assignments of the lipid peaks are given.

absence and in the presence of [Ala^{31}, TOAC^{32}]-NPY. In the presence of 1 mol% spin labeled NPY, the NMR signals of the phospholipids are only slightly broadened. However, the influence of the TOAC label of NPY on phospholipid relaxation is significant.

From measurements of phospholipid relaxation in the presence and absence of the spin labeled peptide, the paramagnetic (i.e., peptide induced) contribution to the relaxation can be determined. This ^{1}H NMR paramagnetic relaxation rate is a measure of the interaction between the TOAC label and the lipid segments [28]. Since the relaxation rate can be determined for each resolved lipid segment, this experiment allows to identify the lipid segments in closest proximity to the spin label of the peptide because these segments will exhibit the largest paramagnetic contribution to the relaxation rate.

In the presence of TOAC labeled NPY mutants, the T_1 relaxation time of the phospholipids is shortened translating into an increased relaxation rate. A plot of the paramagnetic relaxation rate as a function of the position of a lipid segment parallel to the membrane normal allows to identify the lipid segments in closest proximity to the respective spin probe. These segments have the strongest interaction with the peptide and define the average position of a TOAC label in the membrane.

Significant paramagnetic relaxation rates are observed for all lipid segments in the presence of the NPY mutants. This is a result of the high mobility of the lipid segments in the liquid crystalline membrane [33,34] and also the membrane bound peptide. For all three peptides, the labels exhibit a broad distribution around their average membrane localization. However, the comparison of the maxima of the distribution functions shows, consistent with the EPR data, that the helical peptide segment ([Ala^{31}, TOAC^{32}]-NPY and [TOAC^{34}]-NPY), Fig. 8B, C) is more deeply inserted in the phospholipid membrane than the N-terminus ([TOAC^{2}]-NPY Fig. 8A). For the helical amino acids of NPY, a localization in the upper chain/glycerol region is obtained, while for the N-terminal part of the molecule a headgroup/glycerol localization is concluded. The localization of the mobile N-terminus of NPY in this measurement might be biased towards the membrane because of the restricted peptide motions possible in

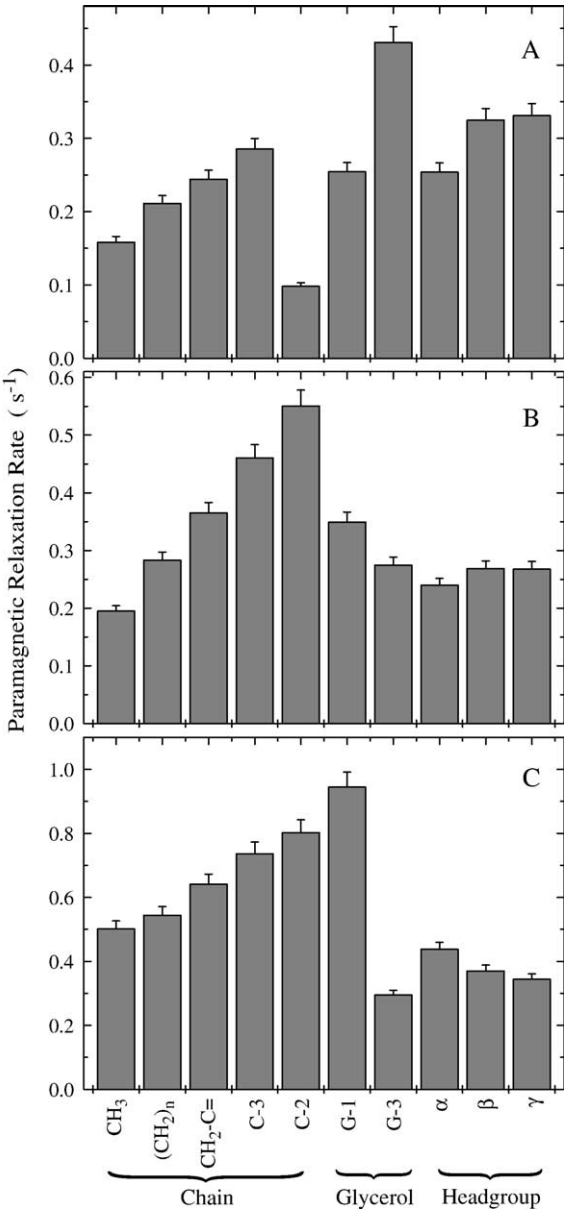

Fig. 8. Paramagnetic relaxation rates of the phospholipid segments in the presence of (A) [TOAC^{2}]-NPY, (B) [Ala^{31},TOAC^{32}]-NPY, and (C) [TOAC^{34}]-NPY. Paramagnetic relaxation rates were determined from T_1 relaxation time measurements. The NPY/POPC/POPS molar ratio was 1:80:20. All measurements were carried out at a water content of 70 wt.% and a temperature of 30 °C.

multilamellar vesicles, where the opposing membrane may restrict the mobility of N-terminus of NPY.

4. Discussion

The major focus of this study was the determination of the localization of TOAC labeled NPY peptides in negatively charged phospholipid membranes. All our results clearly show that the α-helical C-terminal part of the peptide is inserted in a negatively charged phospholipid membrane at the upper chain/glycerol region, also referred to as the lipid water interface of the membrane [34]. This is in agreement with recent X-ray studies of the localization of amphipathic α-helices in lipid membranes [35,36]. The NMR data show a small difference between [Ala^{31}, TOAC^{32}]- and [TOAC^{34}]-NPY suggesting that the TOAC label at the 32nd position of the peptide is slightly deeper inserted than the TOAC label at the 34th position. These differences are also observable from the EPR data using vesicles, which are composed of PS/PC (molar ratio 1:2). This could be related to the helical structure of the C-terminal part of NPY. The EPR results of NPY binding to PS vesicles, however, indicate that the 34th position is slightly deeper inserted in the phospholipid membrane than the 32nd residue of the peptide.

A transmembrane orientation of a single NPY amphipathic α-helix can be excluded because of the high energy penalty for inserting charged amino acid residues into the hydrophobic core of the membrane due to the Born repulsion. However, the formation of a multimeric pore state of transmembrane NPY peptides should be considered. Such a scenario would induce high amounts of vesicle leakage, which has not been observed in our fluorescence measurements (data not shown).Therefore, all transmembrane orientations of NPY can be excluded on the basis of our results.

The structural model resulting from these findings is depicted in a cartoon shown in Fig. 9. The idealized helical wheel is inserted into the lipid membrane with the hydrophilic side facing the aqueous phase and the hydrophobic side inserts into the membrane. The two TOAC labels at positions 32 and 34 are located right at the borderline between the hydrophilic and hydrophobic side of the helix. To place them both in the upper part of the hydrocarbon chains, the amphipathic α-helix is slightly twisted with respect to the membrane normal. The charged residues are localized in the headgroup/glycerol region of the membrane and the hydrophobic residues are buried in the hydrocarbon chain region. Although this cartoon tempts one to imagine a rather rigid structural arrangement, our data suggest a more dynamic distribution of the NPY helix in the membrane. This is motivated by the broad distribution functions obtained for the TOAC label in the liquid-crystalline membrane revealed from the NMR measurement (Fig. 8). Further, the EPR spectra of [Ala^{31}, TOAC^{32}]- and [TOAC^{34}]-NPY are indicative of slowly tumbling immobilized peptides. The N-terminus of the peptide may move freely in a cone undergoing large amplitude motions with short correlation times. The EPR spectra of membrane bound [TOAC^{2}]-NPY show a broader line width then the free peptides. Therefore, the N-terminus of NPY tumbles with the entire vesicle but undergoes further large amplitude reorientation that averages out the anisotropic line features observed for the helical part of bound NPY.

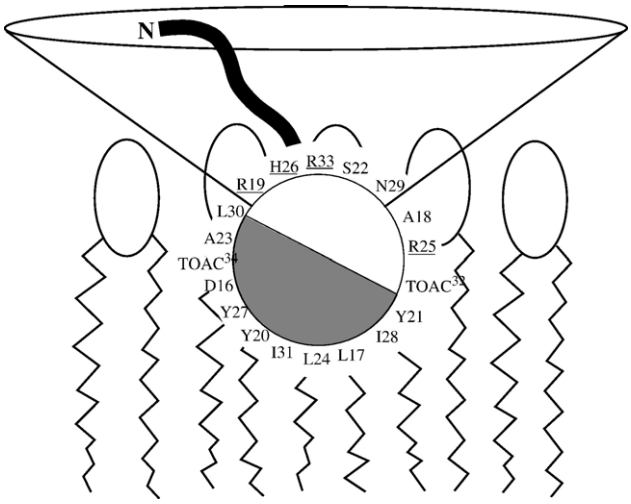

Fig. 9. The cartoon summarizes the structural model derived from the EPR and NMR data of this study. An idealized helical wheel plot of the amino acids 16–34 of NPY is shown. The hydrophobic side of NPY (gray) faces the hydrocarbon chain region and the hydrophilic side is localized in the headgroup/glycerol region of the membrane. The positively charged amino acid residues are underscored. The N-terminus of the peptide is unstructured undergoing large amplitude motions with short correlation times and occasional contacts with the membrane.

From the NMR results, interactions of the N-terminus with the headgroup region can be concluded. This may in part be the result of the spatial constraints imposed to the sample in multilamellar vesicles. On the other hand, this indicates the high mobility of the N-terminus that includes interactions with the lipid headgroups as well as large amplitude motions in the aqueous phase.

Of course, these structural implications are based on the assumption that the structure of membrane-associated NPY is similar to the structure of micelle bound NPY. This seems to be a reasonable assumption at least for the helical part of NPY since α-helices are typical well-conserved structural motives, which require a lot of energy to be denatured. The N-terminus has been found to be unstructured in the solution NMR studies [7] and does not show more structure in the phospholipid membrane.

Further, our results support the model that the binding of NPY to its receptor occurs in two steps [6]. First NPY binds to the membrane as a monomer using the same hydrophobic face of the α-helix as for dimerization to the phospholipid membrane. Second, it diffuses over the membrane surface and finds its receptor [5]. The advantage of such a membrane mediated receptor binding is in the reduced dimensionality. By the membrane binding, the diffusional

motions are restricted to two dimensions, which effectively increases the peptide concentration by a factor of ~1000 and thus increases the probability of the ligand binding to the receptor [37].

The binding of NPY to zwitterionic phospholipid and cellular membranes was determined by McLean et al. [38]. These authors have used a very high peptide/lipid ratio for leakage measurements. Our measurements demonstrate the influence of negative charge of the phospholipid membrane on the binding ability of NPY. The binding of NPY to negatively charged vesicles is observable even at much lower peptide/lipid ratios compared to zwitterionic phospholipid membranes. Thus, NPY binding to negatively charged liposomes is not associated with vesicle leakage (unpublished results).

The thermodynamics of peptide membrane interaction are well understood [39–42]. The increase of the apparent lipid binding constant K caused by electrostatic interactions between the basic amino acids (R^{19}, R^{25}, H^{26}, R^{33}) and the acidic phospholipids can be expressed as $K=K_0 \exp(zF\Phi/RT)$, where K_0 is the intrinsic binding constant to zwitterionic phospholipid membranes, z the effective charge of the peptide, Φ the membrane surface potential, F the faraday constant, and RT the thermal energy. Using vesicles composed of PS instead of PC the binding of NPY to the membrane is much increased. K_0 can be easily determined using $K_0=\exp(\Delta G_0/RT)$, where ΔG_0 is the energy of the hydrophobic interaction. Because our results show that NPY is located in the membrane interface we used the Whimley and White hydrophobic interface scale to determine ΔG_0 [43]. The sum of the each single amino acids of NPY at pH 6 results in ΔG_0= (9.7±2.5) kcal/mol, which is highly unfavorable. The depolarization of the peptide bound in the helical part of NPY (13–36) leads to a reduction of ΔG_0 by 0.4 kcal/mol per residue, which is assembled into a α-helix [44]. Therefore, the hydrophobic contribution to membrane binding of NPY amounts to approximately ΔG_0=(0.5±3.0) kcal/mol and is consistent with the very weak interaction of the peptide with zwitterionic phospholipid surfaces. Therefore, it can be concluded that the electrostatic interactions play the decisive role in peptide binding to the membrane.

The amount of membrane bound NPY can be measured by EPR techniques. The determination of free and membrane bound NPY is carried out by the deconvolution of the EPR spectra and does not require manipulations of the sample (e.g., centrifugation). This is a clear advantage of the EPR method compared to measurements, which require a separation of bound and free peptide. The calculated binding constants are dependent on the pH of the solution, caused by the change of the peptide overall charge. The binding constants at different pH should have the same relation to each other like the term $\exp(zF\Phi/RT)$, which describes the electrostatic effects. However, the calculation of this term is quite difficult because the successive binding of the positively charged peptides to negatively charged membranes reduces the membrane potential of the vesicles. The possible reduction depends on the effective charge of the peptide and molar ratio between peptide and lipid at the saturation point of the binding. Further, the effective charge of the peptide is not known. The reduction of the membrane potential can be determined using the experimentally determined binding constants. At pH 6 and a ratio of 1:100 of peptide/lipid, the reduction of the membrane potential will be about 20% in comparison to the membrane potential of PS-LUV in absence of peptide.

The positive net charge of NPY increases by decreasing the pH value. This leads to a stronger electrostatic interaction of the peptide and the vesicles and causes an increase of the apparent binding constants at low pH. The isoelectric point of NPY is 7.4. The net charge of NPY at pH 9 is about −1e and would cause a weak electrostatic repulsion of the peptide and the vesicle. The analysis of the contribution of the charge of NPY shows that most positively charged amino acids are located in the helical part, whereas most negatively charged residues are located in the loop. This further supports the binding model concluded from our study.

In summary, the techniques applied in this study support a model of NPY binding to negatively charged membranes, where the structured α-helical part of the molecules (residues 13–36) inserts to the lipid water interface of the membrane and the unstructured N-terminus extends into the aqueous phase with occasional contacts with the lipid headgroups.

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