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ORIGINAL PAPER

The interaction of neuropeptide Y with negatively charged and zwitterionic phospholipid membranes

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Abstract The interaction of the 36 amino acid neuropeptide Y (NPY) with liposomes was studied using the intrinsic tyrosine fluorescence of NPY and an NPY fragment comprising amino acids 18–36. The vesicular membranes were composed of phosphatidylcholine and phosphatidylserine at varying mixing ratios. From the experimentally measured binding curves, the standard Gibbs free energy for the peptide transfer from aqueous solution to the lipid membrane was calculated to be around −30 kJ/mol for membrane mixtures containing physiological amounts of acidic lipids at pH 5. The effective charge of the peptide depends on the pH of the buffer and is about half of its theoretical net charge. The results were confirmed using the fluorescence of the NPY analogue [Trp^{32}]-NPY. Further, the position of NPY's α-helix in the membrane was estimated from the intrinsic tyrosine fluorescence of NPY, from quenching experiments with spin-labelled phospholipids using [Trp^{32}]-NPY, and from ^{1}H magic-angle spinning NMR relaxation measurements using spin-labelled [Ala^{31}, TOAC^{32}]-NPY. The results suggest that the immersion depth of NPY into the membrane is triggered by the membrane composition. The α-helix of NPY is located in the upper chain region of zwitterionic membranes but its position is shifted to the glycerol region in negatively charged membranes. For membranes composed of phosphatidylcholine and phosphatidylserine, an intermediate position of the α-helix is observed.

Keywords Liposomes · TOAC · ^{1}H MAS NMR · Peptide–lipid interaction · Membrane partitioning · GPCR

Abbreviations

5-doxyl-PC	1-Palmitoyl-2-stearoyl-(5-doxyl)-*sn*-glycero-3-phosphocholine
10-doxyl-PC	1-Palmitoyl-2-stearoyl-(10-doxyl)-*sn*-glycero-3-phosphocholine
16-doxyl-PC	1-Palmitoyl-2-stearoyl-(16-doxyl)-*sn*-glycero-3-phosphocholine
AMPSO	3-[(1,1-Dimethyl-2-hydroxy-ethyl)amino]-2-hydroxy-propanesulfonic acid
LUV	Large unilamellar vesicles
MAS	Magic-angle spinning
MES	2-[*N*-Morpholino]ethanesulfonic acid
MLV	Multilamellar vesicles
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

In memoriam Dr. Olaf Zschörnig (1958–2009).

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Tempo-PC	1,2-Dioleoyl-*sn*-glycero-3-phospho-(TEMPO)-choline
TOAC	2,2,6,6-Tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid

Introduction

The neurotransmitter neuropeptide Y (NPY) is a 36 amino acid peptide that is widely distributed in the peripheral and the central nervous system (Heilig and Widerlov 1990). NPY contains five tyrosine residues ("Y") and is C-terminally amidated. It was first isolated from porcine brain in 1982 (Tatemoto 1982). NPY is found in all mammals as well as in a wide variety of other animal species including birds, reptiles, amphibians, and fish. Together with pancreatic polypeptide and peptide YY, it forms a highly conserved family, which is named PP family or more recently NPY family (Sundström et al. 2008).

NPY acts both as a regulator of hormone secretion and as a neurotransmitter (White 1993). The effects of NPY are initiated by the Y receptors, which belong to the family of heptahelical G protein-coupled receptors (GPCR) (Ingenhoven and Beck-Sickinger 1999). The regulation of blood pressure and food uptake, which can lead to fat deposition and obesity, is also transmitted by NPY (White 1993). Further, the control of the metabolism and the involvement in cardiovascular regulation, as well as in the promotion of some remarkable aspects of tumour progression, including cell proliferation, matrix invasion, metastatization, and angiogenesis are important functions of NPY (Korner and Reubi 2007; Ruscica et al. 2007). It potentiates the effects of other vasoactive neurotransmitters and vasoconstrictors, and inhibits vasodilators in the sympathetic nervous system. Furthermore, NPY plays an important role in the immune system (Wheway et al. 2007).

NPY is found in the extracellular space, from where it can bind to its GPCR with high affinity. Receptor binding can occur directly from solution or after earlier association with the plasma membrane. Usually, the accessible membrane area is significantly larger than the accessible area for direct receptor binding of the peptide. Therefore, initial membrane binding could be preferred as long as this step is energetically favourable. Consequently, it has been suggested that binding of NPY to the phospholipid membrane is an important prerequisite for its interaction with the receptor (Bettio et al. 2002; Grandt et al. 1994). Binding of NPY to membrane surfaces appears to take place as a monomer, as shown by Bettio et al. (2002). After binding, the peptide diffuses two dimensionally over the membrane surface to the receptor, which drastically increases its local concentration and the probability to find the receptor. Such two-step models have been discussed for neurohormones like NPY in the literature for more than 20 years (Sargent and Schwyzer 1986; Schwyzer 1986, 1992, 1995; White and Wiener 1996). For a more quantitative interpretation of such a two-step model for NPY detailed knowledge about the thermodynamics of NPY binding to zwitterionic and charged membranes is required.

NMR data in aqueous solution at low pH suggest that NPY consists of a C-terminal amphipathic α-helix, which includes residues 13–36. The flanking helical regions and the rest of the peptide have been found to be rather flexible. In particular, the N-terminal segment 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 (Bader et al. 2001; Monks et al. 1996).

It was already shown that the binding efficiency of NPY to lipid membranes is strongly increased in the presence of negatively charged phospholipids. This increase in membrane binding strength is caused by an additional electrostatic interaction between the positively charged amino acids of the peptide and the negatively charged lipids at acidic pH. NPY contains basic residues and has a theoretical net charge of $+1.5e$ at pH 5 and $+0.2e$ at pH 7. The non-integer charge values result from the summation of the pH-dependent charges for each single amino acid of NPY considering the pK_a value of each charged group and the external pH. Further, the α-helix of membrane-bound NPY is located in the membrane–water interface, whereas the N-terminal part of the peptide sticks out from the membrane and is highly flexible (Thomas et al. 2005).

Detergent micelles are widely used as a model to study the interaction of peptides with membrane surfaces (Sanders and Oxenoid 2000). However, micelles comprise an extremely high curvature and represent highly dynamic entities with fast exchange between monomeric and micelle bound detergents. Therefore, micelles fail to reproduce the important properties of the lipid water interface of bilayer membranes (Henry and Sykes 1994). In contrast, liposomes are much better membrane models, since they are composed of true phospholipid bilayers although their applicability to solution NMR studies is rather limited.

Fluorescence techniques are well established to study lipid–peptide interactions. This method has been very successfully applied to build models of the interaction of peptides and proteins with phospholipid membranes (Dempsey 1990). Further, the membrane partitioning of peptides can be easily observed from the fluorescence spectrum (Ladokhin et al. 2000). Solid-state NMR provides a very promising technique to obtain peptide structures and models of the peptide–membrane interaction of such

systems (Huster 2005; Torres et al. 2003). 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.

In this paper, we have studied the binding and localization of NPY and a C-terminal NPY fragment comprising residues 18–36 in different lipid environments by fluorescence and NMR techniques combining the various advantages of each biophysical method. The tyrosine fluorescence of NPY represents a non-invasive probe to study the interaction of the peptide with model membranes without requiring any peptide modification (McLean et al. 1990). The intrinsic fluorescence of NPY and the NPY fragment (18–36) was measured at different lipid peptide ratios to obtain binding curves. The hydrophobic binding energy of NPY was calculated from these curves for NPY, the NPY fragment (18–36) and the NPY analogues [D-Trp^{32}]-NPY and [Ala^{31}, TOAC^{32}]-NPY. The position of NPY's α-helix in the membrane-bound state was investigated by fluorescence quenching techniques for membranes composed of varying mixing ratios of zwitterionic phosphatidylcholine (PC) and phosphatidylserine (PS). These results were confirmed by ^{1}H MAS paramagnetic relaxation enhancement measurements using [Ala^{31}, TOAC^{32}]-NPY.

We have previously shown that NPY binds to negatively charged membranes at pH 7.4, although its theoretical net charge is around 0 (Thomas et al. 2005). In contrast, the binding of NPY to zwitterionic membranes is negligible at pH 7.4. In order to understand the interesting physicochemistry of NPY binding to membranes and to see sizable electrostatic effects at lower PS concentrations, we decided to carry out our study at a somewhat unphysiological pH of 5. Our results may provide a quantitative basis for the discussion of the two-step model for the receptor binding of NPY.

Materials and methods

Materials

Human NPY, the NPY fragment (18–36) and [D-Trp^{32}]-NPY were purchased from Bachem AG (Bubendorf, Switzerland). The amino acid sequence of NPY is Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Met-*Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH$_2$* (the NPY fragment (18–36) is italics). The TOAC-labelled NPY analogue [Ala^{31}, TOAC^{32}]-NPY was synthesized by solid-phase synthesis as reported in detail in literature (Bettio et al. 2002). 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), 1-palmitoyl-2-stearoyl-(10-doxyl)-*sn*-glycero-3-phosphocholine (10-doxyl-PC), 1-palmitoyl-2-stearoyl-(16-doxyl)-*sn*-glycero-3-phosphocholine (16-doxyl-PC) and 1,2-dioleoyl-*sn*-glycero-3-phospho-(TEMPO)-choline (Tempo-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 at pH 5 in the presence of 100 mM NaCl.

Sample preparation

Large unilamellar vesicles (LUV) were prepared by extrusion according to standard procedures (Hope et al. 1985). In brief, aliquots of lipids were dissolved in chloroform, which was subsequently evaporated under high vacuum. The lipid film was resuspended in a defined buffer solution followed by vortexing. After ten freeze–thaw cycles, the lipid buffer solution was extruded trough two stacked polycarbonate filters of 100 nm pore size in a thermostated extruder (LIPEX Biomembranes, Vancouver, BC, Canada). The final lipid concentration after extrusion was determined by a phosphate determination (Chen et al. 1956).

The peptides [D-Trp^{32}]-NPY, native NPY and NPY fragment (18–36) were dissolved in water to yield stock solutions of 1 mM, which were further diluted to obtain the desired concentration. The prepared LUV and solved peptides were mixed in the desired ratio in quartz cuvettes, which were used for the fluorescence measurements.

For ^{1}H MAS NMR measurements, the NPY analogue [Ala^{31}, TOAC^{32}]-NPY was mixed in chloroform with the desired molar ratio of POPC and POPS. After evaporating the chloroform, 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 high-resolution MAS rotors with spherical Kel-F inserts providing a spherical volume of ~15 µl.

Fluorescence measurements

The fluorescence spectra were acquired on a Fluoromax 2 spectrometer (Jobin Yvon, Edison NJ, USA). An excitation wavelength of 278 nm for tyrosine and 290 nm for tryptophan was used, respectively. The fluorescence emission was observed between 300 and 400 nm for tyrosine and

between 310 and 450 nm for tryptophan. Binding data for NPY to various membranes were acquired as follows: aliquots of liposomes were titrated to a peptide solution with a concentration of 3.75–15 μM to achieve the desired lipid–peptide molar ratio. The lipid–peptide ratio was increased by adding more liposomes to the lipid–peptide solution. The spectra of tryptophan fluorescence could be measured without accumulation. Five spectra of tyrosine fluorescence were accumulated for a reasonable signal-to-noise ratio. Additionally, fluorescence spectra of each liposome concentration were acquired in the absence of peptide. These background spectra were subtracted from the original spectra to minimize the effect of light scattering in the presence of LUV. The background corrected fluorescence intensities at 304 nm for tyrosine and at 342 nm for tryptophan were used for the calculation of membrane-bound fraction of peptide according to (Ladokhin et al. 2000):

$$f_b = \frac{I - I_0}{I_\infty - I_0}, \tag{1}$$

where f_b is the membrane-bound fraction of the peptide, I the fluorescence intensity of the peptide at specific lipid peptide ratio, I_0 the fluorescence intensity in the absence of lipid and I_∞ is the fluorescence intensity that corresponds to complete binding of all peptides to the membrane. The value I_∞ is unknown and has to be determined from the fit of the data.

^{1}H MAS NMR measurements

^{1}H MAS NMR spectra were acquired on a Bruker DRX600 NMR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) operating at a resonance frequency of 600.1 MHz for ^{1}H using a 4 mm high-resolution MAS probe at a MAS frequency of 8 kHz and a spectrum width of 10 kHz. Typical $\pi/2$ pulse length was 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 [Ala^{31}, TOAC^{32}]-NPY, 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 with native NPY (no spin label present), $R_{1,p}$ can be easily determined from a relaxation measurement in the presence of [Ala^{31}, TOAC^{32}]-NPY for each molecular segment of the phospholipids (Brulet and McConnell 1975; Polnaszek and Bryant 1984; Thomas et al. 2005; Vega and Fiat 1976; Vogel et al. 2003).

Binding model

The standard Gibbs free energy of transfer of NPY from aqueous solution to the membrane can be determined directly from the binding curve (Ladokhin et al. 2000) using:

$$I([L]) = 1 + (I_\infty - 1)\frac{k_{obs}[L]}{[W] + k_{obs}[L]}. \tag{2}$$

[L] and [W] are the concentrations of lipid and water, respectively.

The Boltzmann equation then yields:

$$\Delta G^\circ_{obs} = -k_B T \ln k_{obs}, \tag{3}$$

in which k_{obs} is the experimentally observed water-to-membrane partition coefficient. These simple equations provide a reasonable and reliable model to calculate the Gibbs free energy of peptide portioning into a membrane, which we refer to as Model I.

If one further seeks to separate the hydrophobic from the electrostatic contributions to the Gibbs free energy, ΔG°_{obs} can be decomposed into

$$\Delta G^\circ_{obs} = \Delta G^\circ_H + \Delta G^\circ_E. \tag{4}$$

The potential problems of separating coulombic from hydrophobic effects are well known and have extensively been discussed (Ladokhin and White 2001). Typically, coulombic and hydrophobic interactions are strongly coupled and the thermodynamic measurement cannot account for electrostatics by means of the Gouy-Chapman theory. Nevertheless, it is instructive to follow this approach in order to obtain an estimate on the various contributions to the free energy of binding. Using $\Delta G^\circ_E = z_{eff} F \Psi$ and $k_H = -k_B T \ln \Delta G^\circ_H$ in Eq. 4, the well-known equation for water-to-membrane partition coefficient follows (Thorgeirsson et al. 1995):

$$k_{obs} = k_H \exp(-z_{eff} e_0 \Psi / k_B T), \tag{5}$$

in which k_B and T are the Boltzmann constant and the absolute temperature, respectively. F is the Faraday constant, e_0 the elementary charge, Ψ the surface potential of a charged lipid membrane and z_{eff} describes the effective charge of the peptide.

It can be easily realized that the observed water-to-membrane partition coefficient k_{obs} will be increased in the presence of negatively charged lipids and positively charged peptides. The surface potential Ψ can be calculated according to the Gouy-Chapman theory:

$$\sigma = \sqrt{8,000 N_A \varepsilon \varepsilon_0 k_B T c_{NaCl}} \sinh \frac{e_0 \Psi}{2 k_B T}. \tag{6}$$

Using a NaCl concentration c_{NaCl} of 100 mM, the surface charge density σ of a pure PS membrane is calculated to be around -0.2 C/m^2.

In aqueous buffer, the surface charge density is reduced by the adsorption of Na^+ and H^+ ions from solution and by binding of peptides to the membrane surface. This reduction of the surface charge density can be calculated from (Murray et al. 1999):

$$\sigma = \sigma_0 \left(1 + \frac{z_{\text{eff}}[\text{p}] e_0 f_\text{b} \exp(e_0 \Psi / k_\text{B} T)}{A_\text{Lipid}[\text{L}]}\right) \times \frac{1}{[1 + K_\text{Na} \exp(e_0 \Psi / k_\text{B} T)][1 + K_\text{P} \exp(e_0 \Psi / k_\text{B} T)]}, \quad (7)$$

where A_Lipid is the required surface area of one lipid molecule. σ_0 is the surface charge density in the absence of peptides and ions in solution. $K_\text{Na} = 1\ \text{M}^{-1}$ and $K_\text{P} = 10^4\ \text{M}^{-1}$ are the binding constants of the Na^+ ions and protons to the phospholipid membrane, respectively.

A straightforward way to obtain the water-to-membrane transfer energy $\Delta G^\circ_\text{obs}$ is to measure a peptide–membrane binding curve. This means that the membrane-bound fraction of peptide f_b is determined from the fluorescence intensities (Eq. 1) at different lipid concentrations. Using this data, the binding constant of the water-to-membrane transfer, which is connected to the partition coefficient by $k_\text{obs} = K_\text{obs} \times 55.5\ \text{M}$, can be determined from (Murray et al. 1999):

$$f_\text{b} = \frac{K_\text{obs} \gamma [\text{L}]}{1 + K_\text{obs} \gamma\ [\text{L}]}, \quad (8)$$

in which the lipid accessibility value γ was set to 0.5 because peptide can only bind to the lipids of the outer membrane leaflet. The observed partition coefficient k_obs in Eq. 5 (and also the binding constant K_obs) can depend on the lipid-to-peptide ratio. This effect is caused by the saturation of the negative charge of the lipids (PS) by the binding of positively charged peptides at small lipid–peptide ratios, which results in a reduction of the surface potential Ψ and a decrease of the observed electrostatic free energy ΔG°_E. The reduction of the surface potential Ψ can be calculated from the reduction of the surface charge density (Eq. 7).

Of course, a number of non-coulombic effects (Arbuzova et al. 2000) can influence the observed Gibbs free energy $\Delta G^\circ_\text{obs}$ at small lipid–peptide ratios, especially when lytic peptides are considered. Since NPY is not a lytic peptide, the influence of non-coulombic effects was neglected in our model. The membrane-bound fraction of peptide f_b can be calculated from Eqs. 8 and 5 to yield:

$$f_\text{b} = \frac{K_\text{H} \exp(-z_\text{eff} e_0 \Psi / k_\text{B} T) \gamma [\text{L}]}{1 + K_\text{H} \exp(-z_\text{eff} e_0 \Psi / k_\text{B} T) \gamma [\text{L}]}. \quad (9)$$

From Eq. 6, it will be possible to calculate the surface potential in the presence of the membrane-bound peptide and ions. Together with Eq. 9, we finally get a system of two equations, in which only the surface potential Ψ and the membrane-bound fraction of peptide f_b are unknown. This system of equations can be solved for the different lipid concentrations. We refer to this theory as Model II. The theoretically calculated binding curve can be translated into fluorescence intensity by using Eq. 1. The values I_∞, z_eff and K_H were fitted in order to get the best agreement with the experimental data. Because the observed fluorescence intensity is decreased for high lipid concentrations (0.75 mM) due to the light scattering of the liposomes, the value $1 - s[\text{L}]$ (s scattering parameter, [L] lipid concentration) was multiplied with the theoretically calculated fluorescence intensity. The scattering parameter was calculated to 0.2 mM^{-1} for all measured binding curves (the observed fluorescence intensity is reduced by 20% at a lipid concentration of 1 mM).

Results

Binding of NPY to zwitterionic and negatively charged phospholipid membranes

The background corrected tryptophan fluorescence spectra of [D-Trp^{32}]-NPY at varying lipid–peptide ratios at pH 5 are shown in Fig. 1a. The liposomes were composed of a 1:1 mixture of PS and PC. The fluorescence intensities at 342 nm for different lipid peptide ratios are shown in Fig. 1b. From this data, a peptide partition coefficient of $k_\text{obs} = 1.9 \times 10^6$ was calculated using Eq. 2, which means that the free energy of peptide transfer to the membrane $\Delta G^\circ_\text{obs}$ is −37 kJ/mol (Model I). The dotted line represents the fit of the experimental data using this model.

The solid line represents the best fit of the data using Model II. The hydrophobic binding energy ΔG°_H of [D-Trp^{32}]-NPY to the lipid membrane was calculated to be −37 kJ/mol. Due to ion binding, the effective charge of NPY was reduced to 0.8e, which is about 50% of the expected net charge of the peptide at this pH value. The electrostatic binding energy $\Delta G^\circ_\text{E} = z_\text{eff} F \Psi$ can be calculated from the effective charge of the peptide z_eff and surface potential Ψ. It amounts to −5 kJ/mol under the conditions of our experiment. Together, the results obtained from the Gouy-Chapman mass action model (Model II) amount to a Gibbs free energy of transfer of $\Delta G^\circ_\text{obs} = -42$ kJ/mol, which compares rather well to the $\Delta G^\circ_\text{obs} = -37$ kJ/mol, calculated from Model I. The differences are only on the order of the thermal energy (2.5 kJ/mol).

Binding experiments using NPY and the C-terminal NPY fragment (18–36) were carried out to determine the contribution of the N-terminal part of NPY to the membrane interaction. Binding measurements using the

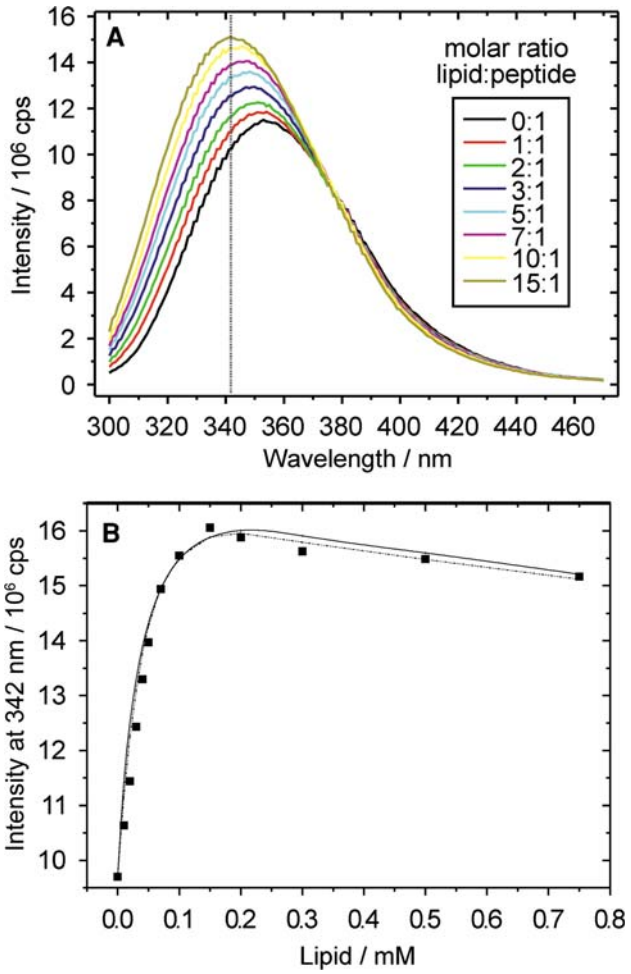

Fig. 1 **a** Fluorescence spectra of [Trp^{32}]-NPY for different lipid-to-peptide molar ratios, acquired at pH 5 and a temperature of 25°C. The used liposomes were composed of a 1:1 PS and PC mixture. **b** The observed bound fraction of peptide was calculated from the fluorescence intensity at 342 nm according to Eq. 1. The *dotted line* describes the fit of the data using Eq. 3 ($\Delta G^{\circ}_{obs} = -37$ kJ/mol) (Model I). The *solid line* represents the best fit using the binding model with parameters $K_H = 18 \times 10^3\ M^{-1}$ and $z_{eff} = 0.9e$ ($\Delta G^{\circ}_{obs} = -42$ kJ/mol) (Model II)

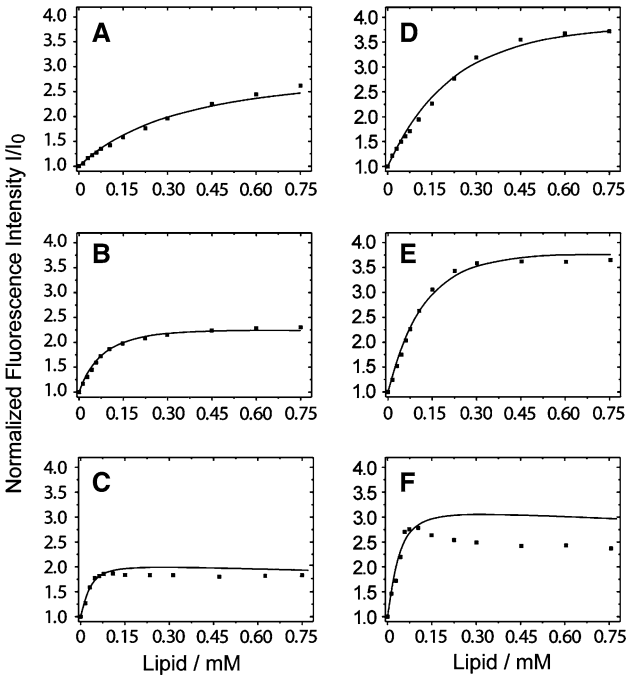

Fig. 2 Normalized intrinsic tyrosine fluorescence intensity of NPY (**a**–**c**) and NPY fragment (18–36) (**d**–**f**) at 304 nm for different lipid peptide ratios, acquired at pH 5 and a temperature of 25°C. The used liposomes were composed of (**a**, **d**) PS/PC (1:3), (**b**, **e**) PS/PC (1:1) and (**c**, **f**) pure PS. The estimated values of z_{eff}, ΔG°_{H} (calculated from k_H) and ΔG°_{obs} are summarized in Tables 1 and 2

N-terminal part of NPY were not possible, because this part itself does not show any membrane activity.

The relative tyrosine fluorescence intensity of NPY and the C-terminal NPY fragment (18–36) at 304 nm (normalized by the fluorescence intensity of the peptides in the absence of lipid) in dependence on the lipid-to-peptide ratio is shown in Fig. 2. The liposomes were composed of a 1:3, 1:1, and a 1:0 molar mixture of PS and PC, respectively. The calculated binding curves for NPY and the NPY fragment indicate that largest total binding energy (sum of hydrophobic and electrostatic) for both peptides is observed for high PS contents, since the peptides are completely bound even at smaller lipid-to-peptide ratios. This effect is caused by the additional electrostatic interaction between the positively charged peptides and the negatively charged phospholipids. The results for the partition coefficient k_{obs} and ΔG°_{obs} calculated according to Model I are shown in Table 1.

Further, the peptide binding curves were analysed by means of Model II; the results for ΔG°_{obs}, ΔG°_{H}, ΔG°_{E}, and z_{eff} are summarized in Table 2. The values for ΔG°_{H} and ΔG°_{obs} indicate that the electrostatic interaction has only a small contribution to the total binding energy ($\Delta G^{\circ}_{obs} = \Delta G^{\circ}_{H} + \Delta G^{\circ}_{E}$) for biologically relevant membrane compositions (fraction of charged lipids on the order of 20%). But due to the long range property of the electrostatic interaction, whereby the peptides are brought in close proximity to the liposomes, ΔG°_{E} still leads to a relevant contribution for peptide binding. Comparing the results of Model I and Model II for ΔG°_{obs} indicates that the differences between the models become even less significant for liposomes, which contain smaller fractions of the charged PS.

The exchange of the hydrophilic threonine in NPY for the hydrophobic tryptophan should result in a slightly larger value for the hydrophobic binding energy ΔG°_{H} for [D-Trp^{32}]-NPY compared to native NPY. However, in our measurements, the hydrophobic binding energy ΔG°_{H} and effective peptide charge of [D-Trp^{32}]-NPY agree with the values of NPY. Perhaps, the hydrophobic energy is used to compensate the energy loss due to the destabilization of

Table 1 Partition coefficients and ΔG^0_{obs} for NPY and the C-terminal NPY fragment (18–36) binding to membranes of varying mixing ratio of PC and PS at a temperature of 25°C calculated according to Model I

Fraction PS	NPY		NPY fragment (18–36)	
	Partition coefficient	ΔG°_{obs} (kJ/mol)	Partition coefficient	ΔG°_{obs} (kJ/mol)
0.00	80×10^3	−28	100×10^3	−29
0.10	100×10^3	−29	470×10^3	−32
0.23	130×10^3	−29	$1{,}900 \times 10^3$	−36
0.37	310×10^3	−31	$4{,}200 \times 10^3$	−38
0.44	$1{,}100 \times 10^3$	−34	$5{,}400 \times 10^3$	−38
0.54	$1{,}900 \times 10^3$	−36	$13{,}100 \times 10^3$	−41
1.00	$2{,}300 \times 10^3$	−36	$17{,}100 \times 10^3$	−41

Table 2 Hydrophobic binding energy ΔG°_H, total binding energy ΔG°_{obs} and the effective peptide charge of NPY and NPY fragment (18–36) in the presence of liposomes composed of varying PS/PC ratios at pH 5 and a temperature of 25°C calculated using Model II

Fraction PS	NPY				NPY fragment (18–36)			
	ΔG°_H (kJ/mol)	ΔG°_E (kJ/mol)	ΔG°_{obs} (kJ/mol)	z_{eff} (e)	ΔG°_H (kJ/mol)	ΔG°_E (kJ/mol)	ΔG°_{obs} (kJ/mol)	z_{eff} (e)
0.00	−28	0	−28	1	−30	0	−30	2.4
0.10	−29	−1	−30	0.9	−30	−6	−36	2.4
0.23	−29	−3	−32	0.9	−31	−7	−39	2.4
0.37	−31	−4	−35	0.9	−33	−10	−43	2.4
0.44	−35	−5	−40	0.9	−35	−10	−45	1.7
0.54	−36	−5	−41	0.8	−36	−11	−47	2.4
1.00	−37	−5	−42	0.8	−38	−9	−47	2.2

The parameters were calculated from the best fit of the experimental binding curves obtained from the intrinsic tyrosine fluorescence of the peptides

NPY's helix by the exchange of the amino acid. The hydrophobic binding energy of the NPY fragment (18–36) is nearly identical to the value of full-length NPY (Table 1), which corresponds to the model, that only helical part of NPY interacts with the membrane (Bettio et al. 2002; Thomas et al. 2005). Therefore, the NPY fragment, which is nearly identical to the α-helical part of NPY, has the same hydrophobic binding energy as NPY. The calculated charge of the NPY fragment (18–36) is larger than for NPY (the negatively charged amino acids of the N terminus are absent), but also about 50% of the expected net charge of NPY fragment (18–36) at this pH value. The interaction of NPY with liposomes composed of pure PS results in a strong aggregation of the liposomes. This effect lowers the quality of the fit dramatically (Fig. 2c, f). The peptide-induced aggregation of the liposomes composed of pure PS was demonstrated by light scattering experiments (data not shown).

Effective charge of NPY at different pH values and varying salt concentrations

The charge status of all amino acids depends on the pH value. Therefore, the charge of NPY also depends on the pH. To study this effect, binding curves of NPY and the C-terminal fragment were observed at three different pH values using liposomes composed of a 1:1 molar mixture of PC and PS. The effective charge of the peptides at the different pH values was calculated from the fit of the binding curves and are shown in Fig. 3. Additionally, the theoretical net charge of NPY (a) and the NPY fragment (b) in dependence of the pH value are given. The net charge of the peptides is reduced by the interaction with ions from the solution. The experimentally observed reduction of the peptide's net charge is about 50% for NPY and 40% for the NPY fragment. Additionally, the effective charge of [D-Trp^{32}]-NPY was calculated from binding curves (data not shown) using varying sodium ion concentrations. The effective charge of the peptide is reduced to $0.1e$ using $c_{NaCl} = 200$ mM and increased to $1.5e$ in the absence of NaCl.

Determination of the penetration depth of [Trp^{32}]-NPY in lipid membranes by fluorescence quenching

Spin probes are able to quench fluorescence over large wavelength ranges (Castanho and Prieto 1995; Chattopadhyay and McNamee 1991). The quenching process

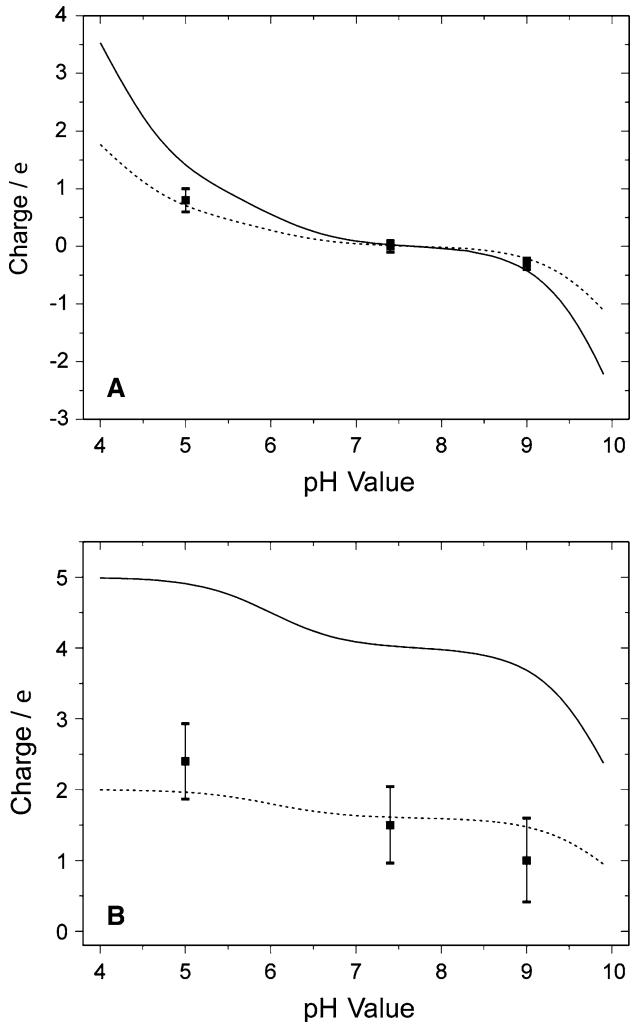

Fig. 3 Experimentally observed effective charge (*filled square*) of NPY (**a**) and the NPY fragment (18–36) (**b**) calculated from the binding curves at different pH values. The used liposomes were composed of a 1:1 mixture of PS and PC. Additionally, the theoretical net charge (*solid line*) is given. The best agreement between experiment and theoretical net charge of the peptides is given for 50% shielding for NPY and a 40% shielding for the fragment, respectively (*dotted lines*)

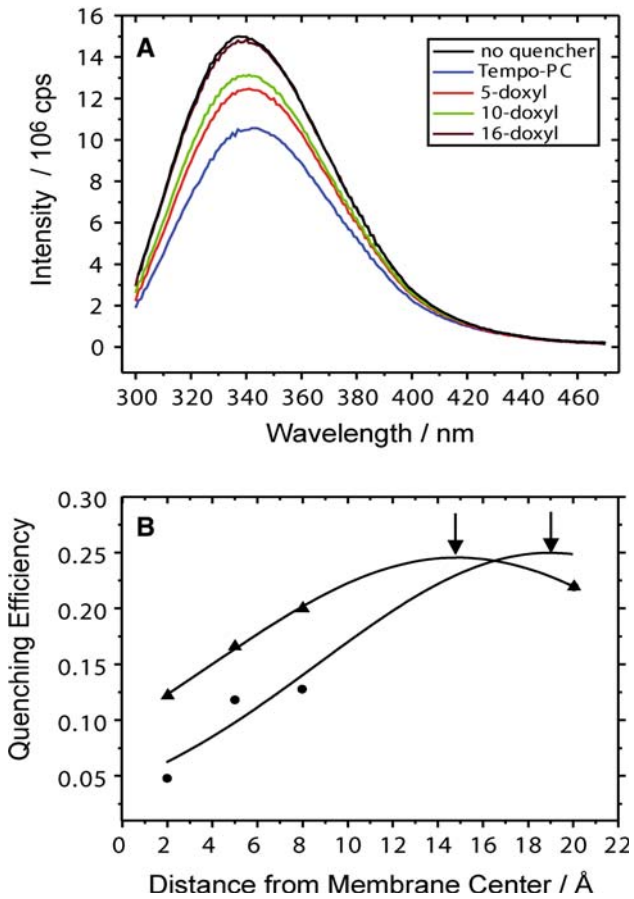

Fig. 4 **a** Background corrected fluorescence spectra of membrane-bound [Trp^{32}]-NPY in the absence and in the presence of spin-labelled phospholipids and a temperature of 25°C. The used liposomes were a mixture of 42:50:8 mol/mol/mol PC:PS:spin-labelled lipid. **b** The quenching efficiency was calculated from the fluorescence intensity (342 nm) in the absence and in the presence of spin-labelled lipid for pH 7.4. The position of the spin label concerning to the membrane normal for Tempo-PC, 5-doxyl-PC, 10-doxyl-PC and 16-doxyl-PC was set to 0, 12, 15 and 18 Å, respectively. These values were estimated from the chemical structure of the lipids. The line is the best Gaussian fit of the data. The used liposomes were composed of 42:50:8 mol/mol/mol (*filled circles*) and of 67:25:8 mol/mol/mol (*filled triangles*) PC/PS/spin-labelled lipid mixture

occurs due to direct contacts between the fluorophore and the spin probe. Therefore, a quenching process will occur for a peptide, which is located in close proximity to the spin probe. Since phospholipid membranes are highly mobile and disordered in the fluid crystalline phase (Huster et al. 1999; White and Wiener 1996; Petrache et al. 1998), there will always be such contacts between membrane-bound peptides and spin probes attached covalently to the lipids. However, the contact probability and therefore the quenching efficiency will decrease by increasing distance between the fluorophore and the spin probe. Since fluid membranes are highly dynamic entities, the distance correlation should rather be expressed in terms of the contact probability.

The phospholipids Tempo-PC, 5-doxyl-PC, 10-doxyl-PC and 16-doxyl-PC were used for the quenching experiments. The spin probes in these phospholipids are located in the headgroup (Tempo-PC) and at the 5th, 10th and 16th position of the phospholipid alkyl chain, respectively. The quenching efficiencies calculated from fluorescence spectra (Fig. 4a) of NPY in the presence of different spin-labelled phospholipids are shown in Fig. 4b. The quenching efficiency q is defined as the difference between the fluorescence intensity of membrane-bound [Trp^{32}]-NPY in the absence I_{ab} and in the presence I_{pre} of spin-labelled phospholipids normalized by the fluorescence intensity in the absence of spin-labelled phospholipids ($q = I_{ab} - I_{pre})/I_{ab}$) (White et al. 1998). It can

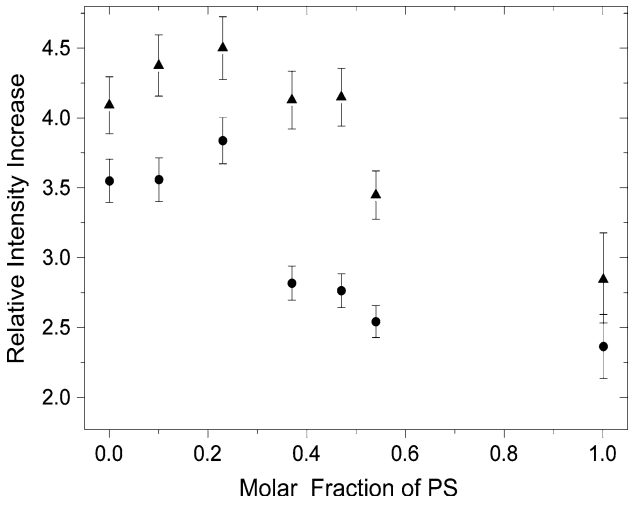

Fig. 5 The ratio I_∞/I_0, which gives a measure of the penetration depth of NPY (*filled circles*) and the NPY fragment (18–36) (*filled triangles*) in the presence of liposomes at varying PS/PC ratios. The fluorescence intensity of the membrane-bound peptide I_∞ was calculated from the fit of the binding curves obtained by the intrinsic tyrosine fluorescence of the peptides. I_0 is the fluorescence intensity of the peptides in solution

be easily realized that the use of Tempo-PC results in the highest quenching efficiency for [Trp^{32}]-NPY. The quenching efficiency is reduced successively for the phospholipids 5-, 10- and 16-doxyl-PC, where the spin probes are located closer to the hydrophobic core of the membrane (Vogel et al. 2003). This is observed for all investigated pH values, but there are no significant differences between pH 5, 7.4, and 9 (data not shown). Accordingly, the most likely position of the peptide is close to the headgroup region of the phospholipids in the membrane–water interface. If the fraction of the charged lipid is reduced to 25%, the quenching efficiency of 5-, 10- and 16-doxyl-PC is increased. From this data, a deeper penetration (about 5–6 Å) of [Trp^{32}]-NPY in weakly charged membranes has to be concluded.

The observed quenching efficiency for membranes composed of 25 mol% PS is about 50% larger than for membranes containing 50 mol% of PS, although the fraction of spin-labelled lipids was the same. This is probably caused by the preferred contact of the NPY analogue with the charged lipids. The reduction of the PS fraction in the membrane leads to an increase of the contact probability with the uncharged spin-labelled lipids. To make the data comparable, the observed quenching efficiency for the weakly charged membranes was normalized to the values for membranes containing 50% PS.

The same experiments were also carried out using the tyrosine fluorescence of native NPY. However, the quenching efficiency for [Trp^{32}]-NPY is easier to describe than the quenching efficiency calculated from the tyrosine fluorescence of native NPY, since the origin of the fluorescence can be related to one definite position in [Trp^{32}]-NPY. Native NPY contains five tyrosines, which results in a broad spatial distribution of the fluorescence over the peptide. However, the smallest quenching efficiency for native NPY is observed in the presence of 16-doxyl-PC, which excludes a localization of NPY in the hydrophobic core of the membrane (data not shown).

Determination of the penetration depth of NPY and the NPY fragment (18–36) in lipid membranes

From the I_∞/I_0 ratio, which can be calculated from the fit of the binding curves, the membrane penetration depth of the peptides can be estimated. The tyrosine fluorescence intensity of NPY in the membrane-bound state is increased compared to the fluorescence intensity in aqueous solution. This is caused by the decreased polarity of the membrane environment to which the peptide binds. Thus, the ratio between the fluorescence intensity of the peptide in aqueous solution I_0 and the fluorescence intensity of the membrane-associated peptide I_∞ provides a measure for the penetration depth of the peptides (Zhang et al. 2003). Figure 5 shows the ratio I_∞/I_0 for NPY and the helical fragment NPY (18–36) in the presence of liposomes with varying PS/PC molar ratios. Because full-length NPY has an additional fifth tyrosine at first position of its sequence, which is not inserted in the membrane (Thomas et al. 2005), the ratio I_∞/I_0 of NPY was corrected by $(I_\infty - 1/5I_0)/(4/5I_0)$.

The ratio I_∞/I_0 is increased by decreasing the fraction of PS in the membrane, which corresponds to a deeper penetration of both peptides in zwitterionic membranes (PC). Additionally, the ratio I_∞/I_0 for the NPY fragment is larger than the value for NPY for each lipid composition, which correlates with a deeper penetration of the NPY fragment in phospholipid membranes.

Since the polarity profiles of charged and zwitterionic membranes are different, the fluorescence intensity can change without an alteration in the penetration depth. To exclude this effect the fluorescence of headgroup-labelled dansyl-PE was measured in a PC and PS matrix. The increase in fluorescence intensity was about 20% in a pure PS membrane compared to a pure PC membrane. This will not explain the change of the I_∞/I_0 ratio from two (PS) to four (PC) as observed for the peptides. This result suggests that there is a shift in the position of the peptides towards the hydrophobic core in zwitterionic membranes. Unfortunately, it is difficult to determine an absolute position of the peptides from the I_∞/I_0 ratio. Therefore, we used ^{1}H MAS NMR to obtain the position of NPY in phospholipid membranes, which are composed of varying fractions of charged and zwitterionic lipids.

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

To investigate the depths of membrane penetration of the NPY analogue [Ala^{31}, TOAC^{32}]-NPY, ^{1}H MAS NMR was used. In this NPY analogue, the threonine at 32nd position is exchanged by TOAC, a paramagnetic artificial amino acid, which is located in the helical part of the peptide. In the presence of paramagnetic molecules, the nuclear relaxation rates are increased. If a peptide with the attached paramagnetic TOAC spin label penetrates the lipid membrane, an increase in the phospholipid relaxation rates can be observed (Thomas et al. 2005).

The ^{1}H MAS NMR spectra are very similar in the presence of the TOAC analogues of NPY compared to the spectra in presence of native NPY, all lipid resonances could be resolved. Example spectra were already shown before, where these kinds of measurements were done for a POPC/POPS 80:20 (mol/mol) membrane (Thomas et al. 2005).

From measurements of phospholipid relaxation rates in the presence and in the absence of the spin-labelled peptide, the paramagnetic (i.e. peptide induced) contribution to the relaxation rate can be determined. This paramagnetic relaxation rate is a measure of the interaction strength between the TOAC label and the lipid segments. Since the paramagnetic relaxation rate exhibits a very strong distance dependence, it can be interpreted as a contact probability between the TOAC label and the respective phospholipid segment (Vogel et al. 2003). By measuring the paramagnetic relaxation rate for each phospholipid segment resolved in the ^{1}H MAS NMR spectrum, a distribution function of the TOAC label in membrane is achieved (Brulet and McConnell 1975; Polnaszek and Bryant 1984; Vega and Fiat 1976; Vogel et al. 2003). A plot of the paramagnetic relaxation rate as a function of the membrane coordinates (Brodersen et al. 2007; Kaczmarek et al. 2008) of the lipid segments is shown for the NPY analogue [Ala^{31}, TOAC^{32}]-NPY in Fig. 6. The lipid membrane coordinates for the lipid groups (for CH_3, CH_2–C=, C-3, C-2, G-1, G-3, Cα, Cβ and Cγ) are plotted on the *x* axis and represent the mean membrane positions of these lipid segments. Note that Cβ and Cγ are not present in a pure POPS membrane. The glycerol G-1 signal could not be analysed in pure POPC membranes since the neighbouring water peak caused phase problems. The lipid coordinates were obtained from a molecular dynamics simulation of a POPC membrane (Scott Feller, personal communication).

For each membrane composition, the label exhibits a broad distribution around its average membrane localization, which is a result of the high mobility and molecular disorder in liquid crystalline membranes (Huster et al. 1999; White and Wiener 1996) that is also observed for the

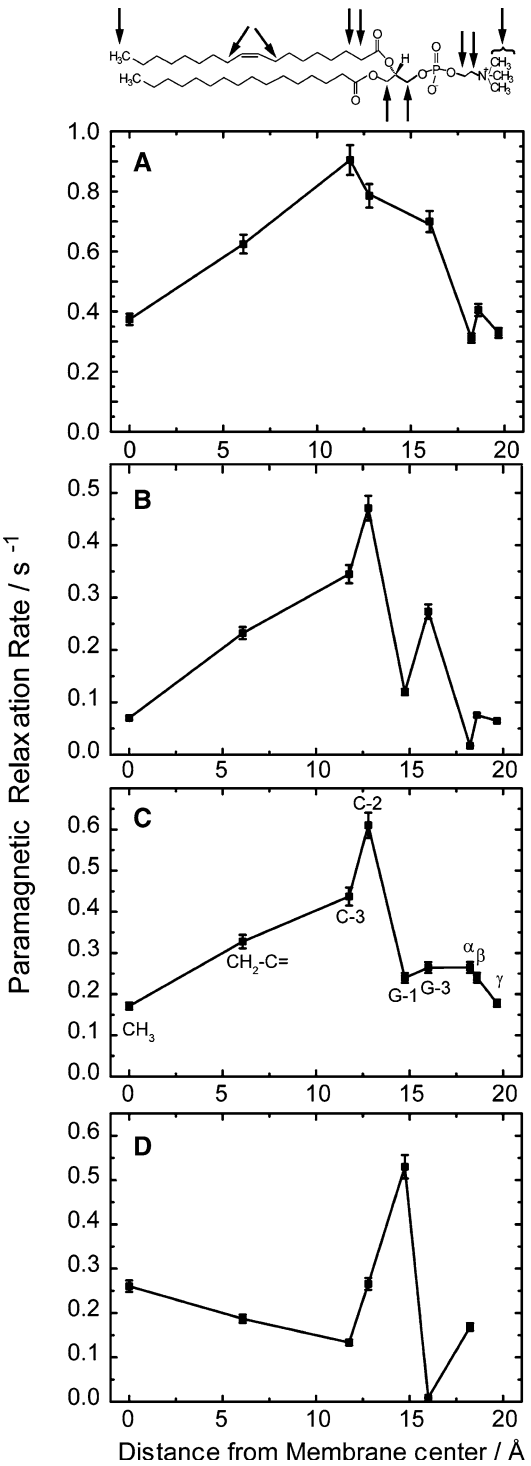

Fig. 6 Paramagnetic relaxation rates of the phospholipid segments in the presence of [Ala^{31}, TOAC^{32}]-NPY as a function of the coordinates of such lipid segments (marked by *arrows* in the lipid molecule above) obtained from a molecular dynamics simulation of pure POPC membranes (Scott Feller, personal communication). Paramagnetic relaxation rates were determined from T_1 relaxation time measurements. The NPY to phospholipid molar ratio was 1:100. The lipid composition of the POPC/POPS membranes was varied between 100:0 (**a**), 80:20 (**b**), 50:50 (**c**) and 0:100 (**d**). All measurements were carried out at a D_2O content of 70 wt% and a temperature of 30°C

Table 3 Hydrophobic binding energy ΔG°_{H}, total binding energy ΔG°_{obs}, and the effective peptide charge of NPY analogues at a temperature of 25°C calculated from the binding curves using Model II (at 100 mM NaCl if not stated otherwise)

pH value	Peptide	ΔG°_{H} (kJ/mol)	ΔG°_{obs} (kJ/mol)	ΔG°_{E} (kJ/mol)	z_{eff} (e)
5.0	NPY	−36	−41	−5	0.8
7.4	NPY	−35	−35	0	0
9.0	NPY	−35	−34	+1	−0.3
5.0	[Trp^{32}]-NPY	−36	−41	−5	0.8
5.0	[Trp^{32}]-NPY	−37	−45	−8	1.5
0 mM NaCl					
5.0	[Trp^{32}]-NPY	−33	−34	−1	0.1
200 mM NaCl					
7.4	[Trp^{32}]-NPY	−35	−35	0	0
9.0	[Trp^{32}]-NPY	−35	−34	+1	−0.3
5.0	[Ala^{31}, TOAC^{32}]-NPY	−34	−38	−4	0.9

The used liposomes were composed of a 1:1 mixture of PS and PC

membrane-bound peptide. However, the comparison of the maxima of the distribution functions shows, consistent with the fluorescence data, that the helical peptide segment is more deeply inserted in zwitterionic membranes than in a pure PS membrane. Further, the distribution becomes somewhat sharper as the PS content of the membrane increases. For the helical amino acids of NPY, a localization in the glycerol region of PS containing membranes can be concluded, whilst the peptide distribution in the PC membranes shifts to a position in the upper chain region.

Membrane binding of the NPY analogues

The fluorescence quenching and the ^{1}H MAS NMR experiments were carried out with the NPY analogues [Trp^{32}]-NPY and [Ala^{31}, TOAC^{32}]-NPY, respectively. The influence of the amino acid exchange using these analogues has to be considered carefully. Therefore, binding curves of these peptides in the presence of liposomes composed of 1:1 molar mixture of PS and PC were measured. The observed results for ΔG°_{H}, ΔG°_{obs} and z_{eff} are presented in Table 3. It can be seen that the amino acid mutations only have a marginal influence on the membrane binding of the peptide.

Discussion

The major focus of this work was to study details of the interaction of NPY with phospholipid membranes composed of mixtures of negatively charged and zwitterionic lipids using the intrinsic tyrosine fluorescence of the peptide. Although NPY has a net charge of approximately 0 at pH 7.4, it only significantly binds to acidic membranes. This suggests that local electrostatics plays a role for membrane binding of NPY, which was assessed in our study. In order to observe the electrostatic effects in bulk measurements, the pH was lowered to 5, where NPY has a (small) positive net charge that facilitates detection of electrostatic contributions to the total binding energy. The results for wild-type NPY were confirmed using the more favourable fluorescence properties of the NPY analogue [Trp^{32}]-NPY and by ^{1}H MAS NMR using a TOAC-labelled NPY analogue.

NPY consists of an unstructured N-terminal part (amino acids 1–12) and an α-helical domain, which comprises the amino acids 13–36 (Monks et al. 1996). It was already shown that only the helical part of the peptide binds to the membrane, whereas the N-terminal segment sticks out from the bilayer and is highly flexible (Thomas et al. 2005). We employed two models to determine thermodynamic data from the peptide binding curves. The Gouy-Chapman/mass action model (Model II) provides an amazingly accurate description for the binding of NPY to lipid membranes. The dependence of the molar fraction of acidic lipids, the ionic strength of the buffer solution, and the effective charge of the peptide is represented very well by this model. However, the discrete nature of the lipid as well as the peptide charges is ignored (Montich et al. 1993). Because of these limitations, the partition coefficients were calculated for the [Trp^{32}]-NPY PC/PS (1:1) system (Model I) to get an impression of the errors using Gouy-Chapman/mass action model.

For membranes containing a physiological concentration of acidic lipids, the free energy of transfer from aqueous environment to the membrane was −29 kJ/mol (determined from the partition coefficient, Model I) and −32 kJ/mol (determined from a model that separates

hydrophobic and electrostatic contributions, Model II). This value refers to strong binding with a fraction of bound peptide >60% at a lipid concentration of ~0.5 mM. For the C-terminal fragment (18–36), the models predict −36 and −39 kJ/mol, which corresponds to >95% bound peptide. The hydrophobic Gibbs energy of NPY's transfer from aqueous solution to the membrane can be calculated from basic physical chemistry. It was already shown that only the helical part of the peptide binds to the membrane, whereas the N-terminal segment sticks out from the membrane and is highly flexible. Interestingly, the experimental hydrophobicity scale does not predict such high binding energies (Wimley and White 1996; Thomas et al. 2005).

We have used the mpex program (Jaysinghe et al. 2006) to calculate the hydrophobic contribution to the binding energy assuming that amino acids 16–36 form a regular α-helix. Under these circumstances, a standard free energy of transfer for aqueous solution to the membrane interface of −18 kJ/mol arises. This is a bit smaller than the value we estimated from our model, but one has to consider that the program assumes that all residues are localized in the membrane interface. However, considering the amphipathic α-helix of NPY is oriented such that the charged residues can point away from the membrane, which decreases the free energy penalty leading to a more favourable free energy of transfer. Because of that, the difference of observed and theoretical values is probably caused by the reduced Born repulsion of the charged amino acids of NPY's helix. Further, not the entire NPY peptide is bound to the membrane, the N terminus, which contributes +30 kJ/mol of binding energy, was found to be highly mobile and localized in the aqueous phase (Thomas et al. 2005).

NPY binding to the membrane is less favourable than direct binding of the peptide to the receptor. The total Gibbs free energy for peptide transfer from solution to a receptor bound state is associated with the K_d value via Eq. 3 (using $k_{obs} = 55.5\ M/K_d$). The K_d values of NPY to the receptors are in the nanomolar range (Merten and Beck-Sickinger 2006), thus the binding energy is in the order of −60 kJ/mol. Accordingly, a value of −30 kJ/mol for the transfer of membrane-bound NPY to the receptor bound state has to be considered.

The calculated values z_{eff} and K_H for membrane binding using [Trp^{32}]-NPY and [Ala^{31}, $TOAC^{32}$]-NPY are nearly identical with the values of native NPY (Table 3). Therefore, it is reasonable to use these NPY analogues to study the peptide–membrane interaction. Because the origin of the fluorescence is related to one definite position using [Trp^{32}]-NPY, the tryptophan fluorescence was analysed in quenching experiments instead of the intrinsic tyrosine fluorescence of native NPY. The results of quenching experiments using spin-labelled lipids show clearly, consistent with previous results (Dyck et al. 2006; Dyck and Losche 2006; Thomas et al. 2005), that the α-helix of NPY is located in the membrane–water interface of the phospholipid membrane.

It was shown in many studies that the amino acids 33–36 play a decisive role in the interaction between NPY and its receptor (Beck-Sickinger et al. 1994; Beck-Sickinger and Jung 1995; Cabrele and Beck-Sickinger 2000; Fuhlendorff et al. 1990; Grandt et al. 1994; Merten et al. 2007). Mutations of these amino acids decrease the binding of NPY to the receptors dramatically. Therefore, the C-terminal NPY fragment (18–36), which shares the amino acids 33–36 with NPY, is able to interact with NPY receptors. However, the final activation of the receptor by NPY, excluding the activation of the Y_2 receptor, is strongly associated with the tyrosine at the N terminus of the peptide.

If the membrane interaction of NPY and the α-helical NPY fragment (18–36) are compared, it is possible to determine the contribution of the N terminus of the peptide to the membrane interaction. Because the N-terminal part of NPY alone shows no interaction with phospholipid membranes, it could be supposed that this part has no effect on membrane–peptide interaction. Since the N-terminal part of NPY comprises some negatively charged amino acids, the effective charge of NPY (0.8*e*) at pH 5 is much lower than the net charge (2.4*e*) of the C-terminal NPY fragment (18–36). Therefore, the observed Gibbs energy for binding of the NPY fragment is increased compared with NPY, especially in membranes with high fraction of PS, due to electrostatic interactions of membrane and peptides.

Surprisingly, the position of NPY's α-helix in the membrane–water interface is influenced by the N-terminal part of the molecule. The fragment is more deeply inserted in the membrane than the helix of the whole peptide. Consequently, the α-helix of NPY is pulled out of the membrane by the hydrophilic and negatively charged N-terminal part. The position of NPY in the membrane–water interface represents an interplay between the hydrophobic helix and the hydrophilic N-terminal part. Because the activation of the receptor by NPY is strongly associated with the tyrosine at the N terminus of the peptide, the pulling out effect will not be a single feature of NPY's N-terminal part.

An additional result presented in this paper is the dependence of NPY's membrane position on the fraction of charged lipid in the membrane. The position of the helix in the bilayer is shifted from the upper chain region in zwitterionic membranes to the glycerol region in bilayers composed of PS. This effect seems to have no meaning in the physiological system, because biological membranes

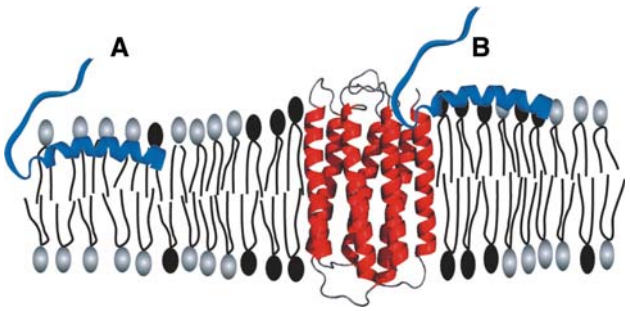

Fig. 7 The cartoon summarizes the structural model derived from the fluorescence and NMR data of this study. The α-helix of NPY is located in the upper chain region in the presence of zwitterionic (*grey*) phospholipids (**a**). In the presence of the charged lipids (*black*), the NPY α-helix is shifted to the headgroup region of the membrane (**b**). A possible accumulation of charged lipids around the receptor can adjust the position of NPY to optimize its binding to the receptor

usually contain not more than 20% of charged lipids and the shift of the peptide position between zwitterionic and weakly charged membranes is very low. However, NPY binds to GPCR proteins, which can model special lipid environment (Soubias et al. 2006). If there was an accumulation of negatively charged lipids around the Y receptor, the peptide would rise in the membrane, which could result in a more effective binding of the peptide to the receptor.

The absolute value of the estimated hydrophobic binding energy of NPY and the NPY fragment to liposomes composed of varying PS/PC ratios increases by increasing the fraction of PS. Basically, such an effect can be associated with a shift of the peptide position in the membrane by different fractions of PS. However, the position of the peptide in the membrane using zwitterionic lipids is optimized by the hydrophobic interaction. Therefore, the highest absolute value of the estimated hydrophobic binding energy should be observed using zwitterionic membranes. Unfortunately, the opposite effect is observed from the binding curves of NPY and the NPY fragment. The main reason for the disagreement is the aggregation of the liposomes at small lipid-to-peptide ratios, especially for membranes composed of more than 30% of PS. Therefore, the calculated binding energy for strongly charged membranes is uncertain. The ratio I_∞/I_0 is not influenced by this effect, because this value is calculated from large lipid-to-peptide ratios.

The data presented in this paper support the following model depicted in Fig. 7: NPY can bind directly to the receptor with a Gibbs free energy of −60 kJ/mol or first binds to the membrane with a Gibbs energy of −30 kJ/mol. Since the available membrane area for binding is normally much larger than the receptor area, the membrane binding will be preferred. The membrane-bound peptide then diffuses close to the receptor and will interact with the special lipid environment around the receptor. If there is an accumulation of negatively charged lipids around the receptor, the peptide will arise in the membrane and can bind more efficiently to the receptor.

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