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Peptide binding to lipid membranes. Spectroscopic studies on the insertion of a cyclic somatostatin analog into phospholipid bilayers

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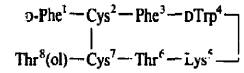
The cyclic peptide SMS 201-995 (+)D-Phe^1-Cys^2-Phe^3-D-Trp^4-(+)Lys^5-Thr^6-Cys^7-Thr(ol)^8 is an analog of somatostatin and binds to lipid membranes by an electrostatic/hydrophobic mechanism. The structural changes accompanying the binding process were investigated with circular dichroism (CD), fluorescence spectroscopy, and phosphorus and deuterium nuclear magnetic resonance. The peptide penetrates into the lipid bilayer and the binding is accompanied by a small change in the CD spectrum suggesting the formation of β-ordered structures. The fluorescence emission spectrum of the tryptophan side chain exhibits a blue shift and an intensity enhancement of the emission maximum, providing evidence that this residue is located in the inner part of the phospholipid headgroup region with a dielectric constant of $\epsilon \approx 7$. The peptide diffuses rapidly in the plane of the membrane, changing the lipid headgroup conformation. This was demonstrated by selectively deuterating the two choline segments and measuring the deuterium spectra as a function of the bound peptide concentrations. A linear variation of the quadrupole splitting with the mol fraction of bound peptide was observed. The molecular origin of this effect is a distinct change in the orientation of the phosphocholine dipole, moving the N^+ end of the dipole away from the membrane surface into the water phase. This type of headgroup rotation appears to be the general response of the zwitterionic phosphocholine headgroup to cationic surface charges. However, peptides appear to be the most efficient modulators of the lipid headgroup structure known to date.

Introduction

The cyclic peptide SMS 201-995



is an analog of the hormone somatostatin. Its biological activity is larger than that of somatostatin and the peptide is of significant clinical interest [1]. The molecule has a formal charge $z = +2$ and binds to neutral and negatively charged membranes. The thermodynamic binding data can be summarized as follows [2]: (i) Monolayer expansion studies demonstrate that the peptide intercalates between the membrane lipids. The surface area requirement of the peptide is approx. 135 Å^2 and the peptide presumably enters the membrane with the long peptide axis parallel to the plane of the membrane. (ii) Binding studies have been performed with monolayers and large multilamellar bilayer vesicles. The peptide binding isotherms and the ζ-potential measurements can be explained by a surface partition equilibrium

$$X_b = K_p C_M$$

where X_b is the molar amount of peptide bound per total lipid, K_p is the partition constant ($K_p = 75\ M^{-1}$ at 0.154 M NaCl), and C_M is the concentration of free peptide immediately above the plane of binding. C_M is distinctly enhanced compared to the equilibrium concentration in bulk solution if the membrane carries a negative surface charge and must be calculated via the Gouy-Chapman theory [3,4].

Abbreviations: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; CD, circular dichroism; NMR, nuclear magnetic resonance.

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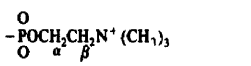
Knowledge of the thermodynamic binding parameters leads to a number of structural questions. Since the peptide intercalates between the lipids, is there a conformational change in the peptide structure? How deep can the peptide penetrate into the lipid bilayer? Is the lipid conformation modified upon peptide penetration? We have attempted to answer these questions by employing different types of spectroscopy. The peptide conformation in water and in the membrane was investigated with circular dichroism (CD); the penetration of the peptide into lipid phase was assessed with fluorescence spectroscopy; finally, the structural properties of the membrane lipids were analyzed with phosphorus and deuterium nuclear magnetic resonance (NMR). The membrane systems were formed from neutral 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and negatively charged 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG). For comparative purposes, CD and fluorescence spectroscopic measurements were also performed with somatostatin. While both SMS 201-995 and somatostatin have been studied in solution (Refs. 1, 5 and 6, and references therein), we are not aware of corresponding spectroscopic studies on the membrane-bound peptides.

Materials and Methods

SMS 201-995 and somatostatin were kindly provided by Sandoz (Basel, Switzerland). The concentrations were determined by ultraviolet spectroscopy using an absorption coefficient of 5700 $M^{-1} \cdot cm^{-1}$ at 280 nm, characteristic of the tryptophan and disulfide absorption [5]. Non-deuterated 1-palmitoyl-2-oleoy-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). For simplification of the discussion, the two methylene segments of the choline headgroup are denoted α and β in the following:



POPC was selectively deuterated at the α- and β-segment as described previously [7].

Preparation of lipid samples. Small unilamellar vesicles (SUV) of POPG were required for CD and fluorescence experiments and were prepared as follows. A lipid dispersion of POPG (approx. 3 mg lipid/ml) was sonicated under nitrogen for about 35 min (at 10°C) until an almost clear solution was obtained. Metal debris from the titanium tip was removed by centrifugation in an Eppendorf centrifuge for 10 min and the vesicle suspension was used without further manipulations.

For NMR measurements about 8-10 mg of POPC and POPG were mixed in dichloromethane or chloroform in the molar ratio of 75:25 and the solvent was removed first in a stream of nitrogen and then under high vacuum. The lipids were dispersed in 200 μl buffer containing 154 mM NaCl, 10 mM Tris-HCl (pH 7.4), plus the desired concentration of SMS 201-995. The samples were strongly vortexted, followed by several freeze-thaw cycles and further vortexing in order to ensure a homogeneous distribution of peptide. The suspension was centrifuged for 120 min at 120000 × *g* (Beckman Airfuge) and the clear supernatant was removed with a pipette. After appropriate dilution the SMS 201-995 concentration in the supernatant was determined with ultraviolet spectroscopy by measuring the absorption at 280 nm. From the difference between the initial and the final SMS 201-995 concentration the amount of SMS 201-995 bound per mol lipid, X_b, could be evaluated [2].

NMR measurements. All ^{2}H-NMR and ^{31}P-NMR experiments were performed with the coarse lipid dispersion as described above. The lipid pellets obtained after centrifugation were used without further manipulation. The ^{2}H-NMR spectra were recorded with a Bruker MSL-400 spectrometer operating at 61.4 MHz, using the quadrupole echo technique [8]. ^{31}P-NMR spectra were recorded at 162 MHz by employing a Hahn echo sequence with gated proton decoupling [9]. The experimental parameters were essentially the same as described previously [10].

Circular dichroism and fluorescence spectroscopy. Circular dichroism measurements were performed with a Cary 61 instrument which was calibrated with d(+)-10-camphor sulfonic acid. The optical length of the cuvette was 2 mm. [θ] represents the mean residue ellipticity in $deg \cdot cm^2 \cdot dmol^{-1}$. Fluorescence spectra were recorded with a Schoeffel RRS 1000 spectrofluorimeter (excitation at 282 nm, emission in the range 300-450 nm). The absorbance in the exciting beam was always chosen to be smaller than 0.1.

Results and Discussion

Peptide conformation in solution and bound to lipid membranes

As mentioned above, the surface partition coefficient for the binding of SMS 201-995 to POPC/POPG (75:25) membranes is only $K_p = 75\ M^{-1}$ (0.154 M NaCl, 10 mM Tris-HCl (pH 7.4)). In order to determine the spectral characteristics of the membrane-bound peptide, all CD and fluorescence measurements were hence made with pure POPG vesicles. The electrostatic attraction between the negative membrane surface and the positively charged peptide considerably enhances the effective binding. In addition, a high lipid-to-protein ratio (cf. legends to figures) was chosen in order to guarantee a complete binding of the peptide to the lipid membrane.

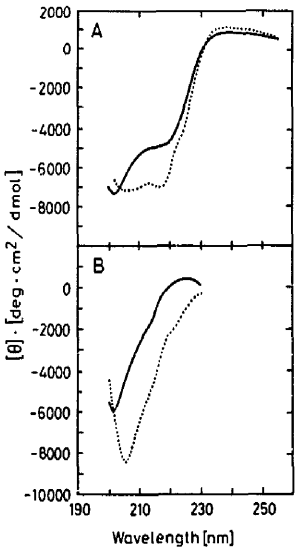


Fig. 1. Circular dichroism spectra of SMS 201-995 and somatostatin in buffer and bound to POPG vesicles. (A) solid line, SMS 201-995 (50 μM) in buffer (154 mM NaCl, 10 mM Tris (pH 7.4)); dotted line, SMS 201-995 (50 μM) and small unilamellar vesicles of POPG (2 mM phospholipid) measured in buffer. (B) solid line, somatostatin (50 μM) in buffer; dotted line, somatostatin (50 μM) and small unilamellar vesicles of POPG (1 mM phospholipid) in buffer.

Circular dichroism spectra of SMS 201-995 in aqueous solution and bound to POPG vesicles are shown in Fig. 1A. In the aqueous phase, the CD spectrum is characterized by a minimum at 203 nm with a shoulder at 220 nm (solid line in Fig. 1A). Upon binding of the peptide to the membrane, the latter transition increases in amplitude from -4500 deg · cm^2/dmol to -6500 deg · cm^2/dmol (dotted line, Fig. 1A). By comparison with CD spectra of elastin (which have no overlapping aromatic residues) in water and methanol [11] this change in the CD spectra of the somatostatin analog suggests the formation of some β-turn structure.

The CD spectrum of somatostatin in buffer (Fig. 1B) is characterized by a minimum at approx. 200 nm and corresponds to published data [5]. For membrane-bound somatostatin (dotted line in Fig. 1B) this minimum is shifted to ~ 207 nm and its amplitude is enhanced. The molecular interpretation of this change is difficult, since the CD absorption of the peptide backbone is masked by contributions from the aromatic amino acid side chains and the disulfide bridge. Nevertheless, the appearance of a weak shoulder at 220 nm again suggests the formation of some β-turn structure.

The transfer of protein segments from water into a non-polar environment leads to a drastic reorganisation of hydrogen bonds since hydrogen bonds between water and amino acid side chains must be replaced by intrachain hydrogen bonds leading to highly ordered protein structures [12]. A typical case in point is melittin which upon binding to lipid membranes undergoes a transition from a random-coil structure in water to an almost 70% α-helical structure in the lipid phase [13]. We have reestimated this value to be 80% [18]. For the cyclic peptides described here the structural changes are much less pronounced, and suggest alternative mechanisms to satisfy the energy requirements. Presumably hydrogen bonds of the peptides to bulk water are replaced by hydrogen bonds to water in the hydration layer of the phospholipid headgroups.

Peptide penetration into lipid membranes

Both somatostatin and its smaller analog SMS 201-995 contain a single tryptophan which can be exploited for polarity studies since the fluorescence spectrum of the indole ring experiences a blue shift upon transfer from an aqueous to a less polar solvent [14,15]. Fig. 2 displays fluorescence spectra of SMS 201-995 and somatostatin in buffer and bound to vesicles. In water, both molecules exhibit a fluorescence emission maximum at 351 nm. However, the fluorescence intensity increases and the emission maximum shifts towards shorter wavelength in the presence of POPG vesicles. Again a high lipid-to-protein ratio was employed in order to completely eliminate spectral contributions of the free peptide in solution. For both molecules the emission maximum shifts by about 12 nm to 339 nm

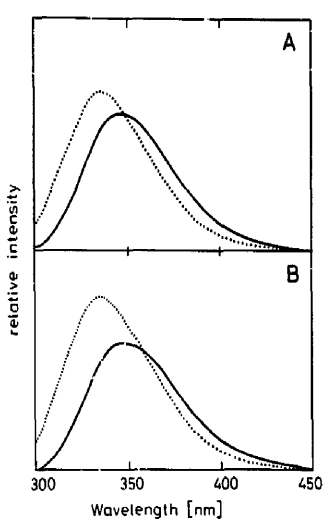


Fig. 2. Fluorescence spectra of SMS 201-995 and somatostatin in buffer and bound to POPG vesicles. (A) solid line, SMS 201-995 (5 μM) in buffer; dotted line, SMS 201-995 (5 μM) and small unilamellar vesicles of POPG (phospholipid concentration 0.7 mM) in buffer (B) solid line, somatostatin (5 μM) in buffer; dotted line, somatostatin (5 μM) and small unilamellar vesicles of POPG (phospholipid concentration 0.4 mM) in buffer.

indicating a polarity comparable to a dielectric constant of about $\epsilon \approx 7$ [15]. Apparently, the tryptophane residue does not penetrate completely into the central part of the bilayer (with $\epsilon = 2$) but remains in the headgroup region. This may be contrasted with the binding of melittin to POPC-like bilayers which is accompanied by a much larger dispiacement in the fluorescence maximum (from 350 nm to 330 nm) suggesting a truly hydrophobic environment in the latter case [16]. Comparably large shifts have also been observed for some small pentagastrin-related pentapeptides [17]. These latter studies provide evidence that it is not the hydrophobicity at the tryptophan residue but the hydrophobicity of the molecule as a whole which determines the location of the tryptophan residue within the membrane.

Peptide binding and lipid head group conformation

^{2}H-NMR studies have demonstrated that melittin binding to pure POPC [10], mixed POPC/POPG [18] and mixed DMPC/DMPS [19] bilayers induces a significant change in the phosphocholine headgroup orientation. A qualitatively similar result was reported for the strongly hydrophobic, synthetic peptide $K_2GL_{20}K_2A$ [20], which carries two positive charges on the C-terminal and three on the N-terminal of its bilayer-spanning helix. We have therefore investigated the influence of the somatostatin-analog SMS 201-995 on the phosphocholine headgroup for both pure POPC and mixed POPC/POPG membranes. ^{31}P-NMR and ^{2}H-NMR spectra were obtained for the phosphate segment and the α- and β-methylene segments, respectively, with and without peptide present. The ^{31}P-NMR spectra (not shown) were always characteristic of liquid crystalline bilayers with no evidence for the formation of non-bilayer phases [21]. Deuterium NMR spectra of pure POPC membranes deuteriated at either the α-CH_2 or β-CH_2 group of the choline moiety are displayed in Fig. 3. All ^{2}H-NMR spectra were characterized by a single quadrupole splitting; under no condition did we observe separate spectra for free lipid and peptide-bound lipid. The deuterium spectra thus indicate a single, time-averaged headgroup conformation at all peptide concentrations. This result requires a rapid two-dimensional translational diffusion of the peptide on the membrane surface, affecting all lipid headgroups to equal extent (within a time of less than 10^{-5} s).

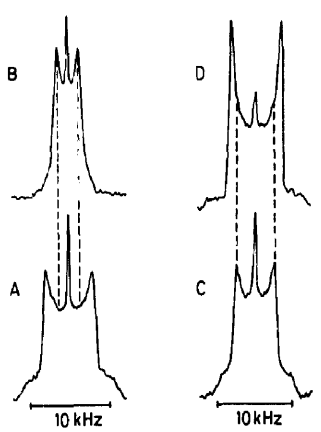


Fig. 3. Deuterium NMR spectra of POPC membranes without peptide (A,C) and with SMS 201-995 (B,D). POPC was selectively deuterated at the α-segment (A,B) and the β-segment (C,D). The amount of bound peptide was $X_b = 0.09$ (mol/mol) in Fig. B and $X_b = 0.07$ (mol/mol) in Fig.D.

TABLE I

Degree of peptide binding, X_b, and corresponding quadrupole splittings, $\Delta\nu_Q$
0.154 M NaCl; 10 mM Tris-HCl (pH 7.4).

Membrane system	X_b (mmol/mol)	$\Delta\nu_Q$ (kHz)
100% POPC		
α-segment	0	5.81
deuterated	15.7	5.21
	42	4.26
	66	3.54
	79.6	2.89
	90	2.60
β-segment	0	5.01
deuterated	14	5.45
	34.5	5.8
	70.4	6.5
75 mol% POPC + 25 mol% POPG		
α-segment of POPC	0	8.45
deuterated	7.3	8.13
	14.8	7.89
	29	7.26
	57	6.59
	104	5.3

The addition of SMS 201-995 to POPC-containing membranes has opposite effects on the two choline segments: the α-splitting decreases, whereas the β-splitting increases. This finding is in agreement with the effect of melittin and of many other positively charged compounds which bind to the membrane surface (cf. below). In order to quantify the observed variations of the deuterium quadrupole splittings, we have measured the amount of bound peptide, X_b, by the centrifugation assay described above [2]; the X_b values and the corresponding quadrupole splittings $\Delta\nu_Q$ are summarized in Table I.

The variation of $\Delta\nu_Q$ with X_b can be explained by the following model [22,23]. The penetration of n_P mol of peptide into a membrane composed of n_L mol of lipid leads to an increase of the membrane surface area

[2]. The total area, A_T, after peptide insertion is given by

$$A_T = N_A(n_L A_L + n_P A_P) \quad (1)$$

where N_A is Avogadro's number and A_L and A_P are the surface area requirements for a single lipid and a single peptide molecule, respectively. At the same time, the electric surface charge, Q, of a pure POPC membrane is determined only by the amount of bound peptide

$$Q = N_A e_o z_p n_p \quad (2)$$

where $z_p e_o$ is the effective charge of the peptide (as seen by the membrane). The surface charge density, σ, is then calculated according to

$$\sigma = Q/A_T = (z_p e_o/A_L) r \quad (3)$$

where r is defined as

$$r = X_b/[1 + (A_P/A_L) X_b] \quad (4)$$

The denominator in the last expression takes into account the bilayer expansion due to peptide intercalation and vanishes for $A_p = 0$ (no penetration). In the following we assume $A_P = 135$ Å^2 [2] and $A_L = 68$ Å^2 [24]. Previous studies have indicated that a change in the membrane surface charge density σ entails a reorientation of the phosphocholine dipole with a concomitant change of the deuterium quadrupole splittings [25]. Since $\sigma \approx r$, we have plotted the variations of the quadrupole splittings $\Delta\nu_\alpha$ and $\Delta\nu_\beta$ for pure POPC bilayer versus r (Fig. 4). For both segments a linear dependence on r was observed with (Fig. 4A)

$$\Delta\nu_\alpha = 5.9 - 41.9\, r \quad (5)$$

$$\Delta\nu_\beta = 5.1 + 22.9\, r \quad (6)$$

($\Delta\nu_Q$ measured in kHz; r given as mol/mol). We have also studied a mixed POPC/POPG (75 : 25, mol/mol) bilayer in which the α-segment of the choline moiety was deuterated. The quadrupole splitting of the pure POPC/POPG membrane was 8.4 kHz due to the presence of negatively charged lipid [26]. Addition of the positively charged somatostatin analogue reversed the effect of PG; again a linear decrease of $\Delta\nu_\alpha$ with r was observed (Fig. 4B):

$$\Delta\nu_\alpha = 8.4 - 36.1\, r \quad (7)$$

The slope m_α is almost identical for pure POPC (Eqn. 5) and mixed POPC/POPG (Eqn. 7) membranes.

Linear relationships between the quadrupole splittings $\Delta\nu_\alpha$ and $\Delta\nu_\beta$ and the mol fraction of membrane bound agents X_b (or r) have been observed previously for a variety of chemically different compounds. Table

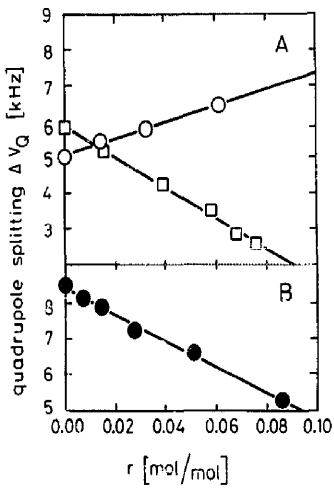


Fig. 4. Variation of the quadrupole splittings of α- and β-deuterated POPC with the amount of bound peptide r (Eqn. 4). (A) pure POPC membranes with either α-segment (□) or β-segment (○) deuterated. The equilibrium concentration of peptide free in solution is 7.3 mM for $r = 0.076$. (B) mixed POPC/POPG (75:25, mol/mol) with the α-segment of POPC deuterated. The equilibrium concentration of peptide free in solution is 0.21 mM for $r = 0.051$. For details see Ref. 2.

II provides a survey of the available data for the phosphocholine headgroup. The experimentally observed quadrupole splittings were evaluated according to $\Delta\nu_i = m_i r + \Delta\nu_i^\circ$ and Table II summarizes the slopes m_i. We note that differences in the binding constants are eliminated by this approach, since X_b(or r) refers to the bound agent only. The slopes m_i thus provide a quantitative measure of the efficacy of membrane-bound molecule to change the quadrupole splittings and, in turn, the orientation of the phosphocholine headgroup. Among the four peptides listed, SMS 201-995 has the smallest effect; nevertheless, its headgroup efficacy is even larger than that of the most efficient hydrophobic local anesthetic. Fig. 5 summarizes the data of Table II in terms of a m_α–m_β plot, i.e., the efficacy of a molecule to change the α-segment (characterized by m_α) is plotted versus its efficacy at the β-segment (characterized by m_β). The surprising result of this plot is a linear correlation between m_α and m_β, independent of the chemical structure of the molecules involved, with

$$m_\beta = -0.48 m_\alpha \quad (8)$$

While the m_i values of the membrane-active agents vary considerably, with melittin (effective charge $z = 2.2$) exhibiting the largest ($m_\alpha = -93.5$ kHz) and Ca^{2+} the

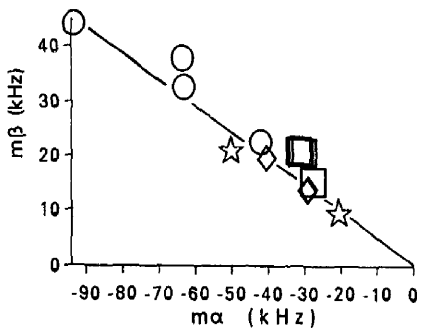


Fig. 5. Variation of the choline headgroup quadrupole splittings upon binding of cationic substances. The variation of the α-segment is plotted vs. the β-segment. ○, peptides; □, cationic amphiphiles; ◇, local anesthetics; ☆, metal ions.

smallest value ($m_\alpha = -20.5$ kHz) the ratio m_β/m_α is always close to -0.5 *. This provides evidence for a strongly coupled motion of the two choline segments.

Experiments with bilayer forming cationic and anionic amphiphiles (which can be mixed with POPC at almost any ratio) have shed light on the molecular nature of the headgroup reorientation [27]: positively charged amphiphiles move the N^+ end of the $^-P\text{-}N^+$ dipole towards the water phase, negative charge moves the N^+ end towards the hydrocarbon interior. The cationic amphiphiles are also included in the m_α–m_β plot and their slopes fit closely into the general scheme

* The only exception known so far is melittin bound to mixed POPC/POPG [18] and mixed DMPS/DMPC membranes [19]. While m_α is identical to the value determined for pure POPC membranes, the ratio m_β/m_α is -0.78 for POPC/POPG membranes and -0.22 for DMPC/DMPS membranes.

TABLE II

Efficacy of membrane-bound agents to change the quadrupole splittings of the choline head group segments

	Effective charge z	m_α (kHz)	m_β (kHz)	$-(m_\beta/m_\alpha)$	Membrane composition	Ref.
Metal ions						
Ca^{2+}	+2	−20.5	+10.0	0.49	POPC	[24]
		−16.7	n.d.		POPC/POPG (80/20)	[26]
		−17.3	n.d.		POPC/POPG (50/50)	
		−14.5	n.d.		POPC/cardiolipin (90/10)	[29]
La^{3+}	+3	−50.1	+21.6	0.43	POPC	[30]
Local anesthetics						
Etidocaine	+1	−40.3	+19.8	0.49	POPC	[23]
Dibucaine	+1	−28.8	+14.0 [a]	0.49	POPC	[23]
Tetracaine	+1	−29.05	+13.82 [d]	0.48	egg PC	[31]
Peptides						
Melittin	+2.2	−93.3	+44.4	0.48	POPC	[10]
		−93.9	+72.3	0.78	POPC/POPG (80/20)	[18]
		−92.0	+20.0	0.22	DMPC	[19]
SMS 201-995	+1.3	−41.9	+22.9	0.55	POPC	this work
$K_2GL_{20}K_2A$	+5 [b]	≈ −66.3 [c]	≈ +33.0 [c]	0.52 [c]	DMPC	[20]
		−57.5	+36.1	0.63 [c]	DMPC/DMPS 80/20	[20]
Gramicidin S	+2 [b]	−63.4	+38.2	0.60	POPC	[32] [33]
Amphiphiles						
$(CH_3)_2N^+(C_{12}H_{25})_2$	+1	−27.4	+15.4	0.56	POPC	[27]
$(CH_3)_2N^+(C_{16}H_{33})_2$	+1	−29.9	+20.9	0.70	POPC	[27]
$(CH_3)_2N^+(C_{18}H_{37})_2$	+1	−31.4	+21.3	0.68	POPC	[27]
$(CH_3)_2N^+(C_{12}H_{25})(C_{16}H_{33})$	+1	−30.9	+20.9	0.68	POPC	[27]

[a] Estimated from Fig. 3 of Ref. 34.
[b] Formal charge; effective charge not determined.
[c] Evaluated from Table I of Ref. 20.
[d] Calculated from Fig. 4A and 5A of Ref. 31.
n.d., not determined.

of Fig. 5. We therefore conclude that the response of the phosphocholine headgroup to cationic peptides, metal ions, and local anesthetics can be described by the same molecular picture as established for the charged amphiphiles. The binding of the somatostatin-like peptide SMS 201–995 hence also induces a rotation of the $^{-}P\text{-}N^{+}$ dipole into the water phase.

This dipole movement has significant implications for the electric properties at the membrane surface. If the headgroup is parallel to the membrane surface in the absence of peptide, the peptide induced reorientation will generate a considerable dipole moment perpendicular to the membrane surface. The alterations in the transmembrane electric field could be strong enough to trigger conformational changes in neighboring proteins, producing, in turn, gating currents due to the movement of charged amino acid side chains [28]. The binding of SMS 201–995 to the membrane surface will thus not only enhance the rate of peptide-receptor interactions but, at the same time the molecule is an efficient modulator of the membrane surface electric properties.

Acknowledgements

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