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Short Communication

Cholesterol prevents interaction of the cell-penetrating peptide transportan with model lipid membranes

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Abstract: Interaction of the cell-penetrating peptide (CPP) cysteine-transportan (Cys-TP) with model lipid membranes was examined by spin-label electron paramagnetic resonance (EPR). Membranes were labeled with lipophilic spin probes and the influence of Cys-TP on membrane structure was studied. The influence of Cys-TP on membrane permeability was monitored by the reduction of a liposome-trapped water-soluble spin probe. Cys-TP caused lipid ordering in membranes prepared from pure dimyristoylphosphatidylcholine (DMPC) and in DMPC membranes with moderate cholesterol concentration. In addition, Cys-TP caused a large increase in permeation of DMPC membranes. In contrast, with high cholesterol content, at which model lipid membranes are in the so-called liquid-ordered phase, no effect of Cys-TP was observed, either on the membrane structure or on the membrane permeability. The interaction between Cys-TP and the lipid membrane therefore depends on the lipid phase. This could be of great importance for understanding of the CPP–lipid interaction in laterally heterogeneous membranes, while it implies that the CPP–lipid interaction can be different at different points along the membrane. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

INTRODUCTION

Cell-penetrating peptides (CPPs) comprise less than 30 amino acids and are able to penetrate cell membranes and translocate various cargoes [1]. The common feature of these peptides appears to be that they are amphipathic and net positively charged. The mechanism of translocation is not known but it appears to be complex, using different endocytotic pathways and other pathways that are apparently receptor and energy independent. One of the effective CPPs is transportan (TP) which consists of the active part of the neuropeptide galanin attached to mastoparan, a component of wasp venom [2]. TP can enter cells at very low concentrations (nanomolar range) and accumulates mainly in cell membranes. Cargoes, internalized by TP, range from small molecules to proteins and supramolecular particles, making TP a promising drug-delivery vector [3]. Most CPPs have limited side effects, but at higher concentrations TP appears to induce pores or bilayer perturbations in the cell membranes that lead to permeation [4–6].

It is clear that peptide–membrane interactions must be of fundamental importance in the mechanism of

translocation of CPPs. One possible way to study these interactions is by spectroscopic methods, for example by fluorescence spectroscopy [7,8], infrared spectroscopy [9], NMR [10], and electron paramagnetic resonance (EPR) with the use of spin-labeled CPPs [11,12].

The aim of the present work was to monitor the CPP–lipid interaction by using EPR with spin-labeled lipophilic probe molecules introduced into the membrane. In addition, the effect on bilayer perturbation was monitored by following the permeation of water-soluble spin probe molecules trapped in liposomes. We studied Cys-transportan (Cys-TP), an analog of TP in which lysine (Lys[13]) is replaced by cysteine (Cys). It has to be noted here that the net charges of TP and Cys-TP differ, which might lead to differences in their interaction properties. On the other hand, the introduction of Cys into the structure of TP is technologically important because the thiol group of Cys enables coupling of the cargo to TP by a disulfide bridge that is readily cleaved in the reductive environment inside the cell, resulting in release of the cargo.

Lipids in different lipid phases display different structural and motional properties. To investigate whether the Cys-TP–lipid interaction could be lipid-phase dependent, the influence of lipid composition on

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the Cys-TP–lipid interaction was studied. Pure dimyristoylphosphatidylcholine (DMPC) membranes were compared to membranes containing different proportions of cholesterol (Chol/DMPC) as they can exhibit lateral phase separation at appropriate temperatures and composition [13].

MATERIALS AND METHODS

Preparation of Model Lipid Membranes

DMPC was obtained from Avanti Polar Lipids (Birmingham, AL, USA) and cholesterol from Sigma-Aldrich (St Louis, MO, USA). For DMPC liposomes, 20 mg of lipids were dissolved in 1.4 ml of chloroform and 0.7 ml of methanol. For liposomes prepared from DMPC and cholesterol (Chol/DMPC), 20 mg of lipid mixtures were used. The organic solvents were removed on a rotary evaporator followed by extended drying using a rotary vacuum pump. One milliliter of phosphate buffered saline (PBS; pH 7.4) was added and the suspension was left to hydrate for approximately 1 h at a temperature around 10° above the temperature of the main phase transition.

Preparation of Cysteine-Transportan

Cys-TP (GWTLNSAGYLLGCINLKALAALAKISIL-amide) was synthesized in a stepwise manner on a 0.1 mmol scale on a peptide synthesizer (Applied Biosystems model 431A, USA) using the *t*-Boc strategy. *Tert*-butyloxycarbonyl amino acids (Bachem, Bubendorf, Switzerland) were coupled as HOBt esters to a *p*-methylbenzylhydrylamine (MBHA) resin (Bachem, Bubendorf, Switzerland) to obtain the *C*-terminally amidated peptide. The peptide was finally cleaved from the resin with liquid HF at 273 K for 45 min in the presence of *p*-cresol. The purity of the peptide was >98% as demonstrated by HPLC on an analytical Nucleosil 120-3 C-18 RP-HPLC column (0.4 cm × 10 cm) and the correct molecular mass was obtained by plasma desorption mass spectrometry (Bioion 20, Applied Biosystems, USA).

EPR Spectroscopy of Spin-Labeled Membranes

A measure of 13 µl of 1 mM solution in ethanol of lipophilic spin probe, methyl ester of 5-doxyl palmitate MeFASL(10,3), was added to a plastic tube and evaporated. A volume of 100 µl of prepared liposomes was added to the tube. For control experiments, PBS was added before measurement, while for other experiments appropriate concentrations of Cys-TP in PBS were used. The tube was then rotated for 5 min (at a temperature above the main phase transition of DMPC) and subsequently hand-shaken for 3 min. The sample was transferred into a quartz-glass capillary (1 mm i.d.) and the EPR spectrum recorded on a Bruker ESP 300E spectrometer (Karlsruhe, Germany) with microwave frequency of 9.59 GHz, 20 mW power, modulation frequency 100 kHz, and modulation amplitude 0.1 mT. The microwave power was nonsaturating, as confirmed by the study of the dependence of signal intensity on the power. The ratio of the number of spin probe molecules to the number of lipid molecules was approximately 1/250.

Existence of different motional patterns of spin labels in different lipid domains, which can be formed due to the bilayer fluctuations, the lipid-phase separation, or the Cys-TP–lipid interaction leads to several superimposed EPR spectra. Consequently, spectra were fitted within a multicomponent fast-restricted wobbling motion approximation using a model that allows us to distinguish up to four spectral components [14]. The model parameters that are provided for each spectral component include the open-cone angle of the wobbling motion, asymmetry angle of the cone, effective rotational correlation time, polarity correction factor, as well as the proportion of each spectral component [15]. Owing to the complex algorithms applied to find the best fitting parameters, a large number of solutions are created, which need to be condensed. This is implemented in the applied condensation algorithm described previously [14]. From the condensed set of information, the open-cone angle and subsequently the order parameter S_z corresponding to particular spectral component can be extracted [15].

Assessment of Membrane Permeability by Spin-Label Reduction

Liposomes were prepared as described above except that they were hydrated with a 10 mM solution of the nitroxide spin probe *N*-1-oxyl-2,2,6,6-tetramethylammonium iodide (ASL) in PBS. For control experiments, PBS was added before measurement, while for other experiments appropriate concentrations of Cys-TP in PBS were used. In the beginning of the experiments the liposome dispersion was mixed with a 100-mM aqueous solution of sodium ascorbate. Decay in the intensity of EPR spectra of ASL with time was a measure of membrane permeability.

RESULTS AND DISCUSSION

Effect of Cys-TP on Lipid Membrane Structure

EPR spectra of liposomes prepared from DMPC lipids and spin-labeled with MeFASL(10,3) are compared in Figure 1(a) before and after the addition of Cys-TP. The study was undertaken at two temperatures, one (295 K) below the temperature of the main phase transition of DMPC (around 297 K) and one (305 K) above. The increased maximal hyperfine splitting, which is a measure of lipid ordering, for the sample with 1.48 mM Cys-TP at 295 K demonstrates that the addition of Cys-TP increases the order of the lipid membranes (compare the distance between the vertical bars in Figure 1(a)). Moreover, the level of ordering depends on the Cys-TP concentration (Figure 1(a)). This is consistent with the ordering effect of TP observed previously by fluorescence polarization measurements for palmitoyloleoylphosphatidylcholine [8]. The effect of Cys-TP was no longer distinguishable for the concentrations around or below 0.09 mM.

The change in lipid ordering upon the addition of Cys-TP can be evaluated also in a quantitative manner through EPR spectra analysis in terms of the multicomponent restricted fast-motion model [14]. Two spectral components have been used in simulations, while this was the minimum number of components
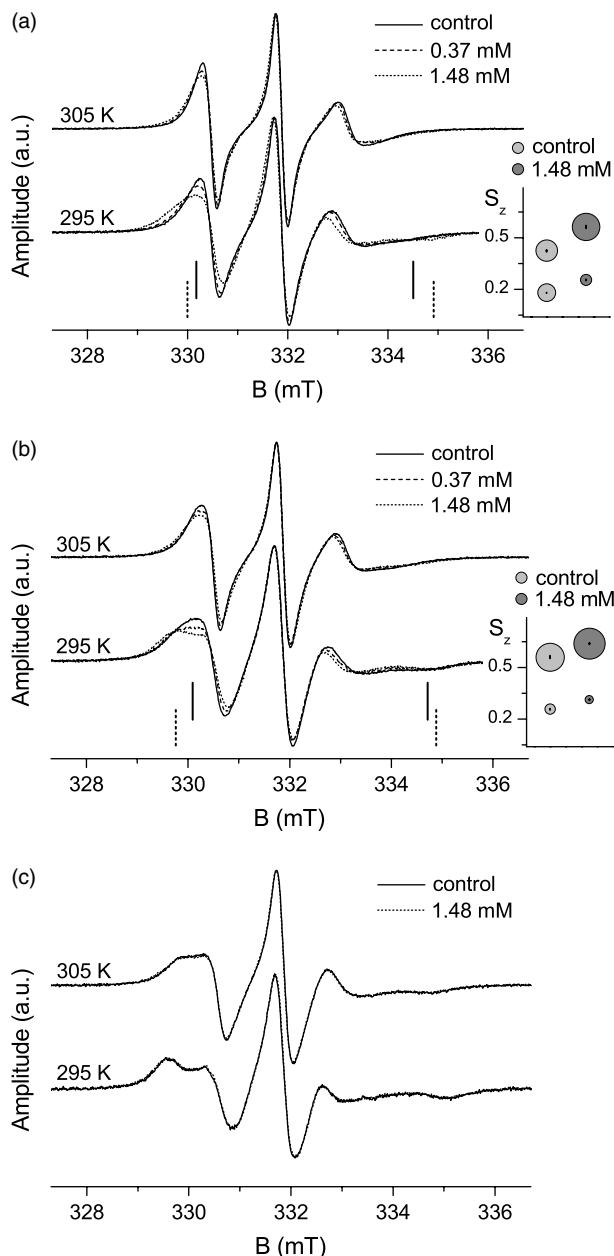
Figure 1 Dependence of the EPR spectra on the Cys-TP concentration and temperature for (a) DMPC, (b) Chol/DMPC 0.2 (mixture with 0.2 mole fraction of cholesterol), and (c) Chol/DMPC 0.4 (mixture with 0.4 mole fraction of cholesterol) liposomes. The horizontal distance between the corresponding vertical bars indicates the maximal hyperfine splitting, a measure of lipid ordering, for samples at 295 K; solid bars are for control samples, and dotted bars are for samples with 1.48 mM Cys-TP. The bubble diagrams in the insets for samples at 295 K show the values of the order parameter S_z (position of the bubbles) and the corresponding estimates of its uncertainty (size of the bar in the center of the bubbles), as well as the proportions of particular spectral components (diameter of the bubbles).

that allowed us to achieve a reasonable goodness of fit. The results for DMPC at 295 K are shown in a bubble

diagram (see inset of Figure 1(a)). Two bubbles for a particular sample represent two spectral components corresponding to two different motional patterns of spin labels. The position of the bubbles indicates the value of the order parameter S_z corresponding to a particular spectral component, while the diameter of the bubbles corresponds to the proportion of the spectral components. Results (see inset of Figure 1(a)) show that after the addition of Cys-TP the order parameter S_z for both components increases and also the proportion of a component having a higher S_z increases. Therefore, both changes are a sign of higher lipid ordering which is in agreement with the qualitative observation of the increased maximal hyperfine splitting.

Effect of Cholesterol on the Interaction of Cys-TP with Lipid Membrane

In order to study the effect of cholesterol on Cys-TP–lipid interaction, experiments were conducted on membranes formed from two lipid mixtures, Chol/DMPC 0.2 and Chol/DMPC 0.4, containing 0.2 and 0.4 mole fraction of cholesterol, respectively. The former mixture can exhibit the coexistence of liquid-disordered and liquid-ordered lipid phases at an appropriate temperature as defined by the corresponding phase diagram [16]. This lipid-phase coexistence is reflected in the two-component EPR spectra, where each component corresponds to a particular lipid phase [13]. On the other hand, Chol/DMPC 0.4 exhibits only the liquid-ordered phase at all temperatures. Model lipid membranes containing lipids in the liquid-ordered phase are valuable model systems for cholesterol-rich lipid domains (lipid rafts) in biological membranes [17].

The addition of Cys-TP to Chol/DMPC 0.2 membranes results in increased lipid order, as seen from the increased maximal hyperfine splitting (compare the distance between the vertical bars in Figure 1(b)) as well as from the higher order parameters and proportions of particular spectral components (see inset of Figure 1(b)). Similarly as in the case of DMPC, the effect is concentration-dependent (Figure 1(b)). The spectrum of Chol/DMPC 0.2 sample at higher concentration of Cys-TP, i.e. 1.48 mM Cys-TP, at 295 K (Figure 1(b)) resembles that of Chol/DMPC 0.4 sample without added Cys-TP at 295 K (Figure 1(c)), where the increased ordering is due to the level of cholesterol. No effect is observed on the addition of Cys-TP to Chol/DMPC 0.4 already at 1.48 mM, as seen from the superimposed lines for sample with or without Cys-TP (Figure 1(c)). This indicates that the more tight lipid packing in Chol/DMPC 0.4 resists the incorporation of Cys-TP in the lipid bilayer. This preventive influence of cholesterol has been observed for other peptides, such as the antimicrobial peptide nisin [18], the membrane-lysing peptides pardaxin and δ-lysin [19,20], the pore-forming peptide melittin [21], and CPP penetratin [7,12], suggesting a general influence of cholesterol.

Moreover, the addition of Cys-TP to Chol/DMPC 0.2 sample exhibiting the coexistence of two liquid-phases results in a spectrum that can be described as the linear combination of spectra for two samples with added Cys-TP, DMPC in the liquid-disordered phase and Chol/DMPC 0.4 in the liquid-ordered phase (Figure 2). Since Cys-TP has no effect on the cholesterol-rich liquid-ordered phase (Figure 1(c)), this result leads to the interpretation that Cys-TP in Chol/DMPC 0.2 samples only interacts with those lipids in the liquid-disordered phase.

Another indication that the above spectrum of Chol/DMPC 0.2 sample can be described as a combination of the spectra for the corresponding DMPC and Chol/DMPC 0.4 samples comes from EPR spectra simulations. The bubble diagram presented as the inset of Figure 2 shows that the order parameter S_z as well
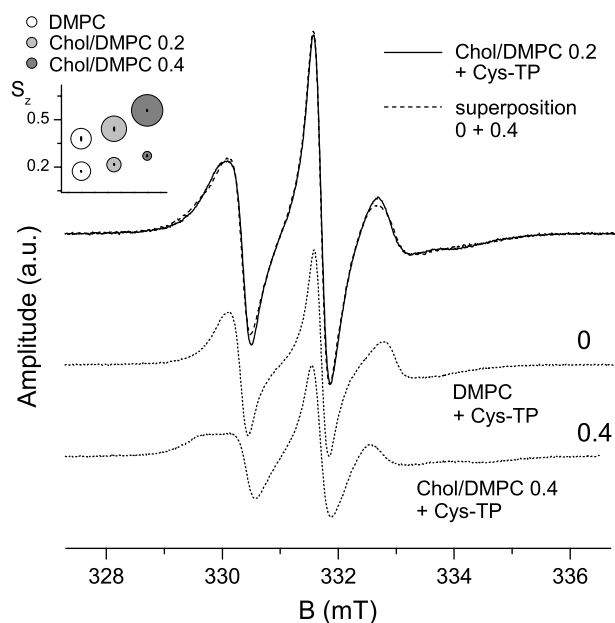

Figure 2 Comparison of the calculated and experimental EPR spectra of a Chol/DMPC 0.2 sample (mixture with 0.2 mole fraction of cholesterol) in the presence of 1.48 mM Cys-TP. The calculated spectrum was a linear combination of the spectrum for DMPC liposomes containing 1.48 mM Cys-TP (weight of spectrum 0.59), with that for Chol/DMPC 0.4 liposomes (mixture with 0.4 mole fraction of cholesterol), containing 1.48 mM Cys-TP (weight of spectrum 0.41). All experimental spectra were recorded at 305 K which is above the temperature of the main phase transition. At this temperature, the Chol/DMPC 0.2 membranes are separated into a liquid-disordered phase and a liquid-ordered phase, while the other two lipid membranes exhibit single lipid phases. The DMPC membranes are in the liquid-disordered phase, and the Chol/DMPC 0.4 membranes are in the liquid-ordered phase. The inset shows a bubble diagram summarizing the results of EPR spectra simulation for different samples where EPR spectra were fitted with two spectral components. See caption of Figure 1 for explanation of the information contained in a bubble diagram.

as the proportion for a particular spectral component exhibits an intermediate value for Chol/DMPC 0.2 sample. This means that Chol/DMPC 0.2 sample reflects properties of both DMPC and Chol/DMPC 0.4 samples.

Similar action of cholesterol that demonstrates the dependence of the peptide–lipid interaction on the form of lipid phase was proposed for δ-lysin and melittin and a mixture of palmitoyloleoylphosphatidylcholine, sphingomyelin, and cholesterol [20,21]. Although diverse models for membrane perturbation by different peptides exist, it is accepted that the perturbation depends on the lipid composition and membrane structure [22]. Taken together with our results, this implies that the CPP-membrane interaction is lipid-phase dependent.

Effect of Cys-TP on Membrane Permeability

The effect of CPPs or other peptides on membrane permeability has been studied by a range of methods. The most common approach is the fluorescence dye leakage assay [5,23]; others include electrophysiological measurements [24,25] and phase-contrast microscopy [26]. In the following it is shown that EPR can also be used to evaluate the effect of a CPP on membrane permeability.

For the EPR-based permeability assay a similar procedure was used as described before [27]. At the beginning of the experiments, liposomes prepared in a solution of the water-soluble nitroxide spin probe ASL are mixed with ascorbate, so the nonincorporated ASL is reduced into the corresponding EPR inactive hydroxylamine. In case of bilayer perturbation, also the incorporated ASL becomes accessible to ascorbate and is reduced by it. Therefore, decay in the intensity of EPR spectra of incorporated ASL is a measure of membrane permeability.

As seen from the decrease of the EPR spectral intensity in Figure 3(a), Cys-TP triggered reduction of the incorporated ASL by ascorbate in DMPC liposomes. Based on the size of ASL and/or ascorbate molecules and taking into account the hydration shell, the bilayer perturbation should have a lateral dimension of the order of magnitude of one to two nanometers in order to allow the passage of ASL and/or ascorbate molecules and their subsequent interaction. The narrow doublet feature superimposed on the nitroxide central line is due to the presence of ascorbate radical. This feature is more apparent for DMPC sample with added Cys-TP, where it causes the central line of ASL triplet to be higher than the other two lines (see the bottom spectrum in Figure 3(a)).

In contrast, the permeability of Chol/DMPC 0.4 membranes was not affected by the addition of Cys-TP, as seen from the comparable intensities of EPR spectra of ASL for samples with and without added Cys-TP (Figure 3(b)). In this case, the ascorbate radical
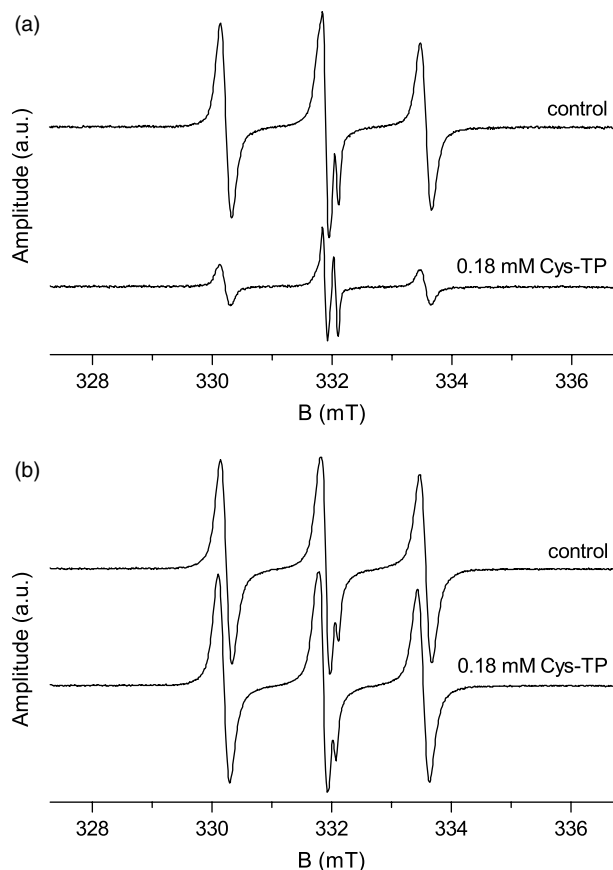
Figure 3 Dependence of the intensity of the EPR spectrum of ASL on the addition of Cys-TP for (a) DMPC and (b) Chol/DMPC 0.4 (mixture with 0.4 mole fraction of cholesterol) liposomes. The spectra were taken 3 min after the addition of the reducing agent ascorbate. The temperature was 305 K. The narrow doublet superimposed on the central line of the nitroxide triplet is due to the presence of ascorbate radical.

signal is due to the reduction of nonincorporated ASL. The absence of an effect on the membrane permeability for Chol/DMPC 0.4 samples is in accordance with the lack of effect on the membrane structure. This further supports the conclusion about the lipid-phase dependence of the Cys-TP–membrane interaction.

CONCLUSIONS

The use of spin-label EPR can offer valuable information about the effect of CPP on membrane structure as well as on membrane permeability. Our observations support the idea that in model membranes the action of Cys-TP is lipid-phase dependent. Specifically, it was shown that a high concentration of cholesterol in Chol/DMPC membranes, for which a liquid-ordered phase structure is induced, prevents structural and permeation effects of Cys-TP. This is important for understanding the interaction of CPPs or other peptides with the cell membrane, while the CPP–lipid interaction

could change along the membrane due to the different local lipid properties.

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