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Counterion-mediated membrane penetration: Cationic cell-penetrating peptides overcome Born energy barrier by ion-pairing with phospholipids

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Abstract

Arginine-rich cell-penetrating peptides (CPPs) can enter cells non-endocytotically, despite that transport of charge across a membrane should be formally associated with an extremely high Born energy barrier. We studied partitioning of several derivatives of the CPP penetratin in a water–octanol two-phase system in presence of natural phospholipids to explore if solvation by ion-pairing to hydrophobic counter-ions may serve as a mechanism for cell internalisation. We demonstrate that anionic lipids can aid peptide partitioning into octanol. Particularly efficient partitioning into octanol is observed with an arginine-rich penetratin compared to a lysine-rich derivative. Substituting tryptophans for phenylalanines results in poor partitioning into octanol, due to decreased overall peptide hydrophobicity. Partitioning into octanol is dependent of phospholipid type and the peptides induced structural changes in the lipid assemblies found in octanol. Attachment of carboxyfluorescein as a model cargo was found to enhance peptide partitioning into octanol. We discuss our results with respect to theoretical electrostatic energies, empirical hydrophobicity scales and in terms of implications for CPP uptake mechanisms. An important improvement of the theoretical transfer energies is obtained when, instead of singular ions, the insertion of ion-paired dipolar species is considered.

Keywords: Arginine; Tryptophan; Octanol; Partitioning; Cargo transport

1. Introduction

Biological membranes are efficient barriers distinguishing the cell interior from the external medium, protecting the cell from a potentially harmful environment as well as assembling the organelles and reactants that are necessary for the cell to function as a living entity. The high selectivity of the membrane, preventing passage of charged and else strongly polar molecules, is accomplished by the low-dielectric and non-polar properties of the hydrocarbon core. Diffusion-controlled transport through membranes often follows the empirical Overton's rule of a direct monotonous correlation between the ability of a molecule to enter cells and its partition coefficient in a hydrophobic medium [1]. Also theoretically, partitioning of a free ion into a low-dielectric reaction field should be associated with an extremely high free energy, primarily originating from the considerable difference in dielectric constant between the aqueous phase and the membrane hydrocarbon core which can be estimated from the Born equation as [2,3]:

$$\Delta G_B = \frac{q^2}{8\pi\varepsilon_0 r}\left(\frac{1}{\varepsilon_M}-\frac{1}{\varepsilon_w}\right) \approx 81\frac{z^2}{r} \quad (1)$$

where q is the charge of the ion, r is its effective radius, ε_M is the relative permittivity of the medium of the membrane hydrocarbon core (~2) and ε_W the relative permittivity of water (~80). If q is replaced by the formal ion charge z and r is expressed in Angstroms the right hand side of equality gives the Born energy barrier in kcal/mol. This energy cost is thus very high even for a large monovalent ion and, consequently, the transport of charge across a membrane in absence of carriers or ion-channels has on this basis long been regarded as impossible. However, this paradigm of membrane impermeability has recently been challenged by two findings. First, it has been shown that certain arginine-rich peptides can cross the plasma membrane by some non-endocytotic, ATP-independent mechanisms [4–6]. The translocation ability of these molecules,

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which belong to a group of peptide-based drug delivery vectors known as cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) is readily recognized although the entry mechanism is not yet understood. Second, it has been suggested, with support from crystallographic and electrophysiological data that the arginine-rich voltage sensor segment of the KvAP potassium channel regulates ion transport by inserting charged peptide residues into the membrane [7,8]. The latter example of mechanism has been vividly debated and even claimed unrealistic since the energy penalty associated with partitioning and transport of charged residues into the membrane hydrocarbon core should be too high to be overcome thermally by the gain in potential energy of a relatively modest translation of charge down the electric field. While the first case of membrane insertion appears generally accepted in the literature, despite the fact that no physical mechanism has yet been proven, the second case is still under strong debate [9–12]. Clearly, if partitioning of charged peptide residues into the membrane hydrocarbon core is indeed possible this suggests that the energy barrier predicted by the Born equation may somehow be surmounted. As will be argued in this paper an extension of the Born equation corresponding instead to the solvation of a dipole in the membrane hydrocarbon core could provide a more realistic model.

Interestingly, arginine-rich peptides have been reported to have superior translocation abilities compared to lysine-rich analogs [13] and there seems even to be an evolutionary bias towards arginines compared to lysines at the positions of cationic residues in the voltage sensor segments of K^+ channels [8]. This has been suggested to indicate that in both these cases arginine side-chains display unique, functional membrane transport characteristics, referred to as "arginine magic" [14]. Still, it should be noted that arginines (and also lysines) are strongly hydrophilic and that their high pK_a values ensure that they do remain protonated at relevant physiological conditions. Additional arguments for the inherent difficulty of partitioning cationic residues into the membranes hydrocarbon core has been proposed by the "inside-positive" rule which states that arginine and lysine residues in trans-membrane proteins are preferentially positioned on the cytoplasmic side of the membrane [15]. It has been suggested that membrane transport of arginine-rich peptides may be accomplished by charge-neutralisation at the membrane surface, possibly by the interaction with sufficiently hydrophobic counter-ions such as anionic phospholipids [13,16,17]. The resulting ion-pairs might be membrane soluble due to the hydrophobic character of the phospholipid tails and hence able to travel across the membrane and enter the cytoplasm.

This study was undertaken to explore the concept of counterion-mediated membrane solubilization of cationic peptides, and the possible applicability of this concept for the understanding of non-endocytotic uptake mechanisms for CPPs. Firstly, we wanted to study to what extent naturally occurring two-tailed phospholipids could be suitable as hydrophobic counterions and what amounts would be needed for efficient solubilization of a cationic peptide with significant charge. Secondly, we wanted to compare arginines to lysines to see if partitioning of cationic peptides into a hydrophobic phase due to ion-pair formation is an ability unique to arginines as has been suggested by Rothbard et al. [13]. The guanidinium moiety on arginine may, contrasting to other cationic amino acids such as lysines, be particularly efficient in forming bidentate hydrogen bonded ion-pairs to oxo-anions such as phosphates. The choice of peptide for our studies fell on the CPP penetratin, a 16 residue basic peptide which originates from the third helix of the Antennapedia homeodomain in Drosophila [18]. Penetratin contains seven positive charges (3 arginines and 4 lysines) and by substituting arginines for lysines and vice versa we created a set of peptides where the total charge is constant but the nature of the basic amino acids varies. The sequences of the four peptides used in this study are given in Table 1. In Fig. 1 are the molecular structures of the charged

Table 1
Peptide sequences (from N-terminal)

Peptide	Sequence	Arg/Lys
Penetratin	RQIK**W**FQNRRMK**W**KK	3/4
PenArg	RQIR**W**FQNRRMR**W**RR	7/0
PenLys	KQIK**W**FQNKKMK**W**KK	0/7
NonPen	RQIKIFFQNRRMKFKK	3/4

Charged residues (arginine and lysines) are underlined and tryptophans are marked in bold. All peptides have in total seven positive charges and the number of arginine and lysine residues is given.

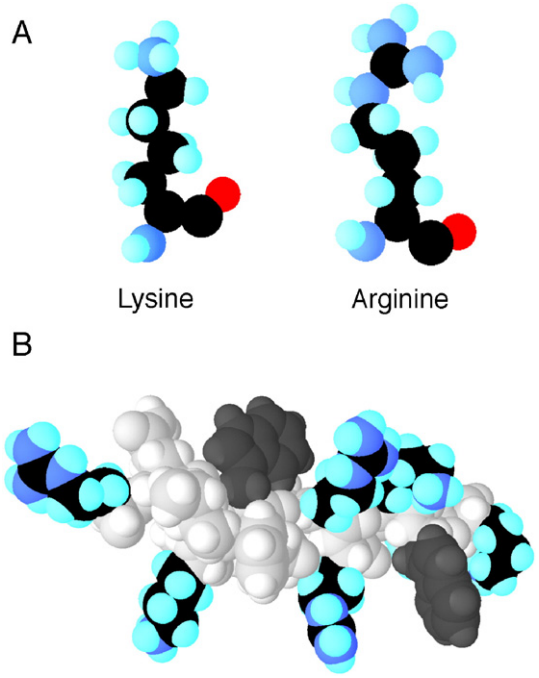

Fig. 1. (A) Ball representation of the molecular structure of lysine and arginine, with carbons in black, nitrogens in blue, oxygens in red and hydrogens in light blue. The atoms are scaled to represent their relative sizes but for clarity the radii are smaller than the van der Waal radii. (B) Van der Waals model of penetratin constructed in SwissPDB Viewer from the structure 1OMQ deposited in the Protein data bank by Lindbert et al. [19] showing the structure of penetratin in a bicellar system. Sidechains of charged residues are colour coded as in (A) and tryptophan residues are marked in grey. The peptide is oriented with the N-terminal to the left.

lysine and arginine side-chains and a van der Waals model of penetratin, constructed in Swiss PDB Viewer from the NMR model of penetratin bound to acidic micelles [19] (deposited in the Protein Data Bank as structure 1OMQ). The van der Waals model of penetratin is given to indicate the bulkiness of this peptide in its folded α-helical state. Penetratin and its analogs bind strongly to acidic membranes [20]. These peptides fold as random coils in solution but adopt partial α-helical conformation upon interaction with membranes [21,22]. However, penetratin may also adopt β-sheet structure at high peptide-to-lipid ratios under conditions where lipid vesicles aggregate [22,23]. Even though arginine-rich peptides are currently among the most efficient CPPs [6,24], most peptide transporters include other amino acid residues as well. For penetratin in particular the importance of two tryptophan residues for efficient uptake has been debated [6,18,25]. Since these two tryptophans add significantly to the total hydrophobicity of penetratin we wanted to investigate if they have a positive effect on the partitioning of penetratin into octanol compared to an analogous peptide where the tryptophans were replaced by phenylalanines. Lastly, while cell-penetrating peptides are frequently used to efficiently deliver cargo molecules into cells, little is known about how the chemical nature and size of the cargo affects uptake efficiency or route of uptake (endocytotic or by passive diffusion). One reason why CPPs and CPP-cargo constructs have not been compared is the lack of reliable cell assays where the internalisation of an unlabelled CPP can be quantified. In most studies peptides are fluorescently labelled and this small cargo molecule is assumed not to affect the characteristics of the peptide. For this reason we shall also compare how the octanol partitioning of penetratin is influenced by an N-terminal carboxyfluorescein cargo.

2. Materials and Methods

2.1. Materials

Peptides were synthesized by standard f-moc chemistry on a Pioneer Peptide Synthesizer (Perspective Biosystems) as previously described [26]. Fluorescent peptides were obtained by reacting the open N-terminus with carboxyfluorescein (CF) succinimidyl ester (Molecular Probes), the corresponding non-labelled versions were acetylated [23]. Peptides were purified by HPLC and the identity was confirmed by mass spectrometry. 1-Octanol (spectrophotometric grade) was from Sigma. 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol (DOPG), 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid (DOPA) and soy-derived L-alpha phosphatidylinositol (soy PI) were from Larodan Fine Chemicals (Malmö, Sweden). The fatty acid content in soy lecithin is approximately 48% linoleic acid, 22.6% oleic acid and 17% palmitic acid [27] which makes the soy PI lipids somewhat more unsaturated than the dioleoyl lipids. 1,2-Dioleoyol-*sn*-glycero-3-phospho-L-serine (DOPS) was from Alexis Corp. and 1,2-dioleoyl-*sn*-Glycero-3-Phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl) (Rh-PE) was from Avanti Polar lipids. The buffer used was 0.1 M Tris–HCl, pH 7.4. Buffer chemicals were from Merck. Deionised water from a Milli-Q system (Millipore) was used.

2.2. Sample preparation

CF-fluorescein labelled peptides were dissolved in 0.1 M Tris–HCl buffer (pH 7.4). The concentration of CF-labelled peptide was determined in pH 9 buffer using an extinction coefficient of 78,000 M^{-1} cm^{-1} at 498 nm. The peptide stock solutions were diluted to 25 μM corresponding to a total absorbance of ~2. 200 μl aliquots of peptide solution were transferred to Eppendorf tubes and an equal volume of 1-octanol was added. Lipid was dissolved in 5–10 μl chloroform and added to the samples which were then vortexed for 30 s and thereafter centrifuged for 5 min at 10,000×*g*.

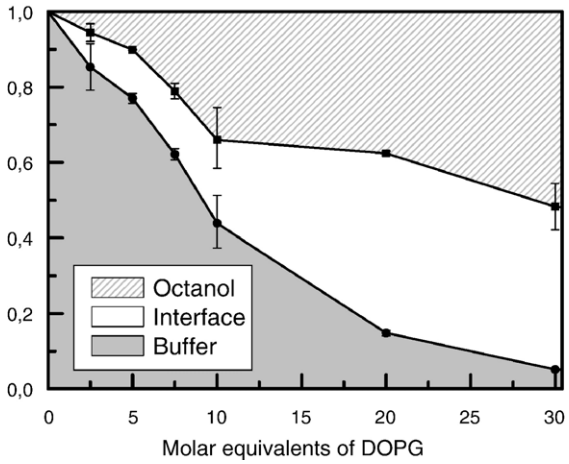

Fig. 2. Partitioning of CF-labelled PenArg as a function of lipid concentration. The fraction of peptide in each phase – aqueous buffer (grey), interface (white) and octanol (hashed) – is plotted versus the molar equivalents of the anionic lipid DOPG. Using a large amount of lipid it is obviously possible to extract practically all peptide from the aqueous phase.

2.3. Analysis of peptide partitioning

The amount CF-peptide in each phase was determined by absorbance. In order to obtain equivalent solvent conditions 100 μl from the water phase was dissolved in 500 μl methanol/octanol (4:1) and 100 μl from the octanol phase was dissolved in 500 μl methanol/buffer (4:1). Absorbance spectra were recorded at room temperature on a Cary 4000 UV-Vis Spectrophotometer (Varian Inc) from 350 to 650 nm in 1 nm increments with a scan speed of 300 nm/min and a band pass of 1 nm. Cationic peptides has a strong propensity to adsorb on glass, plastics, pipette tips, etc. and to avoid overestimating the total concentration in the samples after vortexing it was necessary to determine the total concentration from blank samples were no lipid was added (100% of the peptide remained in the aqueous phase). Thereafter the absorbance in octanol and water was measured. The sum of the absorbance in octanol and water did not equal the total concentration of the blank samples and the remaining part was thus assumed to have accumulated at the interface. This accumulation could also be seen visually when placing the sample under a UV lamp. The partitioning of unlabelled peptides (acetylated N-terminus) was determined using the intrinsic fluorescence emission from its two tryptophan residues. The extinction coefficient of tryptophan is too low to allow for accurate absorbance measurements in the concentration range used in these experiments. Tryptophan emission was measured on SPEX Fluorolog τ-3 spectrophotometer (JY Horiba) using a reduced 0.4×1 cm quartz cell. Emission was recorded at 340 nm upon excitation at 280 nm using an excitation bandpass of 1 nm and an emission bandpass of 4 nm. 15 data points were recorded during 30 s and subsequently averaged. Data were corrected for background contributions.

2.4. Analysis of peptide-induced lipid redistribution

In order to investigate how the lipid partitions between octanol and interface in the two-phase system, experiments were performed where 10 molar equivalents of a lipid mixture of DOPG and Rh-PE (9/1 molar ratio) was added to samples containing CF-labelled PenArg. The rhodamine-labelled phosphatidylethanolamine lipid is net negatively charged. Partitioning of CF-peptide and lipid was quantified simultaneously by recording absorbance spectra between 400 and 620 nm using the same settings as above.

2.5. Dynamic light scattering (DLS)

DLS was used to characterize peptide–lipid complexes formed in the octanol phase during the partitioning experiments. The experiments were performed on a ALV CGS-8F DLS/SLS-5022F instrument (ALV, Germany), equipped with an ALV-6010/160 correlator and dual APD detectors at 25 °C. The wavelength of the incident light was 632.8 nm and the scattering angle was set to 90°. Data were collected for a period of 300 s.

3. Results

3.1. Partitioning of peptide as a function of lipid concentration

The penetratin peptides investigated in this study do not partition into the non-aqueous phase of the octanol–water system when in absence of hydrophobic counter-ions. Fig. 2 illustrates partitioning of the most effective peptide in this study, PenArg, as a function of lipid concentration in an interval ranging from 0 to 30 molar equivalents of DOPG. Peptide in the non-aqueous phase is distributed between octanol and an interface region. The latter phase takes up a very small volume at 10 molar equivalents of lipid (< 1%), but could increase modestly in volume at high lipid concentrations (20 molar equivalents and above). In either case the peptide concentration in the interface is far higher than in the octanol or water. The partitioning into the octanol phase and interface increases linearly with the number of lipid equivalents added at low ratios until approximately 50% of the peptide has left the aqueous phase. Thereafter the increase in octanol partitioning levels off, whereas partitioning to the interface still increases steadily. We conclude that it is indeed possible to extract effectively all peptide from the aqueous phase using a high amount of lipid counter-ions.

3.2. Peptide-induced redistribution of lipid in the non-aqueous phase

The appearance of two seemingly distinct non-aqueous phases, namely octanol and interfacial region, has been further examined in terms of intrinsic and peptide-induced lipid partitioning between these two phases. Fig. 3A shows partitioning of carboxyfluorescein-labelled (CF) penetratin and rhodamine-labelled (Rh) lipid measured by absorbance. In absence of peptide the lipid distribution between octanol and interface is typically 80/20. The amount of lipid in the interface can form a surface approximately 25 times the area of the interface between water and octanol in an Eppendorf tube if we assume that each lipid has a cross-sectional area of 70 $Å^2$ [28]. Addition of peptide to the system results in lipid accumulation in the interfacial region (seen as a decrease in the rhodamine absorption in the octanol sample in Fig. 3A). The amount of lipid transported from octanol to the interface corresponds to 8 lipids per peptide molecule which is very close to the number of lipids needed to formally neutralize the seven positive charges in penetratin. Still, the number of lipids per peptide is almost twice as high in the octanol phase (21:1) as in the interfacial region (12:1). To get insight about the lipid–peptide complexes in the octanol phase, dynamic light scattering (DLS) was used. Fig. 3B shows autocorrelation functions (ACFs) for DOPG (solid line) and DOPG-PenArg (dotted line) complexes in octanol. The anionic lipid is found to assemble in aggregates in the octanol. The irregular shape of the ACF suggests that these aggregates are large and polydisperse and as they may not be spherical it was not found meaningful to characterize their size using the Stokes–Einstein approach. However, addition of peptide obviously decreases the decay time of the ACF which indicates formation of smaller aggregates. An almost exponential shape of the ACF also suggests that these complexes are

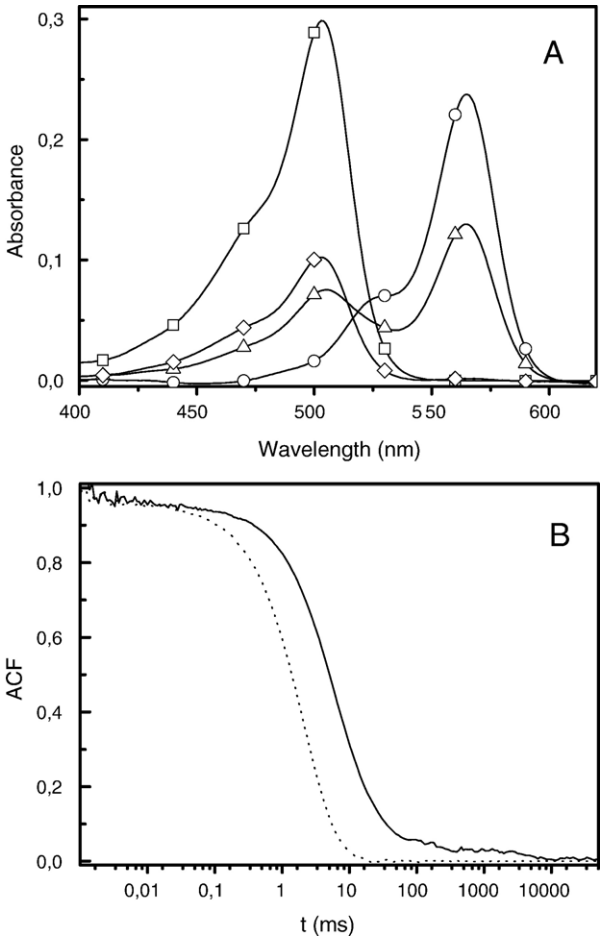

Fig. 3. (A) Partitioning of CF-labelled penetratin (λ_{max} ~498) and rhodamine-labelled lipid (λ_{max} ~565) measured by absorbance. The lipid to peptide ratio was 10:1 and rhodamine-labelled lipid constituted 10% of the total lipid. (–□–) corresponds to the total peptide concentration measured in absence of lipid. (–○–) corresponds to the initial concentration of lipid in octanol phase measured in absence of peptide. This amounts to approximately 80% of the total lipid added, the remaining fraction was found to accumulate at the interface. and (–Δ–) is the absorbance in buffer and octanol, respectively, for a sample containing both peptide and lipid. (–◇–) shows the complete absence of lipid in the octanol phase, but also that a significant amount of peptide has been transferred from water compared to in absence of lipid (–□–). (–Δ–) shows the amount of peptide transferred into octanol, but also that the lipid concentration in octanol decreases in presence of peptide. (B) Dynamic light scattering (DLS) intensity auto-correlation functions (ACFs) for DOPG (solid line) and DOPG/CF-PenArg (dotted line) in octanol. DOPG lipid assembles into large polydisperse aggregates in the octanol phase. Addition of peptide causes a considerable shift of the ACF towards shorter lagtimes, indicating formation of significantly smaller aggregates.

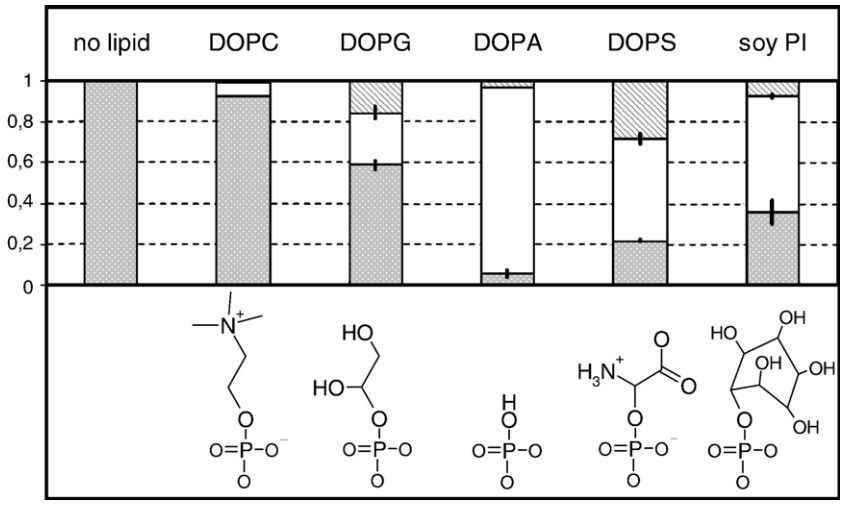

Fig. 4. Water–octanol partitioning of CF-labelled penetratin in the presence of 10 peptide-to-lipid molar equivalents of lipid. The columns show the fraction of peptide found in buffer (grey), 1-octanol (hashed) and interface region (white). The error bars show maximum and minimum values from 2 to 4 independently prepared samples and have been added where deviations from the arithmetic mean exceeds 1%. The lower panel shows the structure of the lipid headgroups.

more well-defined, with a diameter of approximately 100 nm under the assumption that the particles are spherical. To exclude the possibility that the lipid structures in octanol encapsulates bulk water we performed control experiments where the partitioning of free water soluble carboxyfluorescein between octanol and water was assessed in absence and presence of DOPG. No dye could be detected in the octanol phase.

3.3. Effect of lipid type on peptide partitioning

It is shown in Fig. 4 how partitioning of penetratin is affected by the presence of different phospholipids. Control experiments with PenArg, PenLys and NonPen showed the trend of partitioning efficacy with PenArg > penetratin > PenLys > NonPen (see Fig. 5) for all lipids (data not shown). These results clearly indicate that hydrophobic counter-ions are necessary for making penetratin partition into octanol, but do also show that the type of lipid head-group affects partitioning between aqueous and non-aqueous phases as well as the distribution between octanol and interface. Fig. 4 illustrates the superiority of anionic lipids as counter-ions, although a small amount of peptide can leave the water phase in presence of zwitterionic DOPC too.

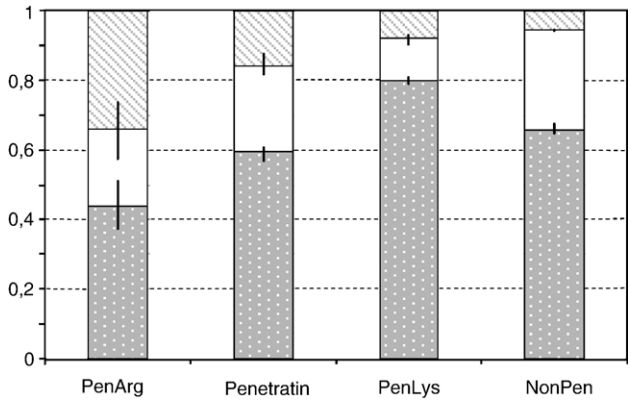

Fig. 5. Partitioning of CF-labelled PenArg, penetratin, PenLys and NonPen in presence of 10 molar equivalents of DOPG. The columns show the fraction of peptide found in buffer (grey), 1-octanol (hashed) and interface region (white). The error bars show maximum and minimum values from 2 to 4 independently prepared samples and have been added where deviations from the arithmetic mean exceeds 1%. Partitioning into octanol increases almost linearly with the number of arginine residues from PenLys to PenArg. The two tryptophan residues in Penetratin appear to have a profound effect on partitioning into the octanol phase (Penetratin compared to NonPen), whereas removal from water appears to be more equal.

3.4. Effect of peptide sequence on partitioning

To address how partitioning in octanol depends on nature of basic amino acid (arginines versus lysines) penetratin was compared to the arginine- and lysine-enriched analogs PenArg and PenLys in presence of 10 molar equivalents of DOPG. Fig. 5 clearly shows that arginine residues favour partitioning into the non-aqueous phases over lysines. The ability to enter the octanol phase increases linearly with the number of arginine residues in the sequence from PenLys to PenArg. For PenArg 34% of the initial added peptide accumulates in octanol which corresponds to a concentration of 8 μM. The fraction remaining in buffer amounts to 44% which corresponds to a concentration of 11 μM. Fig. 5 also shows measurements on the peptide NonPen which is less partitioned into octanol compared to penetratin. By contrast, if partitioning between water and the octanol and interface phases is considered, NonPen and penetratin behave very similarly. We have calculated the free energies of transfer from the experimental data in Fig. 5 and these amounts to −0.14 kcal/mol for PenArg, +0.21 kcal/mol for penetratin, +0.82 kcal/mol for PenLys and +0.38 kcal/mol for NonPen, which in turn indicate how much the energy barrier for transfer from water has been lowered by interaction with lipids. These partition coefficients correspond to Boltzmann factors of 7, 0.05, 10^{-6} and 0.004 respectively, showing clearly that lipid–arginine interaction leads to favourable partitioning in octanol.

Table 2
Comparison of partitioning of peptide with acetylated N-terminus compared to its corresponding CF-labelled version

Peptide	Percentage (%) in octanol			
	Acetylated		*CF-labelled*	
PenArg	23	(±3.3)	34	(±8.5)
Penetratin	10	(±3.2)	16	(±2.5)
PenLys	4	(±0.4)	8	(±1.5)

Partitioning efficiency is given as the percentage of peptide in the octanol phase after addition of 10 molar equivalents of DOPG. The partitioning was determined from tryptophan fluorescence emission experiments for the acetylated peptides and from carboxyfluorescein absorbance measurements for the CF-labelled peptides.

3.5. Effect of carboxyfluorescein on peptide partitioning

Conjugation of CPPs to cargos is generally known to substantially reduce the cellular uptake efficiency or to redirect uptake to endocytotic routes. By comparing partitioning of acetylated and CF-labelled peptides into octanol we show that even a small cargo can significantly affect the ability of the peptide to enter a hydrophobic phase. These data are presented in Table 2 as the percentage of initial added peptide found in the octanol phase for acetylated and CF-labelled peptides respectively. The partitioning of acetylated peptide was measured using fluorescence from the tryptophan residues, wherefore this experiment could not be performed for NonPen. Interestingly, the carboxyfluorescein covalently tethered to the penetratin peptides enhances octanol partitioning compared to the corresponding unlabelled peptide with acetylated N-terminus.

4. Discussion

We have shown that natural, net negatively charged phospholipids can mask the positive charges of arginines and lysines of the CPP penetratin making this peptide soluble in octanol despite its overall hydrophilic character. Our results demonstrate that the free ion Born charging energy barrier may be surmounted by ion-pairing of cationic residues at the membrane surface. Albeit the conditions in a bilayer lipid membrane are from several aspects different from the octanol–water model system, the concept that intrinsic lipid molecules may accompany a peptide molecule to 'grease' its way through the membrane is intriguing and deserves serious consideration. Central for understanding the results from this study are the interactions between lipids and peptides: First, we shall discuss the characteristics of the octanol–water model system and the nature of the lipid–peptide complexes that form in the non-aqueous phase. Second, the peptide–lipid interactions will be considered in terms of how different lipids may affect the strength of the ion-pairs differently and the possible origins of variations observed in transfer efficiency between arginines and lysines. Lastly, we will address the potential application of our results for understanding uptake mechanisms for cationic CPPs in general.

4.1. Lipid–peptide interactions in octanol — characteristics of the model system

We demonstrate in Fig. 2 that partitioning of peptide (PenArg) into octanol can be accomplished by adding negatively charged DOPG, and that it is possible to extract almost all peptide from the aqueous phase in presence of a large excess of lipid. This implies that natural phospholipids can ion-pair to positive charges on a peptide with a high charge density. Seven lipid equivalents would in principle be sufficient to ion-pair all cationic residues of PenArg, but at this ratio as much as 60% of the peptide is found to be left in the aqueous phase and only around 20% is dissolved in octanol, indicating that the equilibrium is still in favour of water-soluble peptide. Peptide that has partitioned into the non-aqueous phase is distributed between octanol and an interfacial region. To further understand the origin of the partitioning of peptide and lipids between the non-aqueous phases let us focus on the peptide–lipid complexes that form in the model system. Lipid redistributes in the non-aqueous phases after peptide addition, resulting in extraction of lipid from the octanol to the interface (Fig. 3A). Despite this extraction the peptide-to-lipid ratio in octanol is almost twice of that in the interface. Lipid dissolves in octanol as large polydisperse aggregates, which is to be expected since exposure of the negatively charged head-group to the hydrophobic solvent would be unfavourable. However, control experiments with free carboxyfluorescein dye confirmed that these aggregates do not contain bulk water. Addition of peptide (PenArg) to the octanol-buffer system with DOPG decreases the size and polydispersity of lipid aggregates in octanol (Fig. 3B). The hydrodynamic diameter of the lipid–peptide aggregates in octanol could be estimated to approximately 100 nm assuming spherical particles. The large aggregate size implies that each peptide is not individually dissolved by lipids, but the importance of peptide hydrophobicity for efficient partitioning in octanol (see below for comparison of penetratin and NonPen) suggests that the peptide is indeed exposed to the octanol phase. Previous studies have shown that penetratin cause aggregation of anionic liposomes at ratios similar to those we have in the interface (12:1) and that this type of aggregation is virtually irreversible [23]. By contrast, for lipid-to-peptide ratios similar to those in octanol (21:1) liposome aggregation does not occur. Therefore, it seems reasonable that penetratin may stabilize formation of compactly packed lipid structures in the interface region. In addition, since penetratin peptides interact strongly through electrostatic interactions with lipid membranes, a possible explanation for tighter packing is that the peptides reduce the local charge density in the lipid aggregates.

4.2. Peptide-lipid interactions and transfer efficiency — effects of lipid

Figs. 2 and 5 clearly demonstrate that DOPG is a potent lipid for ion-pairing and transfer of the penetratin peptides from water to octanol, and the subsequent step in our study was to compare DOPG to other naturally occurring phospholipids. Fig. 4 illustrates the absolute requirement of counter-ions for

transfer, but also that a net negative charge on the lipid head-group is necessary for transfer into octanol. Consistent with the notion that arginine residues can interact strongly with phosphates [29], we notice some accumulation of peptide in the interface also when using DOPC as a "counter-ion" although penetratin has been reported not to associate to zwitterionic lipid membranes [30]. The importance of the negative charge is underscored from the experiments with DOPA which almost completely removes penetratin from the water phase when added at ten molar equivalents. Since the pK_a for ionization of a second OH group on the phosphate in DOPA is 8.0, approximately 20% of the lipids will carry two negative charges at the pH used in this study (7.4), which would allow ion-pairing to two cationic amino acid residues simultaneously. At the same time the divalent negative charge on DOPA is localised on a small lipid head-group making DOPA less hydrophobic than for example DOPG. We believe this may explain why DOPA–peptide complexes are prone to accumulate at the interface. In fact, DOPA has the least hydrophobic head-group of all the lipids in this study, followed by DOPS. In addition it may be argued that the PI head-group is more hydrophilic than that on DOPG due to a larger number of OH-groups. From Fig. 4 we conclude that the peptide partitioning between aqueous and non-aqueous phases qualitatively follows the same pattern as the head-group hydrophobicity. In addition, partitioning into octanol is less efficient using lipids with more hydrophilic head-groups. Although DOPS is an exception, this indicates that the lipid head-group hydrophobicity is an important parameter for peptide–lipid ion-pairing that leads to solvation in octanol. It is noteworthy that DOPA, DOPS and PI all seem to interact more efficiently with penetratin than does DOPG since it has been reported that there is no difference in the binding affinity of penetratin to DOPG or DOPS liposomes [20].

4.3. Peptide-lipid interactions and transfer efficiency–arginines versus lysines

Comparison of penetratin and its arginine-to-lysine and lysine-to-arginine substituted derivatives PenLys and PenArg reveals a clear connection between the number of arginine residues and transfer efficiency (Fig. 5). However, a significant observation is that our arginine-deficient peptide PenLys shows significant transfer from the water phase (20% using DOPG as counter-ion). We have previously reported that PenLys cannot enter cells [6] and others have emphasized that peptide transfer into membrane-mimicking phases aided by counter-ions should be an exclusive property of arginines compared to other cationic amino acids [13]. Rothbard et al. observe almost complete (>95%) transfer of a fluorescein-labelled octa-arginine in presence of sodium laurate compared to no detectable transfer at all for the lysine analog ornithine. Our data suggests that membrane transport aided by ion-pairing should be possible also for lysine-enriched peptides, albeit less efficient. As a first simplified comparison between arginine and lysines one can calculate the Born energy barrier (Eq. (1)) for the transfer of one guanidinium ion compared to one ammonium ion from water into the membrane. Both are monovalent, but the delocalisation of charge on the guanidinium results in this ion having a larger effective radius. Since the Born energy is inversely proportional to the radius this indicates that arginines may have less difficulty to partition than lysines. If the radii of the guanidinium ion on arginine and the ammonium ion on lysine are taken as, respectively, 2.5 Å and 2 Å [15], the Born energies amount to 32 kcal/mol and 40 kcal/mol. A second way of comparing arginines to lysines is by using the inherent hydrophobicity of PenArg, penetratin and PenLys, calculated from the empirical Wimley and White whole-residue-hydrophobicity scale for partitioning into octanol [31,32]. The transfer free energies are 8.9 kcal/mol, 12.9 kcal/mol, and 15.9 kcal/mol for PenArg, penetratin and PenLys, respectively, again showing that an increasing number of arginine residues should decrease the free energy of transfer from water to the hydrophobic phase. Importantly, these values are significantly smaller than those calculated using the Born equation. It is important to recognize that the Born equation comprises a simplified treatment of ion solvation in the sense that it considers the aqueous phase and the membrane hydrocarbon region as infinite dielectric continuums. Already in the simple example of transmembrane movement of cations such as potassium, it has been reported that the ion flux is indeed much greater than predicted by the Born energy. All theoretical explanations for this behaviour involve the formation of narrow transient holes or water-channels, which are presumably too small to allow transport of peptides. We here explore an alternative explanation for the partitioning of charged polypeptides into a non-polar medium, namely the reduction of the Born energy barrier imposed by considering solvation of a dipole rather than a naked ion [33,34]:

$$\Delta G_{\text{dipole}} = \frac{\mu^2}{12\pi\varepsilon_0 a^3}\left(\frac{\varepsilon_w - 1}{2\varepsilon_w + 1} - \frac{\varepsilon_M - 1}{2\varepsilon_M + 1}\right) \approx 32\frac{R^2}{a^3} \qquad (2)$$

with μ the molecular dipole moment and a the effective dipole size. This equation may be rewritten for the special case of interaction of two identical opposite monovalent charges, separated by the distance R. With R and a in Angstroms the right-hand side of equality gives the energy of transferring a dipole from water into the hydrocarbon core in kcal/mol. According to this equation, the energy barrier for partitioning a guanidinium–chloride dipole is 0.93 kcal/mol and the corresponding barrier for the partitioning of the ammonium–chloride dipole would be 1.05 kcal/mol if the radius of the chloride ion is 1.8 Å and the ions are treated as adjacent spheres. These values are indeed reasonably close to what is empirically estimated by the Wimley and White hydrophobicity scale where the free energies would be 1.81 kcal/mol for arginine and 2.8 kcal/mol for lysine. The great discrepancy between the Born theory for isolated charges and the Wimley and White experimentally determined hydrophobicity scale suggests that amino acids never dissolve into the membrane hydrocarbon core as free ions, but rather enter as ion-pairs with an accompanying counter-ion. If, as shown above, this ion-pair is treated as a permanent dipole, the calculated theoretical

energies of transfer become in reasonable agreement with experiment. However, the theoretical energy barrier to be overcome for a peptide like penetratin with seven positive charges is still extremely high, corresponding to a Boltzmann factor (at room temperature) in the order of 10^{-6} per residue for a guanidinium–chloride pair (or $<10^{-50}$ for the PenArg peptide using the free energy of transfer according to Wimley and White). Comparison of these theoretical values to the experimentally determined free energies in the Results section may be made in terms of the number of ion-pairs to lipid counter-ions that are needed in order to obtain transfer. A relevant estimate of the reduction in transfer free energy due to ion-pair formation is 4 kcal/mol which has been empirically determined for intra-peptide ion-pair formation between arginine or lysines and the carboxylic c-termini of a pentapeptide [35].

4.4. Peptide-lipid interactions and transfer efficiency–tryptophans

Tryptophan is the most hydrophobic amino acid in the empirical Wimley and White hydrophobicity scale. Clearly the tryptophan-to-phenylalanine substituted peptide NonPen has less ability to enter the octanol phase than either penetratin or PenLys (Fig. 5). This is in good agreement with the theoretical free energy of transfer from water to octanol calculated using the Wimley and White hydrophobicity scale (13.6 kcal/mol for NonPen compared to 12.9 kcal/mol for penetratin). On the other hand, when our result for partitioning between aqueous and non-aqueous phases is considered there is little difference between penetratin and NonPen. Thus, the hydrophobicity is seemingly not a decisively important parameter in the interaction with negatively charged phospholipids implying that these interactions are largely of electrostatic character. The fact that the overall peptide hydrophobicity is important for solvating the peptide–lipid complex in octanol indicates that parts of the peptide are exposed to the octanol phase, suggesting in turn that the peptide is not completely buried in lipids although we have shown above that peptide and lipids assemble into large aggregates.

4.5. Carboxyfluorescein cargo enhances octanol partitioning

Studies of CPP uptake in cells involve, with few exceptions, the conjugation of the CPP to a cargo which in the simplest case is the fluorescent dye used to detect cell entry. It is established that large cargo molecules generally slow down uptake efficiency and in the case of oligoarginines it has been shown that largo cargos redirect the route of entry to be endocytotic [16,36,37]. Studies comparing uptake characteristics of labelled and unlabelled CPPs are rare. The water–octanol system in this study offers a good possibility to study how the negatively charged carboxy-fluorescein (CF) tag attached to the N-terminus of penetratin peptides influences partitioning compared to the corresponding acetylated peptide. The data in Table 2 show that the peptide partitioning in octanol is markedly enhanced for the CF-labelled peptide, indicating that the hydrophobic carboxyfluorescein anion functions as an endogenous charge neutraliser by forming an ion-pair with one of the positive amino acids in the peptide.

4.6. Implications for non-endocytotic delivery of CPPs

Octanol partitioning experiments show that there is an energetic possibility for the charged penetratin peptides to become introduced into a hydrophobic environment by ion-pairing, but it does not offer any explanation to whether uptake through a biological membrane could occur via such a mechanism or how it physically happens. We propose that the lipid–peptide interactions in octanol may serve as a primitive model of the "transition state" interactions that occur during membrane crossing, and thus the free energy of transfer calculated using the Wimley and White hydrophobicity scale can be thought of as an activation barrier. Transmembrane electric potentials (negative inside) across the plasma membrane have been suggested as a driving force for cell entry of penetratin and subsequently also oligoarginines [38], even though later studies have produced contrasting results [39]. Under the influence of an electrical potential, peptide entry might be regarded as an electrophoretic phenomenon and incomplete charge neutralisation of the peptide would be required. As noted when discussing hydrophobicity and ion-pair formation in relation to the Born equation (vide supra), it is not likely that peptides enter the membrane carrying naked charges and, thus we find a purely electrophoretic transport very unlikely. A model based on formation of inverted micelles was an early suggestion for the entry mechanism of penetratin [40], but no experimental evidence for such a mechanism has ever been presented. Using DLS we searched for evidence of inverted micelles in the octanol phase (Fig. 3B) but without any positive result. On the other hand, we have here shown that the penetratin peptides clearly have ability to induce structural changes of the lipid aggregates in the octanol phase. From our conclusions in this study, we propose that a cationic peptide cannot enter the membrane unless it is ion-paired to a counter-ion. By replacing solvent counter-ions for phospholipids the peptide may be able to cross the membrane in a step-wise manner assisted by a few lipids that sit at the rim of a membrane invagination which is formed by the initial peptide membrane interaction and which can transiently break the membrane bilayer structure. Such a mechanism would enable the peptides to move across a membrane without directly exposing their charged residues to the hydrocarbon core, thereby overcoming the Born energy barrier. Arginine-rich peptides are better than lysine-rich peptides in entering cells via such a passive diffusion mechanism probably simply because fewer hydrophobic counter-ions are needed to increase the peptide hydrophobicity.

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