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REVIEW

Cell Penetrating Peptides: How Do They Do It?

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Abstract Cell penetrating peptides consist of short sequences of amino acids containing a large net positive charge that are able to penetrate almost any cell, carrying with them relatively large cargoes such as proteins, oligonucleotides, and drugs. During the 10 years since their discovery, the question of how they manage to translocate across the membrane has remained unanswered. The main discussion has been centered on whether they follow an energy-independent or an energy-dependent pathway. Recently, we have discovered the possibility of an energy-independent pathway that challenges fundamental concepts associated with protein-membrane interactions (Herce and Garcia, PNAS, 104: 20805 (2007) [1]). It involves the translocation of charged residues across the hydrophobic core of the membrane and the passive diffusion of these highly charged peptides across the membrane through the formation of aqueous toroidal pores. The aim of this review is to discuss the details of the mechanism and interpret some experimental results consistent with this view.

Keywords Cell penetrating peptides · Drug delivery · Arginine-rich peptide · Cellular uptake mechanism · Intracellular protein delivery · Membrane translocation · Antimicrobial peptides · Peptide uptake

1 Introduction

Cell penetrating peptides (CPPs) can be defined as short sequences of amino acids (<30 amino acids) capable of translocating across the cell membrane in a seemingly energy-independent manner [2–4]. Here, we are interested in CPPs that are highly cationic and hydrophilic, exhibiting relatively low or no amphiphilicity when compared to antimicrobial peptides. CPPs have the special property of carrying with them cargoes of a wide range of molecular size such as proteins, oligonucleotides, and even 200 nm large liposomes [5–7].

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They are also referred to as protein transduction domains, membrane translocating sequences, or with the more pictorial name, Trojan peptides. The first discovered peptide of this family was the HIV-1 TAT peptide (YGRKKRRQRRR). It was recognized first that the recombinant HIV-1 TAT protein was taken up by cells [8, 9] and, a few years later, the minimum sequence responsible for the uptake was reduced to 11 amino acids [10]. During the same period, it was found that a fragment derived from the Drosophila Antennapedia homeodomain (RQIKIWFQNRRMKWKK), also called penetratin, had similar properties [11–13]. These two discoveries spurred further research that led to the finding of this family of peptides. Ten years has passed since then and several other CPPs have been discovered, some naturally occurring such as penetratin and some synthetic peptides such as transportan (GWTLNSAGYLLGKINLKALAALAKKIL) or polyarginines and polylysines of different lengths [14, 15].

A large difficulty for achieving therapeutic efficiency in many drugs lies in reaching and crossing the cell membrane. Since these peptides are able to cross these barriers and at the same time are extremely soluble, they are very attractive candidates for delivering therapeutic agents into cellular compartments. They also seem to bypass the endocytotic pathway for translocation, which is known to degrade drugs and DNA. In recent years, several groups have obtained positive results using these peptides for the delivery of therapeutic agents in animal models, with low toxicity. Usually, these peptides are attached covalently to the molecule that needs to be delivered. An interesting method has been developed by Torchilin et al. [5–7] in which they created 200 nm liposomes with several TAT peptides attached to the liposome surface and used this complex to deliver embedded drugs. The power of these peptides to translocate into almost any cell type may represent today their main weakness [16]. Drugs usually need to be transported to specific cells and intracellular compartments and this lack of specificity needs to be overcome before they could be used effectively for this purpose.

How Are These Peptides Able to Cross the Cell Membrane? The answer to this fundamental question has remained elusive [2, 15, 17]. Conflicting results have been reported; some experiments seem to indicate an energy-dependant or endocytotic pathway and others an energy-independent pathway. Both possibilities have conceptual problems that need to be solved. For example, the lack of cell specificity does not correlate well with the idea that these peptides could be targeting any specific receptor on the cell. On the other hand, the idea that they might be able to passively diffuse across the cell membrane is not easy to accept. These peptides are highly hydrophilic. Therefore, direct translocation across the hydrophobic core of the membrane could cost an excessive amount of energy. It is usually assumed that it would require a great amount of electrostatic free energy for these peptides to go from a high dielectric medium (water) to a low dielectric medium (core of the membrane). Furthermore, there is also a free energy cost of translocating the big cargoes that they carry along with them as they translocate to the cytosol.

It is known that antimicrobial peptides are able to translocate across the bacterial plasma membrane in an energy-independent manner [13, 18, 19]. It has been proven in some cases that stable pores of the order of milliseconds [20–24] are formed during translocation and these pores are used to kill the bacteria [25]. Since CPPs seem to translocate in an energy-independent manner, a similar mechanism could be possible. However, this has been conceptually difficult to understand for two main reasons: (a) if CPPs form pores, leakage should be observed, possibly killing the cell, and (b) they are extremely hydrophilic and many of them lack the high hydrophobic content present in antimicrobial peptides. An attractive aspect of the formation of a pore is that it can explain the translocation of the

wide variety of cargoes that can be attached to these peptides, since after the pore is formed the cargo can go through the pore without any extra energetic cost.

Recently, we have proposed a mechanism for the translocation of the HIV-1 TAT peptide based on molecular dynamics simulations. This mechanism shows how these peptides are able to passively diffuse across the cell membrane [1]. The mechanism for translocation can be described as composed of four basic steps (illustrated in Fig. 1): (a) the peptides bind to the surface of the bilayer, attracted by the phosphate groups of the phospholipids. (b) As the surface concentration of peptides increases, the arrangement of lipids is strongly distorted compared to the resting membrane. (c) An arginine side chain translocates to the distal layer,

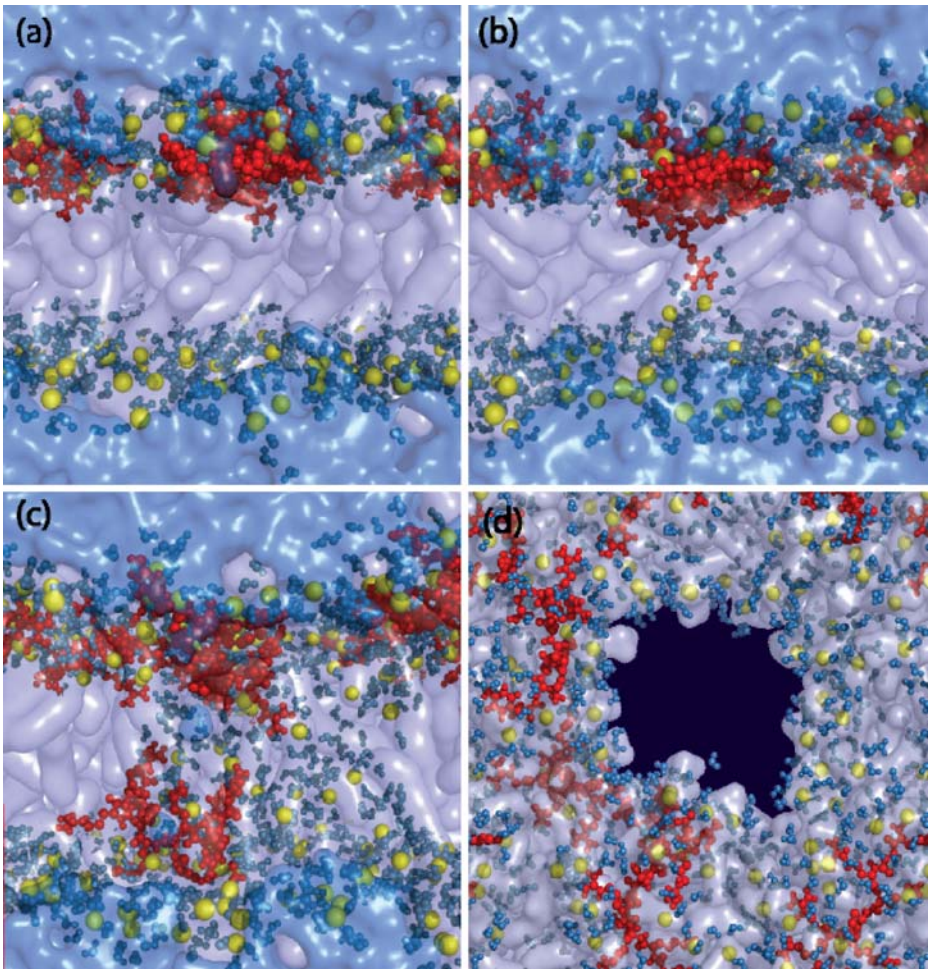

Fig. 1 Four snapshots of a molecular dynamics simulation. **a** A lateral view that shows how the peptides are bound to the membrane before translocation. **b** The translocation of an arginine amino acid surrounded by water molecules that nucleates the formation of a water pore. **c** Lateral view of the pore and the translocating peptide. **d** Top view of the pore. Details of the simulations can be found in [1]. The simulated system consists of 92 lipids and 8,795 water molecules. The phospholipid molecules are represented with *transparent white surfaces*, the phosphate atoms are in *yellow spheres*, the peptide molecules are in *red*, any water molecule at a distance of less than 3.5 Å from any phospholipids or amino acid atom is colored in *solid blue*, and the rest of the water molecules appear as a *transparent blue surface*

nucleating the formation of a water pore. (d) A few lipids translocate by diffusing on the surface of the pore and the pore closes. In the present review, we will describe this mechanism and interpret a few experiments under this light.

2 Binding of the TAT Peptides to the Phosphate Groups

The first question we asked is if these peptides would actually prefer to bind and remain bound to the surface of the membrane instead of being solvated in water. We observed that the arginines and lysines are strongly attracted by the negatively charged phosphate groups in the phospholipids and that after the peptide binds, it stays bound. It has long been speculated that these peptides could be strongly attracted by the phosphate groups on the hydrophilic surface of the cell membranes and that this could be the initial step for the uptake. This was experimentally recognized in [26] where it was shown that the peptides bounded to the surface of the cells cannot be removed by simply washing them with NaCl/Pi. To remove the peptides from the cell surface, the cells needed to be treated with trypsin that hydrolyzes proteins into smaller peptides or amino acids. In other reports, it was also shown that the increase in surface concentration of the peptides grows in parallel with the peptide uptake [27].

The authors of [26] also show that a reduction of the extracellular heparan sulfate correlates with a reduction of peptide uptake. The heparan sulfate molecules are a type of glycosaminoglycans distributed over the surface of the cell. They are used by some receptors to recognize their ligands or regulate their activation. They are negatively charged and contain sulfate groups to which the peptide, especially the arginines, might bind in a similar way as the binding to the lipid phosphate groups over the surface of the bilayer. Since the heparan sulfates are negatively charged, they might generate a long-range attraction of the peptides and this could trigger the binding of the peptide to the cell. The interaction of arginines with phosphate or sulfate groups is not exclusive of this mechanism. It has been implicated in a wide variety of biochemical process. For example, in voltage-regulated potassium ion channels, the arginines are embedded in the membrane and play a critical role in the opening and closing of the channels [28–31].

3 Decrease in the Thickness of the Bilayer with the Increase in the Surface Density of Peptides

We showed that the peptides bound to the surface of the membrane create large distortions of the phospholipid ordering. The TAT peptides create regions with higher density of phosphate groups around the peptide and regions with lower density of phosphate groups away from the peptide. The location of the peptides (underneath the phosphate groups and away from bulk water) also creates strong attractions with phosphate groups on the opposite site of the membrane. These perturbations produce a local thinning of the lipid bilayer, reducing the free energy barrier imposed by the hydrophobic core of the membrane.

The change in the thickness and area of bilayer membranes as a function of the concentration of peptides has been extensively studied in the context of antimicrobial peptides [32–35]. It has been reported that there is an increase in the area per lipid and a reduction in the thickness of model membranes with the increment of peptide concentration. This effect has also been observed in phospholipid monolayers where full penetration or translocation is naturally excluded, making these systems especially

attractive to isolate the effects on the membrane of the initial binding of the peptides. Ishitsuka et al. [36] used lipid monolayers to study protegrin 1, an arginine-rich antimicrobial peptide that also forms pores on microbial membranes [37, 38]. They characterized membrane selectivity, insertion, and the changes in lipid ordering upon the attachment of the peptides to the surface of the monolayer.

Similar observations have been made by Ziegler et al. [39] by creating suspended monolayers of phospholipids in a water–air interface and changing the concentration of CPPs in solution. However, in this case, they observed only partial insertion of the peptide between the phospholipids. They also studied the nuclear magnetic resonance spectra of the structure of the membrane of a vesicle with TAT peptides bound on the surface and observed an alignment of the P–N dipole perpendicular to the surface of the membrane. In the resting membrane, this dipole is approximately parallel to the surface of the membrane. When positively charged molecules bind to the zwitterionic head group, this dipole aligns perpendicular to the membrane with the choline group located further from the carbon chains. The same change in alignment was observed with the TAT peptide. This is consistent with our observations. We see that when a peptide binds to the head group of the phospholipids, it locates between the phosphate and the glycerol groups. Since the peptide and the choline group are positively charged, they repel each other, and the P–N dipole reorients perpendicular to the membrane surface such that the choline group is away from the peptide. This location of the peptide also favors interactions with phosphate groups on the opposite side of the membrane and this interaction might contribute as well, at least partially, to the total absorbance of the peptide in the surface of the membrane. This might also explain why the peptide does not insert completely when the opposite layer of the bilayer is not present, as observed by Ziegler et al. [39].

4 Translocation of an Arginine and Pore Formation

From a conceptual point of view, the most striking finding is the translocation of a loosely bound arginine towards the distal side of the bilayer attracted by phosphate groups on that side (Fig. 1b). As mentioned earlier, it is a common belief that the free energy cost for this event should be extremely high and, therefore, this should be an extremely unlikely event. The membrane is generally viewed as a rigid region of low dielectric constant surrounded by water, which has a very high dielectric constant. This view could be a good approximation in some cases, but in many others it does not give a good qualitative description (i.e., voltage-gated ion channels [30, 31, 40, 41]). The membrane is very flexible and can suffer strong deformations in response to different molecules.

To understand how an arginine can diffuse across the hydrophobic core of the membrane, we can make this simplified model of the membrane slightly more complex as shown in Fig. 2. In this model, we still have the two dielectric mediums, but we also have the charged layers at the interface (represented by the P–N dipole) and we have to allow local deformations of the dielectric mediums such as to capture the local thinning produced by the peptides that attract phosphate groups in the opposite layer. Since the P–N vector is almost on the plane of the membrane, the resting membrane can be approximated by just two mediums of different dielectric constants. When the peptide gets closer to the membrane, the negative surface that models the phosphate groups comes slightly outside to grab the positive amino acids with the P–N vector pointing towards the interior of the bilayer. After the peptide binds to the phosphate groups, there is a switch in the P–N vector towards the exterior of the lipid bilayer and the peptide locates in the interior of the bilayer

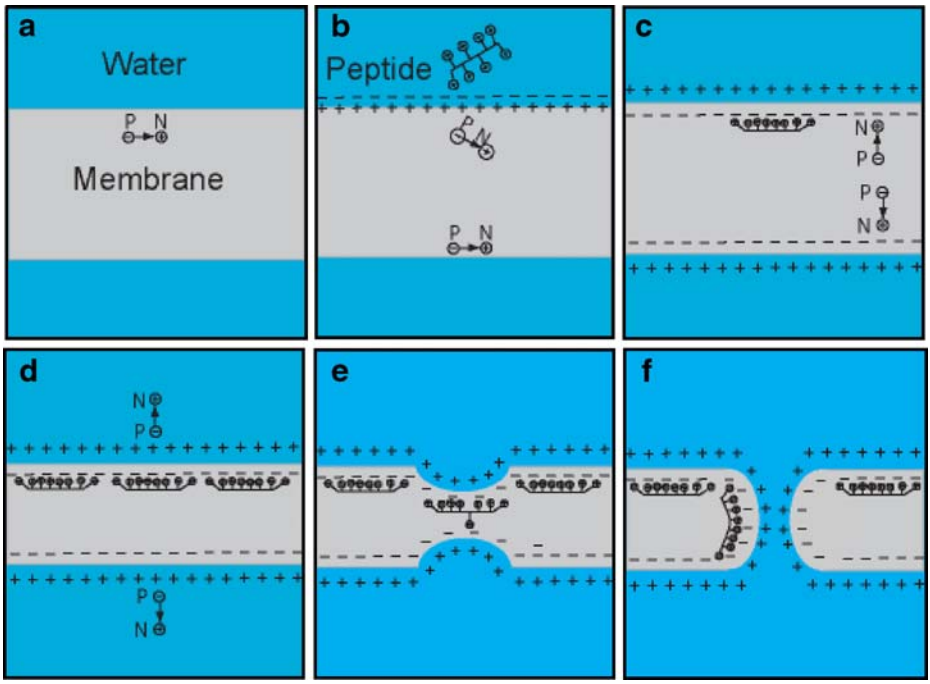

Fig. 2 Cartoon that describes qualitatively the steps involved in the translocation mechanism. In particular, we show the orientation of the dipole moment vector formed by the phosphate group (negatively charged) and the choline group (positively charged) denoted by *P* and *N*, respectively. **a** The P–N dipole vector is in the plane of the membrane, and therefore the resting membrane can be approximated by a dielectric medium of low dielectric constant surrounded by water, that can be approximated as a continuous medium of high dielectric constant. **b** As the peptide approaches the surface of the membrane, it attracts the phosphate groups, and the dipole bends towards the interior of the membrane, exposing locally the phosphate groups to the peptide. **c** Since this is not the best conformation for the system, the peptide inserts into the interface between the hydrophilic head groups and the carbon chains. This allows the peptide to be away from the choline groups. This position also favors strong interactions with the phosphate groups on the opposite side of the membrane since now the dielectric constant of the medium is much lower than in the aqueous medium. **d** As the density of peptides increases, the membrane gets thinner. **e** An arginine amino acid translocates with a local bending of the membrane on both sides of the bilayer. This bending reduces the energetic barrier of the translocation. **f** A pore forms and the peptide translocates bound to the interior of the bilayer surface

close to the P layer and away from the N layer. This location enhances the interaction of the peptide with the P layers on both sides of the membrane. As the surface concentration of peptides increases, the membrane becomes thinner and it becomes more likely that a peptide will reach the distal surface of the bilayer. In this way, it will minimize the free energy of the system. However, the free energy could still be very high if the opposite side is not flexible. As the peptides increase in concentration, it is more likely that a charged amino acid chain, such as an arginine, will translocate to the opposite side attracted by phosphate groups, represented in this case by the negative P layer. As the amino acid translocates, the opposite side of the membrane also deforms locally by the mutual attraction between the phosphate groups and the guanidinium group. To capture this effect, the membrane has to be thought as a very flexible object which allows local deformations of its resting structure. This nucleates a toroidal pore and the peptides diffuse on the surface of the membrane.

Wender et al. [42] have observed that if the length of the arginine side chain is increased (by adding methylene groups to the side chains), the uptake also increases. They also observed that this enhancement was not related to the increase in the hydrophobic content of the side chain (by adding cyclohexyl instead). Our simulations reveal that, in fact, the length of the arginine side chain is very important for the guanidinium group to reach the phosphate groups on the opposite side of the membrane. If the arginine side chain was shorter, either the thickness of the membrane should be smaller or the translocation of an individual side chain should be escorted by the translocation of other side chains at the same time. This would require a larger energetic cost than the translocation of a single amino acid.

After the arginine translocates, water starts to diffuse across the membrane and the hydrophilic groups of the phospholipids bend to avoid contact with the carbon chains. This nucleates the formation of a toroidal pore across the membrane (Fig. 1c and d). Mishra et al. [43] conducted crystallographic studies and showed that aqueous solutions of simple phospholipids and TAT peptides undergo a phase transition from a lipid bilayer to a Pn3m double diamond phase where the lipid molecules surround large (6 nm) toroidal water pores similar to those observed in our simulations.

The formation of toroidal pores on membranes is a common characteristic of many antimicrobial peptides [25]. This is a mechanism used by organisms to kill microbes by producing leakage of bacterial cells and destroying their chemical gradients. The question is why leakage is not observed with the TAT peptide. An explanation could be the rate at which these pores open and close. In the case of antimicrobial peptides, the time that the pore remains open is on the order of milliseconds or more [20–24] while in the case of the TAT peptides, we have observed that the rate is of the order of a microsecond. If the peptide has a cargo attached to it, such as a protein or a fluorescent dye, the cargo could temporarily block the pore as it translocates, reducing the leakage even further. The difference in the rate of translocation between cell penetrating peptides, or more specifically the TAT peptide, and antimicrobial peptides might be partially related to differences in the hydrophobic content of amino acids, combined with an average reduction in the charge.

5 Peptides Diffusion and Closing of the Pore

After the pore opens, a few peptides diffuse over the surface of the membrane as they translocate to the opposite side. This means that the peptides do not have to pay all the energetic cost that would result from bringing all the charged amino acids through the hydrophobic core. The system only has to pay the initial cost to nucleate a pore.

The peptides are strongly bound to some phospholipids which remain bound to the peptides as they translocate, producing lipid flip flop on the bilayer. This lipid flip flop has been measured experimentally with antimicrobial peptides [24, 44] and could, in principle, be experimentally tested with CPPs. The pore closes after a balance between the peptides on both sides is reached. The total process takes less than a microsecond and the maximum diameter of the pore we observed was approximately 5 nm. The rate at which these pores open and close contrasts with the rate of antimicrobials peptides (order of milliseconds or more [20–24]) whose sequences might be specifically designed to maintain the pore open in microbial membranes for enough time to destroy the ionic concentration gradients and consequently kill the cell. This fast rate could explain why sensible leakage has not been detected [39] and why CPPs are able to translocate by forming pores without killing the host cell.

6 Translocation across Unilamellar Vesicles

The cell environment is complex and contains many elements that cannot be easily controlled when performing experiments. For example, binding of arginine-rich CPPs to negatively charged groups over the cell surface (e.g., glycosaminoglycans) may enhance the localization of CPPs on the cell surface. Also, non-homogeneous lipid composition and non-uniform charge distribution in the interior and exterior of the cell [45] that results in the presence of an electric transmembrane potential may help drive CPPs into the cell interior. To be able to understand the basic steps and main ingredients necessary for peptide translocation across the cell membrane, it is important to simplify the system and deal with different effects separately. One common simplification is to study model systems consisting of giant unilamellar vesicles (GUV). Then, the question remains if CPPs will translocate across these model systems and what is the effect of the bilayer composition on the translocation. There is ample evidence that CPPs translocate across model bilayers and exhibit the same sequence selectivity as in cells [46–52]. This gives further support to the idea that these peptides do not need to target any receptor on the surface of the cell and also that the asymmetric distribution of lipids on mammalian membranes (where there is a bigger content of negative lipids in the cytosolic leaflet [45]) is not strictly necessary for translocation. Of particular importance are a series of recent experiments that show that, in particular, the TAT peptides translocate across GUVs [43, 53]. Experiments by Mishra et al. [43] used rhodamine-labeled TAT to show that the peptide translocates spontaneously across GUV without the need of an electric potential gradient across the lipid bilayer.

7 Salt, Counterions, Acidic Phospholipids, and Molecular Dynamics Simulations

Electrostatic attraction is the first step for the attachment of the peptides to the membrane [1]. Consistent with this view, increased salt concentration could shield the interaction and reduce the attraction between the peptides and the cell. If the guanidiniums of a CPP form bonds with anions such as the phosphate groups on the cell surface, then counterions associated with the guanidinium groups must be exchanged at the cell surface. This exchange will depend on the specific counterion associated with the peptide and the phospholipid composition of the cell membrane [54–56].

The electrostatic interactions can also be strongly affected by adding negative lipids to the model bilayer [57]. The concentration can be symmetrical (both layers with the same densities of lipids) or asymmetrical (more negative lipids in the distal layer); this last case might better represent the charge content in mammalian membranes [45] and will enhance translocation.

Simulations allow the possibility to make simplifications that are usually very difficult to achieve in experiments. These simplifications are always made, explicitly or implicitly, to develop theoretical models that can refine our understanding and guide further experiments. At the same time, any experimental or theoretical simplification has to face the cost to distance from the biological complexity. In this sense, the lack of salts, counterions, and acidic phospholipids may represent the main strength and at the same time the main weakness of our work. Depending on the choice of these elements, the translocation could be enhanced or reduced. For example, if we would like to add just enough counterions to reach electroneutrality, then we could add acidic phospholipids to the distal side of the bilayer and this will enhance translocation or we could add simple ions into the solution that, depending on the ion, could screen the peptide charges, reducing the partition of the peptides into the membrane [54, 55]. A better compromise to obtain electroneutrality can

be used in simulations under periodic boundary conditions where, as in our case [1], there is included a homogenous neutralizing background charge [58]. There are several conditions that need to be simulated, i.e., different lipids, counterions, and excess salt. However, all the experimental evidence seems to point out that the essential components to describe the translocation of CPPs are the phospholipid bilayers, the arginine amino acids, and water. Therefore, any model needs to first isolate and understand the cooperative behavior of these elements in detail. At a second layer of complexity should be studied the effects of salts, different lipid head groups, and mixtures of lipids to further refine our understanding.

8 Conclusions

The mechanism suggested by molecular dynamics simulations of TAT peptides in lipid bilayers does not rule out the possibility that the uptake could also follow energy-dependant pathways [59]. However, these pathways might not be as general and could depend on the specific sequence of the CPP and the cargo attached to it. Several recent reports indicate that these peptides get inserted into the cell through endocytosis and pinocytosis [60–64]. We observed in our simulations that these peptides interact very strongly with the membrane, introducing lipid rearrangements and stress. This could lead initially to either pinocytosis or endocytosis, depending on the lipid composition of the membrane. In any case, the peptide escapes from the endosome [65] and this escape could also be described by the mechanism we propose.

The resemblance of CPPs to antimicrobial peptides is very strong. In fact, antimicrobial peptides are also charged and they might use a similar mechanism to nucleate the formation of pores. The hydrophobic content in those peptides has been used partially to justify how these peptides are able to get into the hydrophobic core of the membrane, form pores, and translocate. Although hydrophobicity can help, we can see that CPPs do not need any hydrophobic amino acids to nucleate a pore. Therefore, the cationic amino acids of antimicrobial peptides may also be responsible for the nucleation of the pore and then the hydrophobic amino acids might be responsible for stabilizing the peptides within the pore.

The mechanism that we propose opens a new view of the translocation of cell penetrating peptides. We believe that this will help to interpret and design future experiments. From a fundamental point of view, these results force us to rethink the idea that charged peptides cannot diffuse passively across the membrane. There are several cases in which charged residues are inserted in the membrane and the membrane can produce strong deformations relative to its resting structure, thereby minimizing the energetic cost of the insertion. In the case of CPPs, we see that charged amino acid side chains can even diffuse across the bilayer and nucleate a pore. These observations dramatically challenge our view of protein-membrane interactions and this might contribute to the understanding of a wider group of biological problems.

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