Membrane Crossover by Cell-Penetrating Peptides: Kinetics and Mechanisms – From Model to Cell Membrane Perturbation by Permeant Peptides

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Abstract Membrane-active peptides are a large family endowed with a wide pattern of biological activities (antimicrobial, viral fusion and infection, cell-penetrating or protein-transduction domain), which share the property of interacting with membranes and being internalized in eukaryotic cells. Apart from pinocytosis internalization pathways, these peptides have the capacity to re-organize lipid membranes and to lead to membrane fusion, disruption or pore formation. In this chapter, we focus on these membrane perturbation processes evoked by cell-penetrating peptides that have been widely studied with membrane models and in cultured cells.

Keywords Cell-penetrating peptide • Membrane • Permeation • Pinocytosis • Translocation

Abbreviations

AMP	Antimicrobial Peptide
Antp	antennapedia, homeoprotein
CHO	chinese hamster ovary cells
CPP	Cell-Penetrating Peptide
CS	Chondroitin Sulphate
DOPC	dioleoylphosphatidylcholine
DOPG	dioleoylphosphatidylglycerol
DPPC	dipalmitoyl phosphatidylcholine
DSC	Differential Scanning Calorimetry
ESR	Electron Spin Resonance spectroscopy
GUV	Giant Unilamellar Vesicle

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HS	Heparan Sulphate
HSPG	Heparan Sulphate ProteoGlycans
ITC	Isothermal Titration Calorimetry
LUV	Large Unilamellar Vesicle
MAP	Membrane Active Peptide
NBD	Nitrobenzo-2-oxa-1,3-diazole
NMR	Nuclear Magnetic Resonance Spectroscopy
PEP-1	hepatite C virus related peptide, SGSWLRDVWDWICTVLTDFK-TWLQSKLDYKD-NH_2
P/L ratio	peptide over lipid ratio
Transportan	galanin/mastoparan chimeric peptide, GWTLNSAGYLLGKINLK-ALAALAKKIL-NH_2
Tat	Trans Activator of Transcription protein
Tat(46–58)	Tat derived peptide, GRKKRRQRRRPQ-NH_2

1 Introduction

Cellular signaling mechanisms in plants and animals include homeoprotein transduction, which is particularly important in developmental and physiological processes (Tassetto et al. 2005; Brunet et al. 2007). Homeoproteins have an important paracrine function, being secreted by and internalized into neighbored cells (Prochiantz and Joliot 2003; Joliot and Prochiantz 2008). Specialized peptide domains that are endowed with the property of membrane translocation have been identified in numerous different proteins (Lindgren et al. 2000; Prochiantz 2008). These peptides are grouped under the generic term of cell-penetrating peptides (CPPs) or, when they are derived from proteins, such as the Antennapedia homeoprotein or Tat transcription factors, protein transduction domains (PTD) (Hansen et al. 2008). These peptides have the ability to convey into cells conjugated cargo molecules that can give a positive biological or imaging read out of the intracellular localization of the peptide (Dietz and Bähr 2004; El-Andaloussi et al. 2004; Morris et al. 2008).

2 Amino Acid Composition of Cell-Penetrating Peptides

Dozens of different cell-penetrating peptides have now been reported, which derive from natural protein sequences or have been rationally designed (Hansen et al. 2008). Those peptides are indeed basic and/or amphipathic with a length of 10–20 amino acids. Some of these permeant peptides are pure basic sequences such as oligoarginine (R8, R9) (Futaki et al. 2001; Wender et al. 2001; Nakase et al. 2004) or Tat(48–59) (GRKKRRQRRRPQ) peptides (Weeks et al. 1995; Vives et al. 1997) when others are more hydrophobic such as transportan (WTLNSAGYLLGKINL-KAKAAKAKKIL).

2.1 Necessity for a Peptide Secondary Structure for Internalization?

It is clear that peptide – membrane interactions must be of fundamental importance for the internalization process. Therefore, whether the secondary structure of these peptides when interacting with membrane, plays a key role in the internalization properties has been widely studied. Moreover different peptide secondary structures have been reported for the same peptide. This is certainly a result of the different experimental conditions used in the studies regarding: peptide/lipid (P/L) ratios and concentrations used, buffer composition (e.g., ionic strength), temperature at which the experiments were performed as well as the method used to determine its structure.

Penetratin, the cell-penetrating sequence derived from the third helix of the Antennapedia homeodomain protein, has been shown to have a strong propensity for α-helix formation in lipid environments, which suggested first that the helical structure was necessary for internalization of the peptide (Magzoub et al. 2002; Letoha et al. 2003; Lindberg et al. 2003; Christiaens et al. 2004; Caesar et al. 2006; Clayton et al. 2006). But, a recent computational study on the molecular structure of penetratin, in interaction with lipid bilayers, and experiments with various phospholipids mixtures, have indicated a high structural polymorphism of penetratin (Polyansky et al. 2009). Penetratin could indeed adopt a α-helical, a β-strand or a β-turn conformation depending on the model membrane composition (Magzoub et al. 2002; Clayton et al. 2006; Su et al. 2008). In addition, it has been underlined that the α-helical conformation was not mandatory and could even be detrimental to the membrane translocation properties of penetratin (Derossi et al. 1996; Christiaens et al. 2004). Finally a recent study in living cells with high concentrations (25–50 μM) of penetratin has shown that the secondary structure of the peptide was found to be mainly random coil and beta-strand in the cytoplasm, and possibly assembling as beta-sheets in the nucleus (Ye et al. 2010). Ye and collaborators report no evidence of α-helical structure formation by penetratin, although it is possible (because of limitations with the signal intensity and the lateral spatial resolution (~0.5 μm) of the Raman microscopy methods) that the peptide could form α-helical or other transient conformations as it crosses the cell membrane (Ye et al. 2010).

Thus, whether a correlation exists between the capacity of a CPP to adopt a specific structure and its membrane translocation ability, is still a matter of debate. A recent study with ten different CPPs attempts to classify the peptides in three subgroups depending on their physicochemical properties (the secondary structure being one of them) and correlates those with different internalization pathways (Eiríksdóttir et al. 2010a, b). It has been suggested that the structural polymorphism and malleability of CPPs could be important for the membrane interaction and internalization route (Deshayes et al. 2008). An aspect that has been briefly evocated in the literature is the relevance of CPP self-assembly in the uptake mechanism. It follows that certain CPPs (penetratin, transportan, Pep-1, MAPs) can self-assemble, suggesting that they can be internalized as monomers or aggregates (Pujals et al. 2006).

Therefore, there is no clear relationship between the structure these peptides might adopt in solution or in contact with biological membranes and their ability to enter cells.

3 Binding of Cell-Penetrating Peptides to Membrane Components

3.1 Role of Proteoglycans

In the majority of cases, and independently of the internalization pathway of the CPP, the initial contact involves interactions between the CPP and cell-surface proteoglycans (PGs). Using model systems, the role of heparan sulphate proteoglycans (HSPGs) in CPP uptake has been investigated using isothermal titration calorimetry (ITC; Ziegler and Seelig 2004; Goncalves et al. 2005), plasmon resonance methods (Duchardt et al. 2009; Ram et al. 2008), ESR spectroscopy (Ghibaudi et al. 2005), and affinity chromatography (Fuchs and Raines 2004). Such studies point to considerably tight binding of CPPs to HSPGs such as heparan sulphates (HS), heparin and chondroitin sulfate B (CS) with dissociation constants in the low micromolar range. A higher affinity was observed for these HSPGs when comparing to anionic lipids. Although, the primary interaction between CPPs and HSPGs was considered to be electrostatic, it is also likely that hydrogen bonding occurs, taking into account the ability of the guanidium group (arginines are often present in CPPs) to form hydrogen bonds with sulfate and carboxylate groups.

3.2 Lipids

Taking into account the nature of CPPs, both electrostatic interactions between the positively charged amino acids and the lipid headgroups, and hydrophobic interactions between residues such as tryptophan and the lipid fatty acid region are possible. A strong electrostatic interaction can be established between the peptides and the lipids due to the large entropy gain that results from the release of counterions both at the level of the membrane (the Gouy-Chapman layer) (Zimm and Le Bret 1983) and the peptide (the Manning layer) (Manning 1969). Negative charges in the lipid can arise from the lipid headgroup itself in case of anionic lipids (phosphatidylglycerol, phosphatidylserine and phosphatidic acid) or from the phosphatidic groups of the fatty acids, which can establish strong ionic interactions with guanidium groups, often present in CPPs (Nakase et al. 2008). Even if the majority of the lipids present in the outer layer of eukaryotic cells are zwitterionic, anionic lipids are also present and their role may become relevant when they cluster in small domains, a process that is induced by CPPs and antimicrobial peptides

(Joanne et al. 2009; Epand and Epand 2009). Several studies have demonstrated the importance of an electrostatic recognition using different approaches. Therefore, to measure the affinity between the CPPs and lipid model systems and provide a thermodynamic characterization of such an interaction, ITC and plasmon resonance methods have been used (Goncalves et al. 2005; Binder and Lindblom 2003; Salamon et al. 2003; Alves et al. 2009; Henriques et al. 2010). ITC studies point to binding affinities in the low micromolar range and positive heat capacities indicating that electrostatics plays a major role in the interaction. Titration experiments with increasing amounts of anionic lipids show that at certain anionic lipid concentration and P/L ratios, penetratin is able to bind to both the outer and the inner leaflet presuming transbilayer distribution of penetratin. Since penetratin has not been associated with perturbations in membrane integrity, this indicates that, penetratin translocates through the vesicle membrane by an electroporation mechanism (this will be further discussed below) (Binder and Lindblom 2003). Plasmon resonance studies performed with penetratin indicate that binding to planar lipid bilayers is a fast and multistep process, primarily governed by electrostatic interactions followed by peptide insertion into the hydrophobic membrane core. The peptide also affected the amount of bound water, lipid-packing density, and bilayer thickness (the latter only at high peptide concentrations) accompanied by a decrease in membrane capacitance. A considerable enhancement of the binding was observed in the presence of anionic lipids (Salamon et al. 2003; Alves et al. 2009; Henriques et al. 2010). Improved binding affinities of penetratin to anionic lipids as compared with zwitterionic lipids (10–100 fold increase) have also been observed by following the intrinsic tryptophan fluorescence intensity of penetratin as a function of the lipid concentration (Christiaens et al. 2002).

4 Kinetics of Internalization in Cells

The kinetics of cell-penetrating peptide internalization has been studied by several groups. The reported data differ from one peptide to another depending on the cell type and the method used for peptide tracking.

One of the first studies has shown that ^{125}I-Biotinyl-transportan internalization was quick in Bowes melanoma cells and reached the steady-state after 20 min. Interestingly, the time course was found similar whatever the concentration (5–500 nM) of the peptide (Pooga et al. 1998). The same group reported the kinetics of internalization of penetratin (RQIKIWFQNRRMKWKK), transportan, Tat(48–60) and MAP (KLALKLALKALKAALKLA) (Hällbrink et al. 2001). The CPPs were labelled with a fluorescence quencher (3-nitrotyrosine) and were coupled to a pentapeptide cargo labeled with a fluorophore (2-amino benzoic acid) via a disulfide bond. The kinetics was recorded by following the increase in fluorescence intensity as the disulfide bridge is reduced into the intracellular milieu (Hällbrink et al. 2001). In those experimental conditions, the more hydrophobic the peptides (transportan and MAP) the faster they internalized in cells, the fluorescence plateau being

reached after 15 min for transportan and after 1 h for penetratin. Similar results were obtained by Drin (Drin et al. 2003), using a NBD-labeled fluorescent penetratin analogue. The kinetics plateau was reached after 1 h incubation at 37°C of non-adherent human K562 leukemia cells with 1 μM NBD-penetratin. We have reported also kinetics of internalization at 37°C and 4°C of a biotin-labeled and photoactivatable penetratin analogue (Jiao et al. 2009). The kinetics was determined in CHO-K1 cells and CHO-pgA745 (GAG-deficient) cells. It was shown that at 37°C the plateau was reached after 1 h incubation of 5 μM penetratin with CHO-K1 and after 30 min with CHO-pgA745 (Jiao et al. 2009). Another study with a Tat-conjugated cargo also reported similar kinetics using a fluorescence assay (Cheung et al. 2009). In addition, Pep-1 (Ac-KETWWETWWTEWSQPKKKRKV-cysteamine), a rationally designed cell-penetrating peptide that can establish hydrophobic interactions with cargo molecules (thus that does not require a covalent link with these latter) is able to convey β-galactosidase into cells with a similar time course (Henriques et al. 2005). However, faster (in the range of seconds) and slower (tens of minutes) internalization kinetics could be measured for different cell-penetrating peptides (Eiríksdóttir et al. 2010a) using a releasable luciferin assay (Jones et al. 2006). These results led the authors to classify cell-penetrating peptides according to the internalization kinetics, which reflect their uptake pathways, as translocation for the fast kinetics and endocytosis for the slower one (Eiríksdóttir et al. 2010a).

However all these studies were done with a population of cells, thus the kinetics observed are not the kinetics of single cells but an average of the internalization kinetics in a population.

5 Pathways of Internalization in Cells

Regarding the internalization pathways of cell-penetrating peptides, there is a huge discrepancy between reported studies. The important point that is now spreading in literature is that any single chemical modification of the peptide sequence severely impacts the internalization pathways of the resulting compounds (Maiolo et al. 2005; El Andaloussi et al. 2007; Aussedat et al. 2008; Walter et al. 2009), as well as the cell-type (Mueller et al. 2008). These observations are quite understandable as any modification in the peptide or in membrane components must also affect peptide/membrane interactions.

Although still controversial for some cell-penetrating peptides, it is nonetheless quite clear that there are multiple internalization pathways for cell-penetrating peptides (Nakase et al. 2008; Jiao et al. 2009; Alves et al. 2010). It came out that the discrepancies between reported studies should have arisen from the experimental conditions used, principally the concentration of peptide and the chemicals used to inhibit internalization pathways, that could also have side-effects (Ivanov 2008).

All pinocytosis pathways have indeed been suggested for cell-penetrating peptide internalization, especially macropinocytosis (Jones 2007) that is a reported mechanism for penetratin (Amand et al. 2008), oligoarginine (Nakase et al. 2004, 2007),

Tat (Wadia et al. 2004), M918 (El-Andaloussi et al. 2007) and other permeant peptides (Sawant and Torchilin 2010).

Clathrin-mediated internalization was initially reported for Tat (Richard et al. 2003) but different results were obtained in further studies. It was first reported that knock down of clathrin-mediated endocytosis or knockout of caveolin-mediated endocytosis did not affect the ability of Tat to enter cells (Ter-Avetisyan et al. 2009). In addition, it was suggested that Tat could internalize at 4°C through a direct translocation mechanism (Jiao et al. 2009; Ter-Avetisyan et al. 2009).

For oligoarginine peptides, it was first reported that the peptide internalized through macropinocytosis (Nakase et al. 2004) or that direct translocation driven by the membrane potential occurred (Rothbard et al. 2004; 2005). It was further proposed by a single-molecule motion study that the mode by which octaarginine penetrates the cell membrane could be either a multi-mechanism uptake process or a mechanism different from passive diffusion and endocytosis (Lee et al. 2008). Other studies suggested that this peptide induces the formation of transient pores in cell membranes in the presence of an electrostatic potential gradient (Herce et al. 2009; Cahill 2010). A recent work reports that oligoarginine can change the lipid composition of cell membrane through the translocation in the outer membrane leaflet of sphingomyelinase and ceramide formation (Verdurmen et al. 2010).

Finally, internalization of the pAntp homeobox and of the derived penetratin peptide was originally described as a temperature and energy-independent process (Joliot et al. 1991; Derossi et al. 1994). Further studies suggested that penetratin enters via an endocytosis pathway rather than a translocation mechanism (Drin et al. 2003; Jones et al. 2005). A recent study highlighted the possibility that penetratin could internalize through transient pores and activate a resealing mechanism, known as a membrane repair response (Palm-Apergi et al. 2009)

6 Model Membrane Perturbation by CPP

To evaluate the energy-independent contribution to CPP uptake, the so-called direct translocation, several biophysical studies using different lipid models systems and a panoply of techniques have been employed in an attempt to elucidate the role of proteoglycans and lipids in the uptake mechanism as well as the peptide and lipid restructuration taking place upon their contact.

The direct translocation of CPPs through liposomes, has been investigated by several laboratories and conflicting results have been obtained. Uptake studies on giant unilamellar vesicles (GUVs) reported a transbilayer movement of penetratin (Thoren et al. 2000) or Tat(48–59) (Curnow et al. 2005), which contradicted studies on smaller lipid vesicles or planar membranes where these CPPs were found not to cross the membrane. Studies on LUVs have established that translocation is dependent on membrane potential and is modulated by the lipid composition (Terrone et al. 2003). One of the reasons for the divergent results may come from the different membrane curvature of the different lipid model systems used.

Despite controversial studies regarding the capacity of CPPs to penetrate through lipid bilayers or liposomal membranes (direct translocation), the initial contact with the cell membrane constitutes an important stage in the internalization process and has been investigated in depth. This initial interaction might be important just to increase the local peptide concentration in the surface before its uptake by either direct translocation (eventually leading to a deeper peptide penetration and lipid reorganization) or endocytosis.

Following the initial contact of CPPs with the cell membrane surface mainly driven by electrostatic interactions and the increase in the local peptide concentration, both peptide and lipid reorganization take place to allow peptide uptake by either endocytosis or direct translocation. The mode of action of the peptide in terms of lipid reorganization is dictated both by the CPP structure and the lipid composition of cellular or model systems. Taking into account the different peptide sequences, CPPs have been classified in three major classes:

1. Primary amphipathic such as transportan (Pooga et al. 1998), Pep-1 (Morris et al. 2001), they comprise sequentially hydrophobic and cationic domains and contain more than 20 amino acids, long enough to span the bilayer. They bind with strong affinities to both zwitterionic and anionic lipids suggesting that membrane interaction is dominated by hydrophobic interaction (Magzoub et al. 2001). They penetrate deeper than other CPPs in the membrane but without spanning the bilayer (Deshayes et al. 2004), the insertion is often accompanied by a secondary structure change. They have a tendency to self associate in the headgroup region. They often have antimicrobial activity and are rather difficult to distinguish from antimicrobial peptides as they can greatly perturb bilayer integrity, although less than AMP because they are less deeply inserted.
2. Secondary amphipathic such as penetratin (Derossi et al. 1996), KLAL (Dathe et al. 1996) and RL16 (Lamaziere et al. 2007), are shorter and display amphipathic property (evident when their amino acid sequence is depicted on a helical wheel) only through a change in their secondary structure upon lipid or HSPG contact. They possess poor affinity to neutral membranes, and their affinity is highly enhanced when anionic lipids are present due not only to electrostatic interaction but to a change in the peptide secondary structure (Binder and Lindblom 2003; Wieprecht et al. 2002). Despite the formation of an amphipathic structure by these peptides, the insertion in the bilayer is not marked and no membrane perturbation is usually observed at low anionic lipid content. Their binding leads to a change in the polar lipid headgroup orientation (Kichler et al. 2006; Roux et al. 1989). For tryptophan-containing cell-penetrating peptides, as examplified by pAntp analogues, in-cell studies support the hypothesis that hydrophobic interactions anchor those peptides in the membrane and might help their translocation into the cytosol (Le Roux et al. 1993; Fischer et al. 2002; Christiaens et al. 2004)
3. Non-amphipathic are generally shorter and comprise almost exclusively cationic amino acids such as R9. They do not bind lipid membranes unless they contain a high fraction of anionic phospholipids. Contrarily to amphipathic CPPs, direct translocation is not observed at low micromolar concentrations and at low

anionic lipid contents (Lamaziere et al. 2007; Thoren et al. 2005; Tiriveedhi and Butko 2007; Hitz et al. 2006). They do not induce liposome leakage or other types of membrane perturbation at low P/L ratios and concentrations around those required for biological uptake (1–10 μM) (Fuchs et al. 2004; Afonin et al. 2006). Additionally, no structure change is associated with their membrane binding and they are only superficially adsorbed on the membrane (Goncalves et al. 2005; Roux et al. 1988).

7 Mechanisms of CPP Direct Translocation

There is much evidence that direct translocation through the lipid membrane plays a significant role in CPP entry into cells. The relative importance of direct translocation and endocytosis seems dependent on conditions such as type of CPP, CPP concentration, temperature, cargo or cell type but the existence of the direct translocation pathways seems now ascertained. This leads to the need to explain by which mechanism(s) these highly soluble CPPs, all bearing several positive charges and few or no hydrophobic residues, can cross the hydrophobic core of the membrane bilayer. The order of magnitude of the activation energy for a naked CPP that would enter this hydrophobic core is given by the Born energy of an ion leaving an aqueous solution (relative permittivity 80) for the layer formed by the aliphatic lipid chains of the lipids (relative permittivity 2). This energy for a guadinium ion (radius r_G = 0.25 nm) is $\Delta E_{ion} = \frac{e^2}{8\pi\varepsilon_0 r_G}\left(\frac{1}{\varepsilon_c} - \frac{1}{\varepsilon_w}\right) \approx 60\,\mathrm{kT}$ per ion (at T = 300K). The spontaneous entry of a naked CPP into the bilayer is thus highly unfavorable from a thermodynamical point of view. Several more refined mechanisms, presented below, have been proposed and experimentally backed up, and it is worth mentioning from the beginning that the question of the translocation mechanisms is currently still debated and may have no single answer, the direct translocation mechanisms being CPP or experimental condition dependent.

A first class of proposed mechanism is the neutralization of the positively charged CPP residues by some hydrophobic counterions that would simultaneously reduce the Born energy stated above and favor the solubilization of the CPP in the hydrophobic core of the membrane (Sakai and Matile 2003, Nishihara et al. 2005; Takeuchi et al. 2006; Wender et al. 2008) (Fig. 1). Several potential candidates for the role of amphipatic counterion lie in membranes such as anionic phospholipids or sulfated proteoglycans. The proof of concept has been given by Sakai and Matile (Sakai and Matile 2003) who showed that polyarginines (~80 residues) initially dissolved in an aqueous buffer can partition into chloroform when phosphatidylglycerol lipids were added. Rothbard and collaborators conducted a similar experiment with an arginine octamer labeled with fluorescein (Rothbard et al. 2004). When they added sodium laurate, the CPP migrated completely from water to an octanol phase. They also demonstrated the role of the two hydrogen bonds that a

guadinium ion can form with a phosphate, sulphate or carboxylate group on its counterion. Mono- (resp. di-) methylation of the guadinium group of a CPP prevents formation of one (resp two) hydrogen bond between the guadinium and its counterion. For the fluorescinated arginine octamer it entailed a 80% (resp. 95%) decrease of its uptake into Jurkat cells (treatment : 5 min, 50 μM). In the framework of the solubilizing counterion mechanism, the strength of the non covalent bonds between a CPP and its counterions would therefore prove important. Interestingly this may give a rationale to the reported lower internalization efficiency of CPPs for which arginine residues were replaced by lysines (Mitchell et al. 2000).

Other proposed mechanisms (Fig. 1) are those that maintain, at least partly, the CPP in a polar environment (aqueous solution or polar layer of the membrane). Crossing the membrane then requires a transient reorganization of the bilayer such as the formation of a pore or the encapsulation of CPPs in an inverted micelle. Both options entail high local curvature of the lipids (on the rim of the pore or in/around the inverted micelle embedded in the bilayer) and a possible mismatch between the lipids ("void" on the rim of the inverted micelle). From an energetic point of view, the saving of the Born energy is balanced to some extent by the cost of the deformation of the bilayer that can amount to tens of kT (Siegel 1993; Glaser et al. 1988). Within these models, the transition is driven by the interaction of several peptides with the surface of the membrane and its consequences on the curvature and stability of the bilayer.

The inverted micelle model (Fig. 1) has been mainly proposed for penetratin (Derossi et al. 1996). In this model, cationic residues of penetratins interact with negatively charged phospholipids in the plasma membrane and subsequent interaction of Trp in the peptide with the hydrophobic membrane is thought to induce an invagination in the plasma membrane. The concomitant reorganization of the neighboring lipids results in formation of an inverted micelle, followed by release of peptide and cargo upon micelle disruption. This explanation for the penetratin translocation is supported by ^{31}P-NMR and differential scanning calorimetry experiments showing that penetratin favors the lamellar to hexagonal inverse transition for certain lipid compositions (Berlose et al. 1996; Alves et al. 2008).

A pore formation could occur through different paths (Fig. 1). One is referred to as the "carpet model". It consists of the binding of numerous peptides in the polar region of the membrane. These peptides destabilize the lipid assembly and lead to disruption of the bilayer and formation of pores. This model was first proposed for some AMPs (Shai 1999). But molecular dynamic simulations have suggested that it applies to TAT and arginine nonamer (Herce and Garcia 2007; Herce et al. 2009). The proposed mechanism was that these CPPs bound strongly to the phosphate and carbonyl groups of the phospholipids deep in the membrane just above the hydrophobic core. This destabilized the membrane and lead to the crossing of few CPPs immediately followed by the opening of aqueous pores. This later event has been experimentally confirmed by Herce and collaborators in the case of arginine nonamer by electrophysiological measurements on planar bilayers and cells. However the simulations have been criticized by Yesylevskyy and collaborators who found no pore formation evidenced by similar simulations (Yesylevskyy et al. 2009).

Nevertheless, they illustrate the possible complexity of the translocation mechanism: the simulations showed an accumulation of peptides at the boundary of the polar region that lead to bilayer destabilization and pore nucleation which was consistent with the carpet model. But they also suggested that the entry of the peptides was lead by the phosphate-basic residue interactions and that the first step of the pore nucleation is the entry of the CPP in the bilayer hydrophobic core to create an interaction with distant phosphates: this is more in tune with the concepts developed in the hydrophobic counterion model mentioned above. This emphasizes the fact that the classification presented here distinguishes models for clarity purposes but their borders may be permeable. Another illustration of this complexity is the lipid segregation model. It starts similarly to the carpet model with accumulation of peptides on the membrane surface. But if the peptide binds preferentially to certain lipids (such as anionic lipids), this binding is likely to entail domain formation or more generally a modification of the lateral organization of the lipids in the membrane. These domains may show packing defects at their boundaries and these sites are likely to be more favorable to peptide entry in the membrane or even act as nucleation sites for pore formation. This model again, suggested for AMPs (Epand et al. 2006), may be relevant for CPPs. Recent DSC measurements have shown that penetratin, by segregating cardiolipin in a DDPC/cardiolipin mixtures, was able to induce the formation of domains in an otherwise homogenous membrane (Joanne et al. 2009). Finally, another model related to the carpet model is the electroporation mechanism proposed by Lindblom and collaborators (Binder and Lindblom 2003): they propose that the destabilization of the membrane by the CPP carpet is due (in the case of penetratin) to the asymmetrical distribution of the charged CPPs between the outer and inner surfaces of the bilayer causing a transmembrane electrical field, which alters the lateral and the curvature stresses acting within the membrane.

Certain mechanisms include the formation of an aqueous pore following CPP addition to a membrane. This step is somewhat easier to check experimentally because it can manifest itself trough leakage of hydrophilic markers or ionic current flow. Lactate dehydrogenase release assays conducted on HeLa cells incubated 3 h with 100 μM with Tat, HIV1-rev-(34–50) or arginine octamers showed no significant leak. Absence of leakage of CHO cells treated with penetratin or RL16 (RRLRRLLRRLLRRLRR) was confirmed with a similar assay (1 h, 10 μM) (Joanne et al. 2009). However electrophysiological experiments conducted on DOPC: DOPG 3:1 planar bilayers and HUA smooth muscle cells in presence of arginine nonamers showed significant ionic current revealing membrane permeation (Herce et al. 2009). These results seem controversial but must be compared with the sensitivity of the technique in mind (electrophysiological measurements being more sensitive). It seemed to date that major leaking induced by CPPs can be ruled out while the possibility of transient, rapid, small aqueous pores cannot.

Finally, another question often mentioned in the literature regarding the mechanism of CPP entry is that of the driving force for the uptake of the peptides. This role is generally attributed to the transmembrane potential across cell membrane, a natural candidate for these polycationic peptides. Experiments on vesicules showing membrane potential dependant translocation of penetratin are consistent with this role of

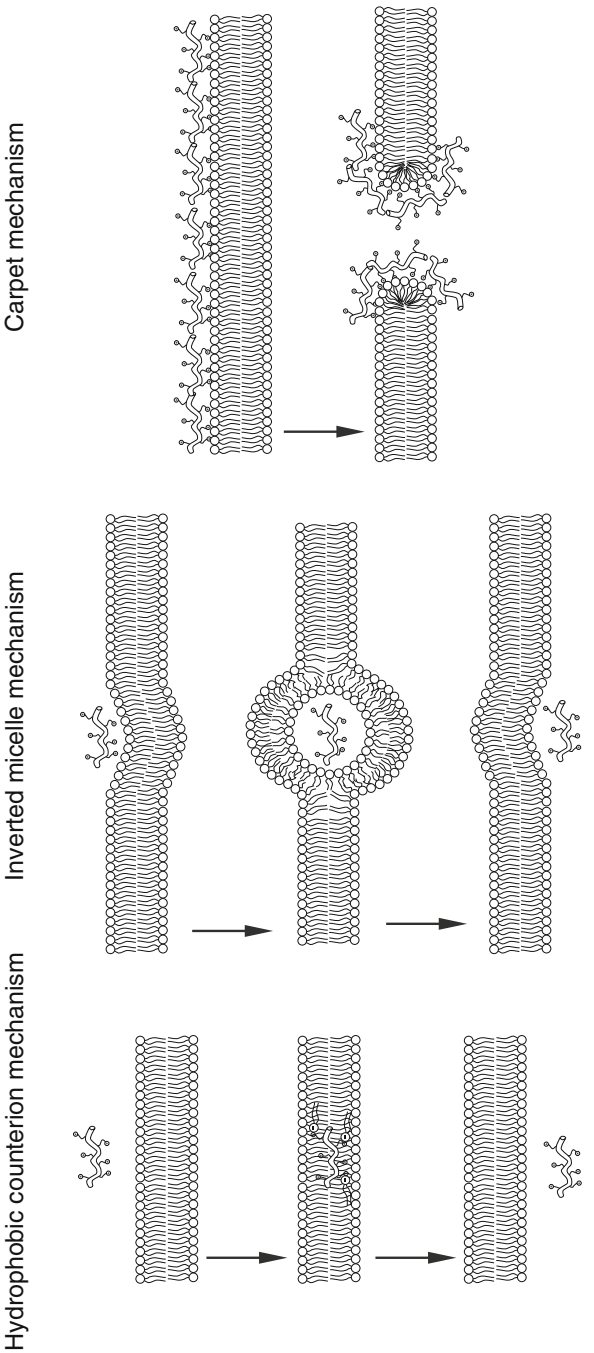

Fig. 1 Schematic representation of the translocation mechanisms reported in literature. Read the Sect. 7 for more details regarding these mechanisms

the potential (Terrone et al. 2003). Modulation of membrane potential trough variation of potassium concentration outside Jurkat cells also showed a strong impact on uptake of fluorescently labelled Tat49–57 or arginine octamer (Rothbard et al. 2004), and of biotin-labelled RW9 (R6W3) (Delaroche et al. 2007). Thus, the role of the potential in cell-penetrating peptide internalization needs to be included in translocation mechanisms. For example, in the frame of the hydrophobic counterion model, the sensitivity of a CPP to the potential requires the absence of neutralization of certain positive charges as suggested by Rothbard and collaborators (Rothbard et al. 2004). Intriguingly, crossing the membrane for a positive elementary charge amounts to a gain of ~5 kT for a –100 mV potential that appears at first sight weak compared to the Born energy of the charge. For models that appeal to aqueous pore formation, an obvious impact of the potential would be a proportional electromotive force on charges in the pore that may be supplemented by a promoting effect on pore formation (the later is not seen on the linear I-V relationship of the ionic current generated in a planar bilayer by arginine octamer in Herce et al. (2009)).

This review of possible pathways for CPP translocation illustrates that direct investigations of the mechanism are difficult because all mechanisms involve nanometric, rare, transient structures. However very significant progress has been recently made in the understanding of the complex interactions between membranes and CPPs and a clarification of the mechanisms of translocation is likely to occur in upcoming years.

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