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Basic cell penetrating peptides induce plasma membrane positive curvature, lipid domain separation and protein redistribution

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ABSTRACT

Basic cell penetrating peptides are tools for molecular cellular internalization of nonmembrane permeable molecules. Their uptake mechanisms involve energy-dependent and energy-independent pathways such as endocytosis, direct translocation or physical endocytosis. These mechanisms are ruled by both, the peptides physicochemical properties and structure and by the membrane lipids characteristics and organization. Herein we used plasma membrane spheres and membrane models to study the membrane perturbations induced by three arginine-rich cell penetrating peptides. Nona-arginine (R9) and the amphipathic peptide RWRRWWRRW (RW9) induced positive membrane curvature in the form of buds and membrane tubes. Membranous tubes underwent rolling resulting in formation of multilamellar membrane particles at the surface of the plasma membrane spheres. The amphipathic peptides RW9 and RRWRRWWRRWWRRWRR (RW16) provoked lipid and membrane associated protein domain separation as well as changes in membrane fluidity and cholesterol redistribution. These data suggest that membrane domains separation and the formation of multilamellar membranous particles would be involved in arginine-rich cell penetrating peptides internalization.

1. Introduction

The interaction of proteins and peptides with membranes usually provokes a biological response leading to activation of intracellular transport mechanisms, protein transduction and antimicrobial defence. Based on arginine-rich peptide properties, cell penetrating peptides (CPPs) have been developed to carry active molecules into the cytoplasm of eukaryotic cells (Mae and Langel, 2006; Murriel and Dowdy, 2006). It is generally considered that cellular delivery of basic peptides mainly results from two different molecular pathways: direct plasma membrane translocation and internalization by endocytosis with subsequent endosomal release into the cytoplasm (Duchardt et al., 2007; Lundin et al., 2008). Moreover, one peptide can use both pathways depending on its extracellular concentration (Alves et al., 2011, 2008) or on temperature (Jiao et al., 2009). It has also been demonstrated that amphipathic and cationic peptides use different endocytic routes for cellular internalization (Lundin et al., 2008). Recently, it was shown that the formation of membranous particles at the cellular surface is involved in direct arginine-rich peptide penetration into the cytosol (Hirose et al., 2012), and that this phenomenon is favoured by hydrophobic groups on the peptides. Thus, peptide sequence and structure specificities determine the nature of peptide-membrane association and in fine the cellular uptake mechanisms. Therefore, the characterization of peptide-membrane interactions is essential to understand the membrane changes occurring during intracellular traffic and antimicrobial activity as well as the mechanisms of cellular uptake that will help to develop efficient vectors for future therapeutic delivery of molecules.

As *in vivo* phenomena linked to peptide-membrane interactions are complex, work on membrane models allowed to study the biophysical mechanisms and the characterization of peptide structure and affinity for different membranes (Eiriksdottir et al., 2010; Maniti et al., 2010; Rydberg et al., 2012; Takechi et al., 2011; Walrant et al., 2012). Several studies showed that basic peptides are able to induce membrane negative curvature resulting in membrane invaginations in the form of vesicles and tubes as well as lipid phase separation (Alves et al., 2008; Lamaziere et al., 2006, 2009,

Abbreviations: CPP, cell penetrating peptide; GUV, giant unilamellar vesicle; LUV, large unilamellar vesicle; PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; PMS, plasma membrane sphere; PS, L-α-glycerophosphatidyl-L-serine; Py-met-chol, pyrene-labelled methyl-cholesterol; R9, nona-arinine; RW9, RWRRWWRRW; RW16, RRWRRWWRRWWRRWRR.

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2010, 2008; Menger et al., 2003). These peptide-induced membrane deformations would result from changes in membrane asymmetry leading to changes in line tension and bending energy of membrane domains (Lipowsky, 1991, 1993). Furthermore, insertion of polymers in the membrane increases bilayer asymmetry and produce positive membrane curvature resulting in budding and outward tubulation in giant unilamellar vesicles (GUVs) (Tsafrir et al., 2003). The induction of positive or negative curvature by association of proteins to the membrane has been well established for the so-called curvature sensing proteins containing Bar-like domains (Antonny, 2006; Dawson et al., 2006; Saarikangas et al., 2009).

The study of cellular implications of peptide membrane-interactions requires the use of cellular models of the plasma membrane. Giant plasma membrane vesicles (GPMVs) or plasma membrane spheres (PMS) are promising models of biological membranes. These vesicles are void of cellular organelles and nuclear fragments (Scott, 1976) and their spherical morphology allows the observation of membrane deformations. Their molecular composition is representative of the plasma membrane. They contain intrinsic and extrinsic membrane proteins (Baumgart et al., 2007). Their overall lipid composition is typical of the plasma membrane containing phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositols and, as for the plasma membrane, they are enriched on sphingomyelin (SM) and cholesterol (Fridriksson et al., 1999; Scott, 1976). Not only the global proportion of lipids is conserved but also the proportion of double bonds in acyl chains (Fridriksson et al., 1999). Only two anomalies have been reported: a decrease in phosphatidylinositol-bis-phosphate (Keller et al., 2009) and the loss of PS asymmetry (Baumgart et al., 2007; Keller et al., 2009; Lingwood et al., 2008). However, partial retention of lipid asymmetry has also been reported (Yavin and Zutra, 1979). PMS are able to undergo protein and phospholipid liquid ordered (Lo) and liquid disordered (Ld) phase separation (Baumgart et al., 2007; Keller et al., 2009; Levental et al., 2009, 2011; Lingwood et al., 2008), they are depleted of metabolic energy and membrane cytoskeleton elements are absent (Baumgart et al., 2007; Lingwood et al., 2008). Recently they have been used to study the penetration of several basic cell penetrating peptides (Amand et al., 2011; Maniti et al., 2012; Saalik et al., 2011) and they allowed us to show peptide capacity to induce negative curvature with the consequent formation of plasma membrane invaginations (physical endocytosis) (Maniti et al., 2012).

In the present study we used plasma membrane spheres to characterize the interaction of three different basic peptides with the plasma membrane. Nona-arginine (R9) that penetrate cells efficiently (Futaki et al., 2001; Mitchell et al., 2000; Wender et al., 2000), the amphypathic peptide RWRRWWRRW (RW9) because it has been shown that the addition of hydrophobic groups and tryptophans to polyarginines increase cell penetration (Futaki et al., 2001; Hirose et al., 2012; Rydberg et al., 2012), and RRWRRWWRRWWRRWRR (RW16) to study the effect of the length of an amphipathic peptide. We show that the association of small basic peptides with the plasma membrane results in the induction of positive curvature with subsequent vesicle bud formation and membrane tube elongation. Moreover, the membrane tubes underwent rolling and formed membranous particles attached to the plasma membrane surface. These effects were accompanied by changes in membrane fluidity, lipid domain separation and membrane-associated protein (annexin 2) redistribution.

2. Materials and methods

2.1. Materials

Nona-arginine (R9), RWRRWWRRW (RW9) and RRWRRWWRRWWRRWRR (RW16) where synthesized and purified as described (Lamaziere et al., 2007). Laurdan was from Molecular Probes, di-4-ANNEPDHQ (ANE) was obtained from Dr Leslie M. Loew (Connecticut, USA) and pyrene-labelled cholesterol (Py-met-chol) was a kind gift of Dr. André Lopez (Toulouse, France). Egg yolk L-α-phosphatidylcholine (PC), egg yolk L-α-phosphatidylethanolamine (PE), brain L-α-glycerophosphatidyl-L-serine (PS) and cholesterol were purchased from Sigma–Aldrich.

2.2. Plasma membrane spheres (PMS)

Standard and Annexin 2-GFP transfected MDCK cells were cultured in 75 cm^2 flasks in DMEM in standard conditions (Ayala-Sanmartin et al., 2004). Plasma membrane spheres were obtained by the method described in (Lingwood et al., 2008) adapted in (Maniti et al., 2012). Briefly, PMS induction was performed by overnight incubation of cells with 1 ml of PMS buffer (1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5 mM Hepes, 1 mg ml^{-1} glucose in PBS pH 7.4). After PMS formation, 1 ml of PMS buffer was added, the culture was agitated gently and the supernatant containing the PMS was recovered and centrifuged at 1000 rpm for 15 min in a standard Eppendorf centrifuge. The PMS were recovered in 100 μl of PBS and conserved at 4 °C for several hours. The experiments presented here were performed with 5 independent PMS preparations.

2.3. Peptide-PMS incubations and treatments

All preparations for microscopic observation were performed on μ-slide 8 well slides (Maniti et al., 2012) containing 160 μl of freshly prepared PMS. For di-4-ANEPPDHQ labelling, a stock solution (200 mM) in ethanol was diluted in PBS. Then, 20 μl were added for a final 5 μM concentration. Peptides were added in 10 μl for a final 10 μM concentration. For experiments in hypertonic solution, 20 or 40 μl of sucrose (500 mM) were added to attain a final concentration of 50 and 100 mM sucrose.

2.4. Confocal microscopy

All confocal images were acquired with a TCS SP2 laser-scanning spectral system (Leica, Wetzlar, Germany) attached to a Leica DMR inverted microscope. Optical sections were recorded with a 63/1.4 or 100/1.4 immersion objectives. We systematically performed 30 confocal slices for each PMS analyzed. Considering that most of the PMS were in the range of 15–20 μm, the acquisition of 30 slices allowed the observation of the PMS surface by steps of 500–700 nm. Images stacking and 3D-projections were obtained using ImageJ software (NIH). For di-4-ANEPPDHQ imaging, the samples were excited at 488 nm (Ar ion laser) and the fluorescence emission was collected at 570–590 nm for the liquid ordered (Lo) contribution and at 620–640 nm for the liquid disordered (Ld) contribution. Imaging of Annexin 2-GFP was performed by excitation at 488 nm and the emission light was collected at 500–520 nm.

2.5. Quantification of plasma membrane deformations

The quantification of the different structures observed: tubes, rolled tubes, buds and deformations were performed by direct observation under the microscope. Protrusions were considered as tubes when the length was higher than the diameter. The experiments were performed two to four times. The number of PMS analyzed by direct microscopy was: 1656 for the control PMS, 409 for R9, 1150 for the RW9 and 322 for the RW16 treatments. The number of PMS analyzed by random confocal images were: 88 for the control PMS, 69 for R9, 96 for the RW9 and 69 for the RW16 treatments. The statistical significance was assessed by ANOVA with GraphPad Prism. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.