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# Membrane permeation of arginine-rich cell-penetrating peptides independent of transmembrane potential as a function of lipid composition and membrane fluidity

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ABSTRACT

Cell-penetrating peptides (CPPs) are prominent delivery vehicles to confer cellular entry of (bio-) macromolecules. Internalization efficiency and uptake mechanism depend, next to the type of CPP and cargo, also on cell type. Direct penetration of the plasma membrane is the preferred route of entry as this circumvents endolysosomal sequestration. However, the molecular parameters underlying this import mechanism are still poorly defined. Here, we make use of the frequently used HeLa and HEK cell lines to address the role of lipid composition and membrane potential. In HeLa cells, at low concentrations, the CPP nona-arginine (R9) enters cells by endocytosis. Direct membrane penetration occurs only at high peptide concentrations through a mechanism involving activation of sphingomyelinase which converts sphingomyelin into ceramide. In HEK cells, by comparison, R9 enters the cytoplasm through direct membrane permeation already at low concentrations. This direct permeation is strongly reduced at room temperature and upon cholesterol depletion, indicating a complex dependence on membrane fluidity and microdomain organisation. Lipidomic analyses show that in comparison to HeLa cells HEK cells have an endogenously low sphingomyelin content. Interestingly, direct permeation in HEK cells and also in HeLa cells treated with exogenous sphingomyelinase is independent of membrane potential. Membrane potential is only required for induction of sphingomyelinase-dependent uptake which is then associated with a strong hyperpolarization of membrane potential as shown by whole-cell patch clamp recordings. Next to providing new insights into the interplay of membrane composition and direct permeation, these results also refute the long-standing paradigm that transmembrane potential is a driving force for CPP uptake.

## 1. Introduction

Cell-penetrating peptides (CPPs) are emerging drug delivery vehicles because of their capacity to induce efficient cellular uptake of molecules that otherwise do not enter cells [1]. In order to enable a rational optimization of CPPs and understand cell type preference, CPP entry mechanisms have been intensely investigated. The mode of entry depends on peptide concentration, cell type and the conjugated cargo [2–5] but also on structural characteristics of the peptide such as amino acid charge and stereochemistry [6]. At low micromolar concentrations, arginine-rich CPPs are mainly endocytosed, whereas rapid cytoplasmic entry can occur at high concentrations [7]. This entry is associated with the accumulation of peptide at certain membrane areas, called nucleation zones (NZ), which are colocalizing with ceramide platforms [8–10]. Uptake via NZ is restricted to arginine-rich CPPs such as nona-arginine [8], with the human lactoferrin-derived CPP being a

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remarkable exception as this peptide only has four arginine residues [11].

Although the mechanism of CPP internalization has been described to be cell line-dependent, the reasons for these differences are unknown [4]. A considerable amount of research has been conducted on the role of glycosaminoglycans (GAGs) in endocytosis since these are the first (negatively) charged molecules that cationic CPPs encounter when approaching a cell [12,13]. Several studies demonstrated a positive effect of GAGs on CPP internalization [8,14–16]. However, the role of GAGs depends on the type of CPP, and GAGs are probably not acting as autonomous receptors but rather as coreceptors as reviewed by Favretto et al. [13]. This hypothesis is further supported by the finding that in the absence of GAGs, CPPs still bind to the cells and are internalized [17]. Furthermore, the membrane potential ($V_{mem}$) has been considered a driving force for the internalization of cationic CPPs [18,19].

In direct comparisons of the frequently used cell lines HEK and HeLa we had observed that HEK cells showed a direct cytoplasmic peptide uptake at concentrations at which HeLa cells only showed endocytosis [20,21]. In this study, we made use of these earlier observations to elucidate the molecular and cellular characteristics underlying the difference in uptake mechanisms between these cell lines with regard to the occurrence of direct translocation at low peptide concentrations and nucleation zone-dependent uptake.

Using electrophysiological recordings in living cells during peptide internalization we show that both cell lines differ fundamentally in their membrane characteristics. In HEK cells, direct membrane translocation was independent of membrane potential. By contrast, an intact resting membrane potential was required for the induction of nucleation zones in HeLa cells and, remarkably, this process was associated with a sudden hyperpolarization of the plasma membrane. Lipidomics revealed that, in contrast to HeLa cells, membranes of HEK cells have a low sphingomyelin content, and conversion of sphingomyelin into ceramide further enhances membrane translocation. A role of membrane fluidity and membrane microdomain organisation was established by demonstrating that in HEK cells direct permeation is abolished at room temperature and upon cholesterol depletion. These results also resolve a long-standing dispute according to which fluorescent labelling is incompatible with direct membrane permeation [22], and shall open new directions of research into the interdependence of membrane composition and permeation of macromolecules.

## 2. Materials and methods

### 2.1. Materials

Unless stated otherwise, all compounds were obtained from Sigma-Aldrich and cell culture materials from Invitrogen. The used sphingomyelinase (bSMase) is derived from *Bacillus cereus* and provided at a concentration of 138 U/mL. Stock solutions of valinomycin (9 mM) and gramicidin A (1.06 mM) were prepared in DMSO and ethanol, respectively.

### 2.2. Cell culture

HeLa and HEK293T cells were obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). HeLa cells were cultured in RPMI supplemented with 10% fetal calf serum (FCS) whereas HEK cells were maintained in high glucose DMEM containing 10% FCS. Both cell lines were cultivated in an incubator at 37 °C with 5% $CO_2$. Cell culture media were purchased from Invitrogen.

### 2.3. Peptides

For translocation experiments in large unilamellar vesicles, peptides were synthesized on an automated Syro II multiple peptide synthesizer (MultiSynTech, Witten, Germany), using standard solid phase Fmoc protocols. Peptide synthesis reagents, Fmoc-protected amino acids, and 7-(9-Fluorenylmethyloxycarbonylamino)-coumarin-4-acetic acid (Fmoc-ACA-OH) were purchased from Merck Biosciences (Darmstadt, Germany) and/or Iris Biotech (Marktredwitz, Germany). Solvents for peptide synthesis were purchased from Merck (Darmstadt, Germany) or from Biosolve (Valkenswaard, Netherlands), and solvents for HPLC purification were obtained from Fischer Scientific (Geel, Belgium). The C-terminal phenylalanine of each peptide sequence carried a 7-amino-4-methylcarbamoylcoumarin (ACC) moiety that experiences a wavelength shift upon chymotrypsin cleavage. ACC-resin synthesis was performed manually prior to the peptide synthesis [23]. The crude peptides were purified using a high-pressure liquid chromatography (HPLC) device from JASCO (Groß-Umstadt, Germany) on a preparative Vydac C18 column, using a water/acetonitrile gradient supplemented with 5 mM HCl. The purified peptides were characterized by using analytical HPLC (Agilent; Waldbron, Germany) coupled to an ESI mass spectrometer (μTOF Bruker, Bremen, Germany), and were found to be > 95% pure.

Fluorescently labelled peptides were purchased from EMC microcollections (Tübingen, Germany). All peptides were C-terminally amidated and N-terminally labelled with 5,6-carboxyfluorescein or Alexa 488. Peptide concentrations were determined based on the absorbance of fluorescein at 492 nm with a Novaspec II spectrophotometer (Pharmacia, New York, USA). Fluorescein-labelled peptides were diluted in Tris-HCl buffer (10 mM, pH 8.8) and an extinction coefficient of 75,000 $M^{-1}$ $cm^{-1}$ was assumed for calculating peptide concentrations, the Alex488-labelled peptide was diluted into deionized water and extinction measured at 495 nm assuming an extinction coefficient of 73,000 $M^{-1}$ $cm^{-1}$.

### 2.4. Confocal laser scanning microscopy

Two days before the experiment 20,000 HeLa cells or 25,000 HEK cells were seeded in chambered coverslips (Nunc, Wiesbaden, Germany or Ibidi, Martinsried, Germany, respectively). Confocal microscopy was performed using a Leica SP5 microscope with an HCX PL APO 63 × N.A. 1.2 water immersion lens (Leica, Mannheim, Germany). During image acquisition, cells were maintained at 37 °C. Fluorescein was excited using an argon ion laser at 488 nm and emission was collected between 500 and 550 nm. Cells were incubated with the indicated peptide concentrations and the specific compounds, as indicated for each experiment. During imaging, cells were maintained in RPMI without phenol red in the presence or absence of 10% FCS.

### 2.5. Flow cytometry

80,000 HeLa cells or 100,000 HEK cells were seeded in 24-well plates one day before the experiment. After incubating cells for 10 min either with 1 μM gramicidin A or 10 μM valinomycin in RPMI or RPMI alone, cells were co-incubated for 20 min with the indicated peptide concentrations. Cells were washed with RPMI twice, trypsinized, centrifuged and resuspended in RPMI + 10% FCS without phenol red. Fluorescence intensities were measured using a flow cytometer (BD Biosciences, Erembodegem, Belgium). Approximately 10,000 intact cells were gated based on forward and sideward scatter. The Summit 4.3 software (Dako, Fort Collins, USA) was used for the analysis of gated cells.

### 2.6. Patch-clamp combined with confocal laser scanning microscopy

For electrophysiological recordings, HEK293T cells or HeLa cells were cultured on 35 mm glass dishes and transferred to a recording chamber (Luigs & Neumann, Ratingen, Germany) on an upright fixed-stage scanning confocal microscope (TCS SP5 DM6000CFS, Leica Microsystems, Mannheim, Germany) equipped with an HCX APO L 20 × NA 1.0 water immersion objective (Leica Microsystems) as well as