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Bilayer interaction and localization of cell penetrating peptides with model membranes: A comparative study of a human calcitonin (hCT)-derived peptide with pVEC and pAntp(43–58)

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Abstract

Cell-penetrating peptides (CPPs) are able to translocate problematic therapeutic cargoes across cellular membranes. The exact mechanisms of translocation are still under investigation. However, evidence for endocytic uptake is increasing. We investigated the interactions of CPPs with phospholipid bilayers as first step of translocation. To this purpose, we employed four independent techniques, comprising (i) liposome buffer equilibrium dialysis, (ii) Trp fluorescence quenching, (iii) fluorescence polarization, and (iv) determination of ζ-potentials. Using unilamellar vesicles (LUVs) of different phospholipid composition, we compared weakly cationic human calcitonin (hCT)-derived peptides with the oligocationic CPPs pVEC and penetratin (pAntp). Apparent partition coefficients of hCT-derived peptides in neutral POPC LUVs were dependent on amino acid composition and secondary structure; partitioning in negatively charged POPC/POPG (80:20) LUVs was increased and mainly governed by electrostatic interactions. For hCT(9–32) and its derivatives, *D* values raised from about 100–200 in POPC to about 1000 to 1500 when negatively charged lipids were present. Localization profiles of CPPs obtained by Trp fluorescence quenching were dependent on the charge density of LUVs. In POPC/POPG, hCT-derived CPPs were located on the bilayer surface, whereas pVEC and pAntp resided deeper in the membrane. In POPG LUVs, an increase of fluorescence polarization was observed for pVEC and pAntp but not for hCT-derived peptides. Generally, we found strong peptide–phospholipid interactions, especially when negatively charged lipids were present.

Keywords: Cell penetrating peptides; Fluorescence spectroscopy; Liposome–buffer partitioning; Lipid bilayer models; Phospholipid vesicles; Peptide–lipid interactions

1. Introduction

Over the past decade, several classes and/or prototypes of cell-penetrating peptides (CPPs) have been characterized in several aspects. Initially, their entry mechanisms were postulated not to be mediated by receptors or transporters [1,2], suggesting a passive, non-endocytic transfer. More recently, however, increasing evidence has been brought up suggesting the involvement of active, endocytic processes [3–7]. The ability of the CPPs to translocate when covalently or physically linked with a cargo, including polypeptides and nucleic acids, renders them of broad interest in cell biology, biotechnology and drug delivery. In

Abbreviations: CPP, cell penetrating peptide; hCT, human calcitonin; pVEC, vascular endothelial cadherin-derived CPP; pAntp, penetratin, Antennapedia homeodomain-derived CPP; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-phosphatidylglycerol; DPC, dodecyl phosphocholine; Br-PC, 1-palmitoyl-2-stearoyl-(11,12-dibromo)-*sn*-glycero-3-phosphocholine; NBD-PE, N-(7-nitrobenzofurazan-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; 5-DSA, 5-doxylstearic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; TFA, trifluoroacetic acid; PBS, phosphate-buffered saline; RP-HPLC, reversed phase HPLC; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; MLV, multilamellar vesicles; DLS, dynamic light scattering; K_{SV}, Stern–Volmer constant

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fact, CPPs have been used as vectors for the cytoplasmic and nuclear delivery of hydrophilic biomolecules and drugs, both in vivo and in vitro [1,2,8]. The exact mechanisms underlying the translocation across membranes are still under investigation. As a consequence of artifactual uptake phenomena due to cell fixation, the hypothesis of an energy independent, direct transport of CPPs trough biomembranes, postulated in earlier CPP studies [9–11], had to give way to concepts involving endocytosis [3–6,12]. Increasing attention is concentrated on the first step of CPP uptake: an initial adherence on the membrane surface leading to an enrichment in the phospholipid bilayer, which may subsequently trigger endocytic uptake [3,5,12–15]. In fact, in the few studies combining cell biological and biophysical approaches, good correlations between membrane affinity and uptake efficiency could be observed [16,17]. This underlines that both the cell biology of the uptake and the biophysical analysis of the interactions between the CPPs and the bilayer membrane are legitimate approaches necessary for a better understanding of CPP translocation mechanisms.

In the present study, we investigate in detail the interactions of two classes of CPPs with several bilayer models with the aim to contribute to the mechanistic understanding of such interactions as a step towards cellular translocation. Four independent methodological approaches were employed, comprising liposome buffer equilibrium dialysis, Trp fluorescence quenching, fluorescence polarization, and determination of ζ-potentials. In particular, we compared weakly cationic C-terminal fragments of human calcitonin, hCT(9–32) and modifications thereof with two oligocationic CPPs, the vascular endothelial (VE)-cadherin-derived CPP, pVEC, and the Antennapedia homeodomain protein-derived penetratin, denoted pAntp (Table 1).

Human calcitonin (hCT) is a peptide hormone that is approved for the treatment of established osteoporosis [18]. N-terminally truncated derivatives of hCT, which lack hormonal activity, represent a novel class of weakly cationic CPPs and have been systematically investigated by Tréhin et al. It has been shown that sequences from hCT(9–32) to hCT(18–32) penetrated the plasma membrane of a fully organized epithelial model, differentiated MDCK monolayers, and resulted in a sectoral, vesicular cytoplasmic distribution. The uptake process was temperature-, time-, and concentration-dependent, indicating that translocation may follow an endocytic pathway; among the investigated derivatives, hCT(9–32) was the most efficient one, and its single, positively charged lysine in position 18 turned out to be essential for uptake [19]. Furthermore, uptake was found to be cell-line specific with a punctuated, cytoplasmic pattern in MDCK cells and paracellular accumulation in Calu-3 cell monolayers. Remarkably, hCT-derived peptides did not show significant permeation across the epithelial models [20]. This owes to their efficient metabolic cleavage when in contact with the epithelial cells [21].

Cadherins are single transmembrane-spanning glycoproteins of about 700 amino acids. pVEC, a peptide derived from the murine VE-cadherin, contains 18-amino acids (residues 615–632), with 13 cytosolic amino acids outside and 5 amino acids in the transmembrane region. It has been shown to translocate efficiently into various cell lines by a receptor-independent mechanism, and to carry macromolecular cargoes through plasma membranes [22,23]. The sequence of pAntp corresponds to the 16 amino acid sequence of the third α-helix (residues 43–58) of the Antennapedia homeodomain protein of *Drosophila* [24,25]. Residues 48 and 56 are tryptophans (Trp) being useful fluorescent probes for biophysical analysis. The third helix was found to be responsible for interaction with DNA by binding specifically to cognate sites in the genome and also for the translocation of the entire protein across cell membranes [24,25]. The fragment pAntp retains the membrane translocation properties of the homeodomain and has, therefore, been proposed as a universal vector for cellular delivery [2]. In fact, there are numerous studies proving the translocation of pAntp into different cell lines [26,27]. More recent studies propose an endocytic uptake [15,16]. Its exact translocation mechanism is still controversially discussed as reviewed by Trehin and Merkle [3].

To our knowledge, the present study provides the first investigation of a direct interaction of hCT-derived peptides and pVEC with bilayer models. Indirectly, interactions of hCT-derived CPPs have been suggested by atomic force

Table 1
Abbreviations, amino acid sequences and molecular weight of the peptides studied in this work

Abbreviation	MW (Da)	Sequence
hCT	3417.8	CGNLS TCMLG TYTQD FNKFH TFPQT AIGVG AP-NH_2
hCT(9–32)	2609.9	LG TYTQD FNKFH TFPQT AIGVG AP-NH_2
W10-hCT(9–32)	2739.0	L<u>W</u> TYTQD FNKFH TFPQT AIGVG AP-NH_2
A23-hCT(9–32)	2583.8	LG TYTQD FNKFH TF<u>A</u>QT AIGVG AP-NH_2
W30-hCT(9–32)	2739.0	LG TYTQD FNKFH TFPQT AIGV<u>W</u> AP-NH_2
hCT(18–32)	1698.9	KFH TFPQT AIGVG AP-NH_2
hCT(21–32)	1286.4	TFPQT AIGVG AP-NH_2
hCT-random	2609.9	FL TAGQN TIQTP VKTGG HFPFA DY-NH_2
pVEC	2208.7	LLIIL RRRIR KQAHA HSK-NH_2
W2-pVEC	2281.7	L<u>W</u>IIL RRRIR KQAHA HSK-NH_2
pAntp	2246.7	RQIKI WFQNR RMKWK K

Amino acid substitutions are underlined.