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Design and characterization of novel cell-penetrating peptides from pituitary adenylate cyclase-activating polypeptide

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The discovery of cell-penetrating peptide opened up new promising avenues for the non-invasive delivery of non-permeable biomolecules within the intracellular compartment. However, some setbacks such as possible toxic effects or unexpected immunological responses have limited their use in clinic. To overcome these obstacles, we investigated the use of novel cell-penetrating peptides (CPPs) derived from the endogenous neuropeptide *Pituitary adenylate cyclase-activating polypeptide* (PACAP). First, we demonstrated the propensity of native PACAP isoforms (PACAP27 and PACAP38) to efficiently deliver a large and non-permeable molecule, *i.e.* streptavidin, into cells. An inactive modified fragment of PACAP38, *i.e.* [Arg^{17}]PACAP(11–38), with preserved cell-penetrating physico-chemical properties, was also synthesized and successfully use for the intracellular delivery of various cargoes such as small molecules, peptides, proteins, and polynucleotides. Especially, its effectiveness as a transfection agent was comparable to Lipofectamine 2000 while being non-toxic for cells. Uptake mechanism studies demonstrated that direct translocation, caveolae-dependent endocytosis and macropinocytosis were involved in the internalization of [Arg^{17}]PACAP(11–38). This study not only opened up a new aspect in the usefulness of PACAP and its derivatives for therapeutic application but also contributed to the identification of new members of the CPP family. As such, inactive PACAP-related analogs could represent excellent vectors for *in vitro* and *in vivo* applications.

1. Introduction

Advances in genomics and proteomics have led to the identification of new molecular targets, notably intracellular enzymes or other types of proteins, for the treatment of various diseases. An effective drug requires access to the intracellular compartments thereby justifying the need for cell-penetrating molecules. The therapeutic potential of peptide-, protein-, and nucleic acid-based drugs is frequently compromised by their limited ability to cross the plasma membrane resulting in poor cellular access. Consequently, the recent discovery of various cell-penetrating peptides (CPPs), mostly synthetic or derived from protein transduction domain, has opened up new possibilities in biomedical research [1,2]. CPPs have been shown to cross cellular membranes and to deliver numerous conjugated biopharmaceuticals including proteins, peptides, siRNA, and DNA both *in vitro* and *in vivo* [1–4]. As such, several pre-clinical and clinical studies demonstrated the potential of CPPs as therapeutic vectors in the treatment of human diseases [3]. However, several drawbacks have impaired their rapid use in clinics. Indeed, CPPs, derived from transduction domain, were linked to an intrinsic gene expression or associated with regulatory processes [5]. Introduction of engineered cell-penetrating peptides was thought to represent a good alternative to circumvent these problems. However, such exogenous carriers might trigger a specific immunological response [5]. Altogether, the extent or consequences of these non-desirable effects raised safety issues regarding their therapeutic use. Consequently, the development of peptide carriers derived from naturally occurring endogenous peptide, like calcitonin, could be a promising way to overcome these obstacles [6].

The pituitary adenylate cyclase-activating polypeptide, a 38- or 27-amino acid C-terminally α-amidated neuropeptide (PACAP38 or

Abbreviations: CD, circular dichroism; CHO, Chinese hamster ovary; CPP, cell-penetrating peptide; FITC, fluorescein isothiocyanate; GFP, green-fluorescent protein; hCT, human calcitonin; HEK293, human embryonic kidney 293; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MβCD, methyl-β-cyclodextrin; MFI, median fluorescent intensity; PACAP, pituitary adenylate cyclase-activating polypeptide; RP-HPLC, reversed-phase high-performance liquid chromatography; Strep, Alexa Fluor® 568 streptavidin.

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PACAP27), was originally isolated from ovine hypothalamic extracts [7]. Both PACAP isoforms exert pleiotropic activities that are all mediated through the activation of three distinct receptors, named PAC1, VPAC1 and VPAC2, ubiquitously distributed within the central nervous system and in peripheral tissues [8,9]. Currently, PACAP agonists represent promising candidates to safely reverse or slow down the course of disabling neurological illnesses or brain trauma [10,11]. As a member of the VIP/glucagon/growth hormone-releasing hormone (GHRH) super-family, PACAP isoforms share structural and physicochemical properties, *i.e.* numerous basic residues dispersed throughout a C-terminal helical domain, with other members of this family [11,12]. As a matter of fact, it was demonstrated that PACAP isoforms, containing between 5 and 11 basic residues, adopt an amphipathic helical conformation when interacting with cellular membrane [13,14]. Interestingly, as recently reported, both PACAP isoforms are able to translocate within the intracellular compartments through a receptor-independent mechanism [15].

In the present study, we first demonstrated the propensity of PACAP38 itself to efficiently deliver large macromolecules, such as streptavidin, across cell membrane. However, the use of PACAP itself as a CPP is hampered by its pleiotropic activities *via* the activation of its cognate receptors. Thus, two shorter and inactive analogs of PACAP, *i.e.* PACAP(11–38) and [Arg^{17}]PACAP(11–38), were designed and characterized for their ability to deliver various cargoes, including large proteins, peptides, ribozymes, or DNAs within the intracellular compartment. Our present investigation demonstrates for the first time the ability of PACAP-based CPPs to efficiently deliver molecules into cellular compartment extending the scope of the usefulness of PACAP derivatives for therapeutic purposes.

2. Materials and methods

2.1. Materials

Fmoc-protected amino acids, Rink-amide AM-functionalized polystyrene resin and BOP (benzotriazol-1-yl-oxy-*tris*(dimethylamino)-phosphonium hexafluorophosphate) reagent were purchased from Chem-Impex (Wood Dale, IL, USA). Common solvents for solid phase peptide synthesis and purification were obtained from Fisher Scientific (Nepean, ON, CAN) whereas trifluoroacetic acid (TFA) was from PSIG (Montreal, QC, CAN). $Na^{125}I$ and DRAQ5™ were respectively purchased from PerkinElmer (Montreal, QC, CAN) and BioSatus Ltd. (Leicestershire, UK). Alexa Fluor® 568 streptavidin was supplied by Invitrogen (Burlington, ON, CAN). Endocytosis inhibitors, *i.e.* nocodazole, nystatin, amiloride, sucrose, ammonium chloride, and chloroquine, as well as other chemicals including fluorescein isothiocyanate (FITC), D-biotin, *m*-cresol, and propidium iodide (PI) were obtained from Sigma Aldrich (Mississauga, ON, CAN).

2.2. Peptide synthesis and purification

All peptides were synthesized on a Rink-amide AM-functionalized polystyrene resin (0.53 mmol/g) using standard Fmoc chemistry and a BOP coupling strategy. Biotinylated peptides were directly obtained by conjugating a biotin moiety to the N-terminal of the peptide-resins. FITC-conjugated peptides were synthesized as previously described [15]. Peptide cleavage was achieved at room temperature using a mixture of TFA/ethanedithiol/phenol/water (92/2.5/3/2.5) for 3 h. The resulting crude peptides were purified on a preparative RP-HPLC using a Phenomenex C_{18} Gemini column and the collected fractions were analyzed concomitantly by analytical RP-HPLC, performed on a Phenomenex C_{18} Jupiter column, and MALDI-TOF mass spectrometry using α-cyano-4-hydroxycinnamic acid as matrix (Voyager DE, Applied Biosystems). Fractions corresponding to the desired product and at a purity higher than 95% were finally pooled and lyophilized (Table S1).

2.3. Circular dichroism analysis

Circular dichroism (CD) spectra were recorded at room temperature from 189 to 250 nm, using a 0.1 mm optical path length with a 0.5 nm step, a 2 nm bandwidth, and an integration time of 0.2 s on a CD6 dichrograph (Jobin-Yvon, Longjumeau, FR). Each spectrum represents the mean of three scans corrected for solvent contribution. A digital low-pass filter was used as a smoothing routine. Peptides were dissolved in 20% 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) at a final concentration of 0.05 mg/mL.

2.4. Point mutation modeling

The Met^{17}Arg point mutation was introduced in the PACAP38 coordinates (PDB ID: 2D2P) using the SCWRL4 software [16]. This software uses a backbone-dependent rotamer library, an energy function based on its library of rotamer frequencies, and a repulsive steric energy term. It also uses a graph decomposition to solve the combinatorial packing problem to find the best side-chain conformation of the newly introduced residue [17].

2.5. Electrostatic potentials

Visualization and calculations were performed with PyMOL 1.3r1 using [Arg^{17}]PACAP(11–38) coordinates. The PQR structure was generated with the Python software package PDB2PQR [18,19] and electrostatic potentials were calculated with the PyMol-implemented Adaptive Poisson-Boltzmann Solver (APBS Tools 2.1) [20].

2.6. Cell culture

CHO-K1 and HEK293 cells were maintained respectively in Ham-F12 and DMEM medium supplemented with 2 mM L-glutamine, 100 UI/mL each of penicillin and streptomycin, and 10% fetal bovine serum (FBS). HeLa cells were grown in MEM media containing 10% FBS, 100 UI/mL each of penicillin and streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. Transfected CHO cells co-expressing the human PAC1, VPAC1 or VPAC2 receptor and a mitochondrial apo-aequorin protein were maintained in Ham-F12 medium with 10% FBS, 2 mM L-glutamine, 100 UI/mL each of penicillin and streptomycin, 400 μg/mL G418 and 250 μg/mL zeocin. Cells were maintained as monolayer at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air and passages were performed by trypsinization once cells reached 70–80% confluence.

2.7. Pharmacological characterization

Binding and intracellular calcium mobilization experiments were performed in CHO cells stably transfected with the human PAC1, VPAC1 or VPAC2 receptor and a mitochondrial apo-aequorin protein as previously reported [21]. Briefly, for competition binding assay, transfected cells, cultured in 24-well plates, were washed three times with binding buffer [25 mM Tris–HCl, 25 mM $MgCl_2$, 0.1% (w/v) BSA, and 5 mg/L bacitracin] and then exposed to increasing peptide concentrations (10^{-12} M to 10^{-5} M) in the presence of 0.05 nM ^{125}I-Ac-PACAP27. Following a 2 h incubation at room temperature, cells were washed twice with binding buffer, lyzed with NaOH (0.1 M), and the cell-bound radioactivity was quantified using a γ-counter (1470 Automatic Gamma Counter, Perkin Elmer). Results were expressed as the percentage of the specific binding of ^{125}I-Ac-PACAP27 obtained in the absence of competitive ligands. Non-specific binding was determined in the presence of 10 μM PACAP38 and averaged 10% of total binding.

For calcium mobilization assay, cells in mid-log phase were detached, re-suspended at 5×10^6 cells/mL in culture media supplemented with 10 mM HEPES and 0.1% BSA. Then, following the addition of Coelenterazine H (at a final concentration of 5 μM), the