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Short communication

Cell-penetrating peptides modulate the vascular action of phenylephrine

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Abstract:
Cell-penetrating peptides (CPP) are a family of peptides able to penetrate the cell membrane. This group of compounds has attracted consideration as potential therapeutic tools for the delivery of various substances into cells. Here, we investigated possible interactions between several CPP synthesized in our laboratory and the vascular action of phenylephrine.
We used isolated rat tail artery and examined the influence of pretreatment by seven different CPP on the concentration-response curve induced by the α_1 receptor agonist phenylephrine. Peptides were synthesized by solid-phase peptide synthesis (SPPS) using the 9-fluorenylmethoxycarbonyl (Fmoc) method.
Among the seven different polypeptides, i.e., TP10 (transportan-10), [Lys(AAc)^{13}]TP, [Lys(CAc)^{13}]TP, [Lys(GAc)^{13}]TP, [Lys(TAc)^{13}]TP, [Lys(UAc)^{13}]TP and [Lys(Ac)^{13}]TP, only TP10 and [Lys(AAc)^{13}]TP, both at a concentration of 1 μM (the lowest concentration inducing a significant change in the contraction of isolated rat stomach in our pilot study), rendered rat tail artery more sensitive to phenylephrine; the relative potency increased significantly. Conversely, [Lys(Ac)^{13}]TP strongly decreased the efficacy of phenylephrine.

Key words:
transportan, phenylephrine, cell-penetrating proteins, rat tail artery

Introduction

One of the fundamental requirements for drug efficacy is the interaction of drug molecules with effectors (usually specific receptors), as only direct drug-receptor interaction ensures appropriate therapeutic effect. Bioavailability is a well-known pharmacokinetic parameter and indicates the fraction of an applied drug dose entering the central compartment, i.e., blood. However, even drugs with high levels of bioavailability may not work properly if the target receptors are located intracellularly and the drug molecules do not penetrate the cells. The techniques currently used for the internalization of different substances such as genes, antibodies and others including electroporation and microinjection are robust and harsh. Therefore, these techniques are impractical for *in vivo* use due to the high level of cell damage [8]. Thus, substances able to penetrate cell membranes have recently attracted consideration in the context of their use as tools for the feasible transportation of various drugs into cells. Cell-penetrating peptides (CPP) consist of short peptide sequences that are able to translo-

cate large molecules into the cells and increase the efficacy of specific drugs as well as reduce their toxicity. However, the mechanism of this transport remains unclear [3, 11, 15]. After synthesizing several CPP in our preliminary study, we performed experiments to determine if these peptides affect phenylephrine vascular action using a typical model, an isolated rat tail artery.

Materials and Methods

Synthesis and purification of peptides

Peptides (Tab. 1) were synthesized by solid-phase peptide synthesis (SPPS) using a Labortec AG model SP 650 peptide synthesizer and the 9-fluorenylmethoxycarbonyl (Fmoc) strategy [1, 10, 12–14]. All amino acids were coupled as active derivatives with the use of *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate with the addition of 1-hydroxybenzotriazole (1:1) coupling method. Deprotection of the Fmoc group was conducted with 20% piperidine in *N,N*-dimethylformamide. In the case of analogues **3–10**, in which previously prepared 2',3'-O-isopropylidenenucleoside-5'-carboxylic acids [4, 5] or nucleobase acetic acids [2] were coupled *via* the ε-amino group of an L-Lys residue to transportan (TP) molecules, a hydrazine-labile ivDde group was used to protect the ε-amino function group of the Lys^{13} residue within the TP structure. Additionally, an acid-labile *tert*-butoxycarbonyl group was used to protect the α-amino function group of the *N*-terminal Gly residue. After synthesis, the peptides were cleaved from the solid support with a trifluoroacetic acid/phenol/triisopropylsilane/water (88:5:2:5) mixture. The crude peptides thus obtained were purified by reverse-phase high-performance liquid chromatography (RP-HPLC), with several isocratic systems and linear gradients of acetonitrile in 0.1% trifluoroacetic acid. Eluates were fractioned and analyzed by analytical RP-HPLC. Peptide fractions with purities greater than 98% were combined and lyophilized. The identities of the peptides were confirmed by MALDI-TOF mass spectrometry.

Tab. 1. Primary structures of the studied peptides

No.	Peptide	Amino acid sequence
1	TP10 (transportan-10)	AGYLLG**K**INLKALAALAKKIL-NH_2
2	[Lys(AAc)$_{1,3}$]TP	GWTLNSAGYLLG**K(AAc)**INLKALAALAKKIL-NH_2
3	[Lys(CAc)$_{1,3}$]TP	GWTLNSAGYLLG**K(CAc)**INLKALAALAKKIL-NH_2
4	[Lys(GAc)$_{1,3}$]TP	GWTLNSAGYLLG**K(GAc)**INLKALAALAKKIL-NH_2
5	[Lys(TAc)$_{1,3}$]TP	GWTLNSAGYLLG**K(TAc)**INLKALAALAKKIL-NH_2
6	[Lys(UAc)$_{1,3}$]TP	GWTLNSAGYLLG**K(UAc)**INLKALAALAKKIL-NH_2
7	[Lys(Ac)$_{1,3}$]TP	GWTLNSAGYLLG**K(Ac)**INLKALAALAKKIL-NH_2

Attached molecules: AdC – adenosine-5'-carboxylic acid, UrC – uridine-5'-carboxylic acid, CyC – cytidine-5'-carboxylic acid, AAc – 9-adeninylacetic acid, CAc – 1-cytosinylacetic acid, GAc – 9-guaninylacetic acid, TAc – 1-thyminylacetic acid, UAc – 1-uracilylacetic acid, Ac – acetic acid

Experimental design

All experiments were performed on Wistar rats weighing between 200 and 300 g and kept under standard laboratory conditions. All animals received humane care in accordance with the recommendations of the local Ethics Committee.

According to a well-established method [7], after anesthesia by pentobarbital (60 mg/kg, *ip*), the segments of the ventral portion of the tail artery were carefully removed, cleaned of adhering tissue and cut into 3–5 mm rings and then mounted under optimal tension in a 10-ml bath containing a solution of the following composition: 119 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 11 mM glucose. The solution was held at 37°C and aerated with carbogen (5% CO_2 and 95% O_2). The isolated arterial segments were attached to a pressure transducer (TAM-D, Hugo Sachs Elektronik, March, Germany), with a preload of 0.5 g. A peristaltic pump (PP1-05, Poland) supported the perfusion of the arterial fragments with flows ranging from 0.4 ml/min at the beginning of the experiments up to 1.6 ml/min during application of the drugs. After incubation for 90 min, all preparations were treated with the following: 1) phenylephrine (at increasing concentrations from 1×10^{-7} M to 3×10^{-4} M) alone, or 2) phenylephrine in the presence of a CPP at 1 μM concentration (perfusion of arterial segments with a solution containing each peptide was started 10 min before the first aliquot of phenylephrine was added). Previously,

a pilot study on CPP-phenylephrine interaction effects on rat stomach fundus contraction was performed to determine the peptide concentration exhibiting a significant effect on the smooth muscle.

Statistics

Pharmacological parameters related to drug efficacy as pD_2, relative potency and Hill coefficient were calculated using the computer program GraphPad Prism ver. 4.0. All results are expressed as the mean ± SE. The concentration-response curves were analyzed using Pharma/PCS version 4 (Pharmacological Calculations System) software. The statistical significance of the differences between the means was determined by Student's *t*-test for paired data (for each point of the curve before and after treatment with CPP) and by ANOVA with a multiple comparison Newman-Keuls test to determine the type of data distribution; $p < 0.05$ was taken as the level of significance.

Results and Discussion

As shown in Figures 1–3, phenylephrine, a selective α_1 receptor agonist, increased the tension of the rat tail artery in a concentration-dependent manner. Moreover, in the presence of TP10 and [Lys(AAc)^{13}] TP, its efficacy was augmented. Interestingly, TP10 enhanced the effects of phenylephrine in the middle of the concentration-response curve (Fig. 1), whereas [Lys(AAc)^{13}]TP did the same but at higher concentrations of phenylephrine, including its maximal effect (Fig. 2). Notably, the relative potency of phenylephrine increased in both cases (from 1 to 1.45 and to 1.49, respectively; $n = 6$, $p < 0.05$); however, the pD_2 values ($-\log EC_{50}$) were unchanged (5.41 ± 0.4 *vs.* 5.56 ± 0.5 and 5.42 ± 0.3 *vs.* 5.4 ± 0.4, respectively; $n = 6$, NS). Additionally, the Hill coefficient of phenylephrine action increased in the case of TP10, from 1.46 to 2.73 ($p < 0.05$), whereas it decreased in the case of [Lys(AAc)^{13}]TP, from 1.43 to 1.22 (NS), see Table 2. Conversely, the addition of [Lys(Ac)^{13}]TP significantly decreased the vascular action of phenylephrine, as demonstrated by the statistically significant lower potency (0.33 *vs*. 1) and efficacy (41.4%) of the drug acting in the presence of this peptide (Fig. 3).

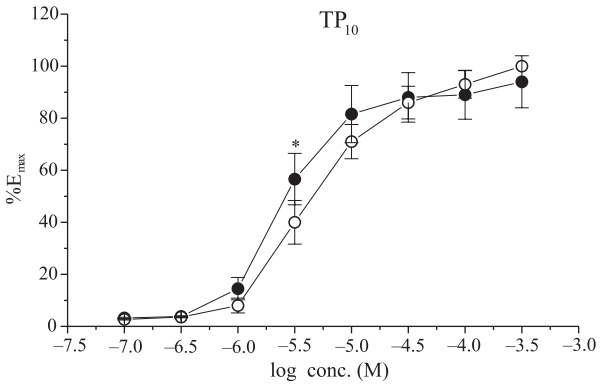

Fig. 1. Concentration-response curve of phenylephrine alone (open circles) and after pretreatment with 1 μM TP_{10} (closed circles). * $p < 0.05$; Student's *t*-test paired data, n = 6, the mean ± SE

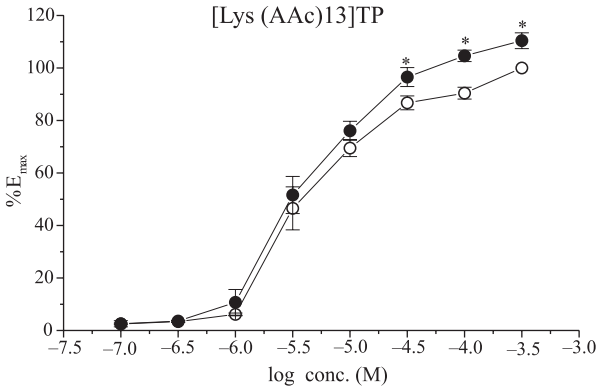

Fig. 2. Concentration-response curve of phenylephrine alone (open circle) and after pretreatment with 1 μM [Lys(AAc)13]TP (closed circles). * $p < 0.05$; Student's *t*-test paired data, n = 6, the mean ± SE

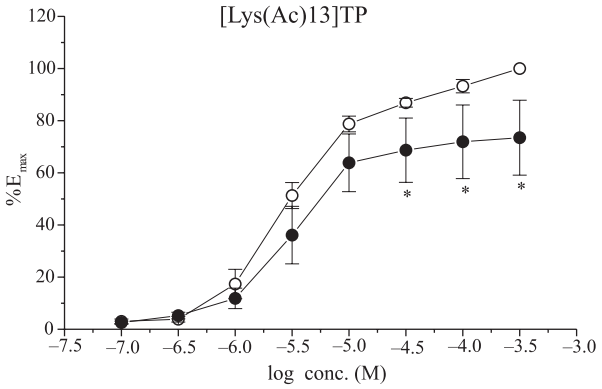

Fig. 3. Concentration-response curve of phenylephrine alone (open circle) and after pretreatment with 1 μM [Lys(Ac)13]TP (closed circles). * $p < 0.05$; Student's *t*-test paired data, n = 6, the mean ± SE

Tab. 2. Some parameters related to phenylephrine vascular action in rat tail artery in the presence of different CPP

CPP	Hill coefficient	Relative potency	p
TP_{10}	2.7*	1.45*	< 0.05
[Lys(AAc)13]TP	1.2	1.49*	< 0.05
[Lys(Ac)13]TP	3.8**	0.34**	< 0.01

CPP – cell penetrating peptides

Phenylephrine vascular action is exclusively mediated *via* α_1-adrenoceptors. The structure of this receptor is well established, consisting of seven transmembrane domains connected by alternating extracellular and intracellular loops; however, there are several subtypes of this receptor. It seems that several specific amino acid residues located in extracellular domains III, IV and V are responsible for the binding of some antagonists such as prazosin and tamsulosin [9]. CPP are generally divided in two classes: the first are amphipathic helical peptides such as transportan and the model amphipathic peptide (MAP), and the second are arginine-rich peptides such as penetratin and the trans-activating transcriptional activator TAT [6]. The peptides examined in this study belong to the first class and contain the peptide sequences from the amino terminus of the neuropeptide galanin. There is one unique characteristic of [Lys(Ac)^{13}]TP that should be taken into account in context of its negative interaction with phenylephrine, namely, this is the only peptide among our series with an acetylated ε-amino group in the lysine side chain; therefore, this peptide is acidophilic, in contrast to all the others, which are basophilic. The acetylated ε-amino group could be very important, as acetylation of some enzymes and receptors has an irreversible character and serious functional consequences. Therefore, this could be an explanation for the diminished effects of phenylephrine on vascular smooth muscle previously treated with [Lys(Ac)^{13}]TP. The high value of the Hill coefficient (Tab. 2) suggests some structural changes to the α_1-adrenoceptors, resulting in the low affinity of phenylephrine under such conditions. Taking into account the effects of [Lys(Ac)^{13}]TP, which is the most important in our study from the clinical viewpoint of the possible use of CPP as a drug carrier, the significantly lower E_{max} of phenylephrine in the presence of this peptide strongly suggests an allosteric interaction. The main limitation of this study is that we did not use higher concentrations of CPP. However, the aim of this preliminary study was to determine the threshold concentration of CPP bearing significant risk of serious side effects were these compounds to be used as drug carriers.

To conclude, in this preliminary study we showed that several peptides in the CPP family can interact with α_1-adrenoceptors. Special attention should be paid to those that, by their inhibitory action on α_1-adrenergic receptors, could induce severe hypotension, which should be taken into account in their future use for the delivery of large molecules.

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