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Structural Requirements for Cellular Uptake of α-Helical Amphipathic Peptides

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Abstract: The structure of the cell-permeable α-helical amphipathic model peptide FLUOS-KLALKLALKA-LKAALKLA-NH_2 (**I**) was modified stepwise with respect to its helix parameters hydrophobicity, hydrophobic moment and hydrophilic face as well as molecular size and charge. Cellular uptake and membrane destabilizing activity of the resulting peptides were studied using aortic endothelial cells and HPLC combined with CLSM. With the exceptions that a reduction of molecule size below 16 amino acid residues and the introduction of a negative net charge abolished uptake, none of the investigated structural parameters proved to be essential for the passage of these peptides across the plasma membrane. Membrane toxicity also showed no correlation to any of the parameters investigated and could be detected only at concentrations higher than 2 μM. These results implicate helical amphipathicity as the only essential structural requirement for the entry of such peptides into the cell interior, in accord with earlier studies. The pivotal role of helical amphipathicity was confirmed by uptake results obtained with two further pairs of amphipathic/non-amphipathic 18-mer peptides with different primary structure, net charge and helix parameters from **I**. The amphipathic counterparts were internalized into the cells to a comparable extent as **I**, whereas no cellular uptake could be detected for the non-amphipathic analogues. The mode of uptake remains unclear and involves both temperature-sensitive and -insensitive processes, indicating non-endocytic contributions.

Keywords: cellular uptake; amphipathic peptides

INTRODUCTION

Most current strategies for translocating polar compounds such as antisense oligonucleotides and peptides across the plasma membrane of living cells rely on endocytic mechanisms [1]. An endocytic mode of uptake, however, poses problems in that the quantity of transported substrates and particularly the rate of their liberation from endosomes are limited; sufficiently high concentrations at the target sites in the cytosol or nucleus are therefore difficult to achieve [1,2].

As promising alternatives, non-endocytic translocation approaches have been presented recently by several authors, based on the use of protein derived vector peptides [3–8]. These natural peptides proved to be capable of introducing covalently tagged peptides and oligonucleotides directly into the cytosol in an non-destructive fashion. The actual mechanism of entry of these peptides and their conjugates remains unclear. The non-endocytic nature of the cellular uptake is indicated by the observation that efficient translocation occurs even at 0°C [3]. In a previous study we could mimic the

Abbreviations: AEC, bovine aortic endothelial cells; CLSM, confocal laser scanning microscopy; DPBSG, Dulbecco's phosphate buffered saline supplemented with 1 g/L D-glucose; FLUOS-, 5(6)-carboxyfluoresceinyl; PBS, phosphate buffered saline.

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permeation behaviour of the aforementioned natural peptides with a simple helical amphipathic model peptide, FLUOS-KLALKLALKALKAALKLA-NH_2 (**I**) [9,10]. Our results indicated that multiple, energy-dependent and -independent mechanisms are involved in the cellular uptake of **I**, and that amphipathicity is crucial. With a view to a more rational design of such vector peptides, we have investigated further the structural requirements for the membrane-translocating activity of helical amphipathic peptides. To this end we varied the structure of **I**, firstly with respect to its hydrophobicity, hydrophobic moment and hydrophilic face, leaving other parameters nearly constant and secondly by varying molecular size and charge. Cellular uptake and membrane destabilizing activity of the resulting peptides were studied using aortic endothelial cells by HPLC combined with CLSM.

MATERIALS AND METHODS

5(6)-Carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS) was purchased from Boehringer (Mannheim, Germany). Release of lactate dehydrogenase was assessed by means of LDH-L reagent from Sigma (Deisenhofen, Germany) Other chemicals and reagents, when not specified, were purchased from Sigma or Bachem (Heidelberg, Germany).

Peptide hydrophobicity (H) and the hydrophobic moment (μ) for an ideal helix were calculated using the Eisenberg scale [11]. The term hydrophilic face of the helix (Φ) refers to the angle subtended by those amino acid residues exhibiting a higher hydrophilicity than alanine according to the Eisenberg scale [11]. Circular dichroism (CD) measurements were carried out using 10^{-5} M peptide solutions in TFE/H_2O 1:1 or 0.015 M SDS, respectively, on a J 720 spectrometer (Jasco, Japan) between 185 and 260 nm at room temperature.

Peptide Synthesis

Peptides were synthesized automatically (MilliGen 9050 peptide synthesizer) by solid-phase methods using standard Fmoc chemistry in the continuous flow mode (TentaGel S RAM resin 0.22 mmol/g (Rapp Polymere, Tübingen), TBTU, 2 equivalents of DIEA, coupling 20 min, deblocking with 20% piperidine in DMF for 10 min, final cleavage with 93% TFA/5% H_2O/2% triisopropylsilane for 3 h, as described previously [12]. To introduce the fluorescent label, the peptides were N-terminally conjugated with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS; Boehringer, Mannheim) (2 equivalents in DMF), and the final cleavage was performed with 95% TFA/5% water for 3 h. Purification of 100 mg samples was carried out by preparative HPLC on PLRP-S 100A, 10 μ, 250 × 20 mm i.d. (Phenomenex, Hösbach, Germany) to give final products >95% pure by RP-HPLC analysis, which gave the expected $[M+H]^+$ mass peaks by MALDI-MS (MALDI II, Kratos, Manchester).

Cell Culture

Calf aortic endothelial cells (AEC), 12.–20. subculture of a cell line (LKB Ez 7), established and characterized by Halle *et al.* [13], were seeded at an initial density of 5×10^4 cells/cm^2 in 12-well culture plates. The cells were cultured at 37°C in a humidified 5% CO_2 air environment in minimal essential medium supplemented with 290 mg/L glutamine and 10% fetal calf serum. After 4 days without replacing the medium, the cells were used for uptake experiments.

Uptake Experiments

After removal of the medium, the cells were rinsed two times at 37°C with Dulbecco's phosphate buffered saline (DPBS; Biochrom KG, Berlin) supplemented with 1 g/L D-glucose (DPBSG) and subsequently exposed, unless indicated otherwise, at 37°C for 30 min to 500 µL of the peptide solutions in DPBSG. Thereafter the incubation solutions were aspirated and the cells washed two times with ice-cold PBS, incubated with 500 µL of ice-cold PBS and treated with diazotized 2-nitroaniline as described previously [14] in order to modify any surface-bound peptide. In brief: to 400 µL ethanol/water 1:1 v/v containing 2-nitroaniline (0.06 M) and HCl (0.125 M), 50 µL 0.6 M $NaNO_2$ were added. After standing for 5 min at ambient temperature, 10 µL of this reagent were added to the ice-cold PBS covering the cell layer and allowed to react for 10 min at 0°C. After aspiration of the diazo reagent the cells were washed two times with ice-cold PBS and finally lysed with 0.5 mL 0.1% Triton X-100 containing 10 mmol/L trifluoroacetic acid for 2 h at 0°C. The resulting lysate was used for HPLC-analysis and protein determination according to Bradford [15]. The average protein content of 10^6 cells assayed by this method was 110 µg. The average volume of the cells was determined to be 1.4 pL by means of a Coulter-ZM counter (Counter Electronics Ltd., Luton, UK).

In each experiment triplicate wells were exposed in parallel for 30 min at 37°C to 1.8 μM **I** to obtain reference values for normalization. The basis values for normalization, determined in eight independent uptake experiments were 50 ± 31, 56 ± 29 and 155 ± 37 pmol/mg protein for the metabolized, internalized and membrane-inserted peptide fractions, respectively.

To measure the pore forming propensity of the peptides, the cells were pre-exposed to a 12 μM solution of fluorescein diacetate in DPBSG (containing 0.5% DMSO) for 15 min at 37°C. After aspiration of the fluorescein diacetate solution, the cells were rinsed four times with DPBSG at 37°C and then exposed to the peptides.

HPLC Analysis

HPLC was performed using a Bischoff-HPLC-gradient system (Leonberg, Germany) equipped with a Polyencap A 300, 5 μm) column (250×4 mm i.d.), precolumns containing the same adsorbent and a Fluorescence HPLC-Monitor RF-551 (Shimadzu).

Up to 200 μL of the cell lysates were passed through a precolumn containing 60 mg of Polyencap (A 300, 5 μm) in the case of fluorescein labelled peptides or 60 mg of the Pinkerton type [16] absorbent polyhydroxyethyl aspartamide (60 mg, 5 μm, 300 A; Ict GmbH, Frankfurt, Germany) for tryptophan labelled peptides, which thereafter was connected to the HPLC column. The elution was carried out with 0.01 M TFA (A) and acetonitrile/water 9:1 (B) at a flow rate of 1.0 mL/min with gradients from 30 to 45% B (0–10 min) and 45 to 80% B (15–20 min). Quantitation was performed by fluorescence measurement at 520 nm (fluorescein) and 350 nm (tryptophan) after excitation at 445 and 285 nm, respectively, using calibration values obtained with the parent peptide under identical conditions.

Confocal Laser Scanning Microscopy (CLSM)

A total of 10^4 cells were plated on 22×22 mm coverslips glued (with Silicones RTV 615A/615B, Paul Hellermann GmbH, Pinneberg, Germany) above the hole (15 mm diameter) of punched plastic Costar culture dishes (35 mm diameter; Tecnomara Deutschland GmbH, Frankfurt/Main, Germany); culture conditions, exposure to peptides and washes were as described above. After subsequent overlaying of the cells with 200 μL PBS, microscopy was performed within 10 min at room temperature using an LSM 410 invert confocal laser scanning microscope (Carl Zeiss Jena GmbH, Jena, Germany). Excitation was performed at 488 nm by means of an Argon–Krypton laser and a dichroitic mirror FT 510 for wavelength selection. Emission was measured at 515 nm with a cut-off filter LP 515 in front of the detector. For optical sectioning in the z-direction, 16 frames with a thickness of 1 μm were made.

RESULTS

Influences of Altered Helix Parameters upon Cellular Uptake

To gain insights into the role of helix parameters in cellular uptake we assessed the uptake into endothelial cells of a series of analogues of the parent amphipathic model peptide KLALKLALKALKAALKLA-NH_2 which had stepwise alterations of hydrophobicity, hydrophobic moment or hydrophilic face but unchanged positive charge and helix forming propensity (Table 1).

The peptide series had been designed earlier in another context [17] and contained tryptophan as an internal fluorescent probe. Impairment of HPLC-quantitation by interference of the tryptophan fluorescence with that of cell-own compounds could be circumvented by means of a precolumn containing a biphasic aspartamide solid-phase of the Pinkerton type [16]. Passage of the cell lysates through this precolunm before connection to the HPLC apparatus allowed wash out of higher molecular weight cell components while retarding the peptides.

Differentiation between surface-bound and internalized peptide fractions was performed as described previously [10,14] using the membrane-impermeable, primary amino group modifying reagent diazotized 2-nitroaniline. Fluorescent products of metabolic breakdown generally showed peak areas lower than 20% of that of the respective intact peptide in the HPLC chromatogram and were neglected.

For measuring pore-forming activity the leakage of fluorescein from the cell interior after preloading cells with diacetylfluorescein was determined. In order to monitor toxic effects of the respective peptides, a generally slightly toxic peptide concentration of 5 μM was chosen; in the case of the parent peptide **I**, 30% fluorescein leakage was observed under these conditions [10].

Clear cellular uptake and also pore-forming activity were found in almost all cases (Table 1). No unambigous correlation, however, either between

Table 1 Internalization of Peptide into Aortic Endothelial Cells and Fluorescein Leakage after Preloading with Fluorescein Diacetate and Exposure for 30 min at 37°C to 4.5 μM Solutions in DPBSG of KLA1-Related Peptides Showing Stepwise Alterations of the Helix Parameters Hydrophobicity (H), Hydrophobic Moment (μ) and Hydrophilic Face (Φ)

Peptide[a]	Peptide composition	pmol internalized peptide/mg protein	Fluorescein leakage related to that of KLA1-exposed cells[b]	H	μ	Φ (°)	α-Helix (%) 50% TFE
KLA1	KLALKLALKAW-KAALKLA-NH_2	161 ± 21	1.0 ± 0.09	-0.025	0.329	80	73
KLA2	KLALKAALKAW-KAAAKLA-NH_2	15 ± 6	0.75 ± 0.14	-0.056	0.329	80	68
KLA3	KLALKAAAKAW-KAAAKAA-NH_2	<10	0.79 ± 0.12	-0.087	0.329	80	59
KLA11	KITLKLAIKAW-KLALKAA-NH_2	47 ± 7	0.98 ± 0.16	-0.027	0.284	80	69
KLA5	KIAAKSIAKIW-KSILKIA-NH_2	68 ± 2	1.46 ± 0.36	-0.026	0.406	80	64
KLA12	KALAKALAKLW-KALAKAA-NH_2	149 ± 20	1.21 ± 0.11	-0.056	0.391	80	67
KLA13	KLALKLALKWA-KLALKAA-NH_2	15 ± 4	0.75 ± 0.14	-0.025	0.320	80	61
KLA14	KLLAKAAKKWL-LLALKAA-NH_2	29 ± 14	0.72 ± 0.10	-0.025	0.300	100	60
KLA9	KLLAKAALKWL-LKALKAA-NH_2	44 ± 10	1.39 ± 0.42	-0.025	0.295	120	55
KLA10	KALKKLLAKWL-AAAKALL-NH_2	218 ± 31	1.42 ± 0.17	-0.025	0.299	140	62
KLA15	KLAAALLKKWK-KLAAALL-NH_2	226 ± 19	0.94 ± 0.12	-0.025	0.297	160	60
KLA8	KALAALLKKWA-KLLAALK-NH_2	341 ± 58	0.89 ± 0.09	-0.025	0.291	180	62

[a] Designation as used by Dathe *et al.* [17].

physico-chemical parameters and uptake behaviour/pore-forming activity or between uptake behaviour and pore-forming activity was apparent from the data obtained. Generally these findings argue against a decisive role for any of the altered helix parameters for cellular uptake.

Influences of Molecular Size, Charge and Primary Structure upon Cellular Uptake and Membrane Toxicity

To investigate further the structural requirements affecting the ability of this type of model peptide to cross or destabilize plasma membranes, we altered chain length, charge and helix forming propensity using FLUOS-KLALKLALKALKAALKLA-NH_2 (**I**) as parent compound. Comparison with the results obtained with the tryptophan labelled series is justified by the similar uptake and toxicity found for the related pairs of tryptophan and fluorescein labelled analogues KLAI and **I** and KLA8 and **II**, respectively (Tables 1 and 2).

Reduction of the chain length by two amino acid residues had no significant influence upon translocation activity (**III**; Table 2). Shortening the molecule by four amino acid residues, either N- or C-terminally, however, resulted in a substantial loss of uptake and also of membrane toxicity (**IV**, **V**; Table 2), suggesting a minimum chain length corresponding to four complete helix turns to be essential.

Replacement of two of the five lysines of the molecule by glutamine residues slowed the uptake markedly, accompanied by a clear reduction in membrane toxicity (**VII**; Table 2). Surprisingly, further elimination of basic lysine side-chains resulted in a significantly enhanced entry rate into the cell interior (**VIII**, **IX**; Table 2), even when the noticeable contributions of surface-bound peptide to the quan-

Table 2 Internalization of Peptide into Aortic endothelial Cells and Fluorescein Leakage after Preloading with Fluorescein Diacetate and Exposure for 30 min at 37°C to 4.5 μM Solutions in DPBSG to **I**-Related Peptides with Alterations in Chain Length, Charge and Helix Parameters, to **I**-Non-Related Amphipathic-Non-Amphipathic Peptide Pairs (**XI**/**XII**, **XIII**/**XIV**), Possessing Reduced Net Charge with Respect to **I**, and to the Natural Cell-Permeable Peptide Ant-P [3,4]

Peptide	Peptide composition	pmol internalized peptide/mg protein	Fluorescein leakage related to that of I-exposed cells[a]	*H*	*μ*	Φ (°)	α-Helix (%) TFE	α-Helix (%) SDS
I	Fl-KLALKLALKAL-KAALKLA-NH_2	228 ± 54	1.0 ± 0.38	−0.0161	0.3339	80	60	68
II	Fl-KALAALLKKLA-KLLAALK-NH_2	371 ± 56	0.66 ± 0.18	−0.0161	0.3001	180	65	72
III	Fl-KLALKLALKAL-KAALK-NH_2	218 ± 23	3.13 ± 0.47	−0.0161	0.3339	80	52	61
IV	Fl-KLALKALKAAL-KLA-NH_2	<30	0.30 ± 0.18	−0.0317	0.3358	60	45	51
V	Fl-KLALKLALKAL-KAA-NH_2	<30	0.43 ± 0.25	−0.0317	0.330	80	50	59
VI	F1-KLGLKLGLKG-LKGGLKLG-NH_2	<30	0.33 ± 0.19	−0.0461	0.313	80	13	8
VII	Fl-KLALKLALKAL-QAALQLA-NH_2	42 ± 12.4	0.59 ± 0.23	0.0294	0.2912	80	53	66
VIII	Fl-KLALQLALQAL-QAALQLA-NH_2	461 ± 44	1.03 ± 0.10	0.075	0.2505	80	78	78
IX	Fl-QLALQLALQAL-QAALQLA-NH_2	5670 ± 3971[b]	0.95 ± 0.62	0.0978	0.234	80	72	72
X	Fl-ELALELALEAL-EAALELA-NH_2	135 ± 14.6[b]	0.34 ± 0.28	0.117	0.216	80	84	21
XI	Fl-LKTLATALTKL-AKTLTTL-NH_2	539 ± 80	0.88 ± 0.35	−0.025	0.3414	160	95	79
XII	FL-LLKTTALLKTT-ALLKTTA-NH_2	n.d.[c]	0.57 ± 0.17	−0.025	0	360	74	39
XIII	Fl-LKTLTETLKEL-TKTLTEL-NH_2	141 ± 54	0.79 ± 0.32	−0.170	0.387	220	78	56
XIV	FL-LLKTTELLKTT-ELLKTTE-NH_2	n.d.[c]	0.42 ± 0.15	−0.170	0	360	52	17
XV	Fl-RQIKIWFQNR-RMKWKK-NH_2	216 ± 58	0.29 ± 0.10	−0.4822	0.1652	280	29	23

[a] Fluorescein content of **I** exposed cells/fluorescein content of peptide exposed cells.
[b] No discrimination between internalized and surface-bound peptide possible with diazotized 2-nitroaniline because of the lack of modifiable side-chain amino groups.

tity of **IX** measured in the cell lysate are taken into consideration. In the case of **IX** (and also of **X**) the lack of side-chain amino groups prevented differentiation between surface-bound and internalized peptide fractions by means of diazotized 2-nitroaniline. That **IX** has been actually internalized at least comparably to **I** is suggested by a threefold increased level of fluorescent metabolites in the cell lysate of **IX**-treated cells compared to that found after exposure to **I** (not shown). CLSM revealed a similar image for both **I**- and **IX**-treated cells also indicating comparable uptake.

Reducing the helix forming propensity by Ala-Gly replacement strongly impaired the degree of internalization (**VI**; Table 2). The same was found for the negatively charged **X**, which while possessing high intrinsic helix forming propensity, showed only low helicity in the presence of SDS-micelles and, therefore, very likely also at the negatively charged surface of mammalian cells (Table 2). Both results

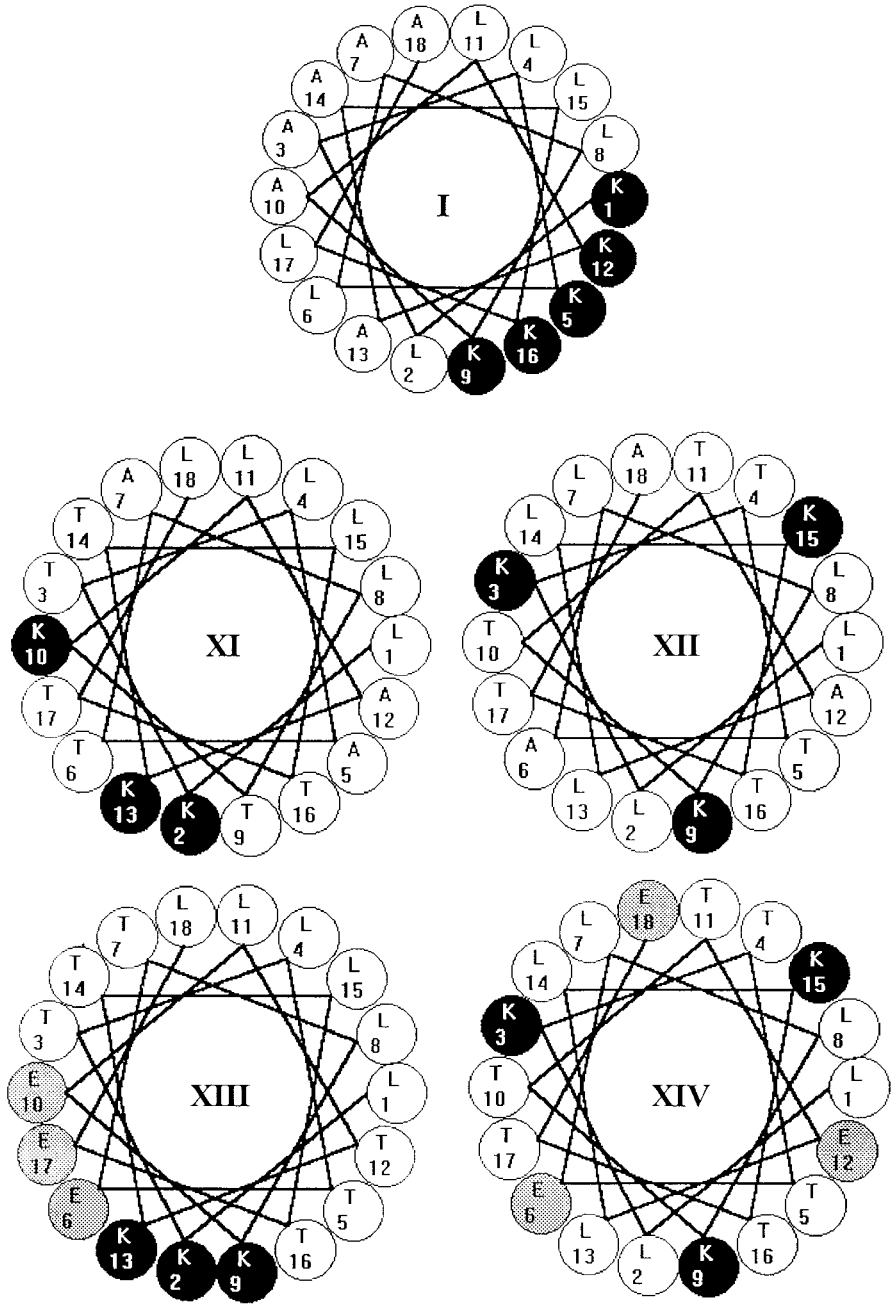

Figure 1 Helical wheel projections of **I** and the amphipathic/non-amphipathic peptide pairs (**XI**/**XII**, **XIII**/**IV**).

suggest helix formation of **I**-like peptides to be crucial for passage across the plasma membrane. This notion is in accord with our previous results implicating helical amphipathicity as an essential structural requirement [9,10].

In order to clarify further the role of amphipathicity and additionally that of positive net charge, regarded by others to be decisive for peptide internalization [8], we included in our investigations two pairs of helical amphipathic/non-amphipathic peptides (designed by E. Krause and S. Rothemund, unpublished) being unrelated to **I** and with a lower net charge (**XI**–**XIV**; Table 2; Figure 1). Clear cellular uptake of a comparable order as found with **I** was observed for the amphipathic peptides **XI** and **XIII**. For the non-amphipathic counterparts, **XII** and **XIV**, containing charged residues uniformly distributed around the helix (Figure 1), no internal-

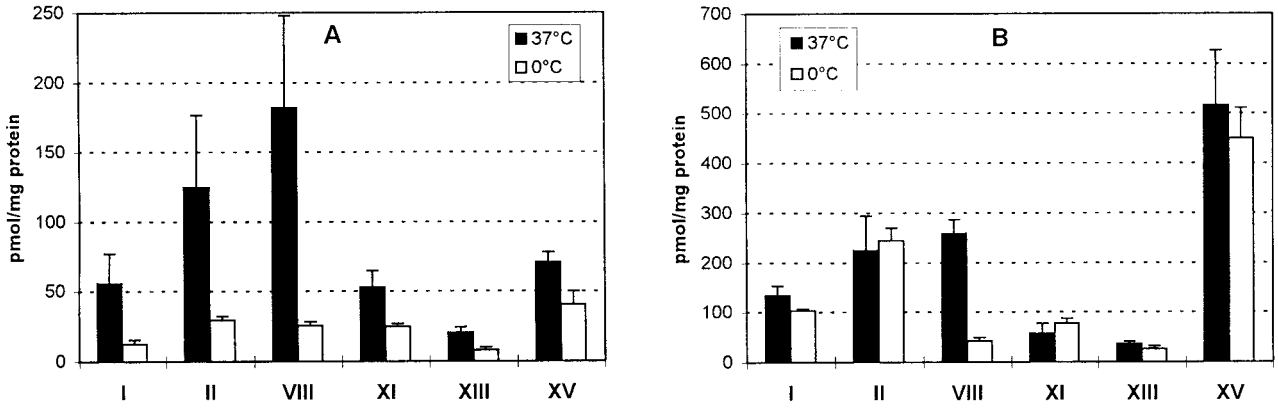

Figure 2 Cell-associated peptide, showing fractions remaining intact (A) and partially modified by diazotized 2-nitroaniline (B), respectively, after exposing AEC to 1.8 μM of various peptides (see Table 2) for 30 min at 37°C or 0°C, respectively. Before exposure to the peptide, the cells were incubated for 60 min at 37°C or 0°C in DPBSG. Each bar represents the mean of three samples ± S.D.

ization was detectable by the HPLC approach. These findings further confirm our previous conclusion that amphipathicity is crucial for cellular internalization and likewise suggest that net positive charge is not decisive in this context. The latter is in accord with the comparability of the cellular uptake of **I** and that of **I**-derived peptides containing reduced numbers of lysine residues cited above. The similar degree of internalization found for **XV**, a natural homoeobox-derived membrane-permeable peptide [3,4] possessing two positive side-chains more than **I** (Table 2) also supports this conclusion.

Cellular Uptake of Altered Concentration and Temperature

To ensure that the results obtained at 5 μM peptide concentration were not biased significantly by partial damage to the plasma membrane and to obtain information about the degree to which non-endocytic mechanisms participate in cellular uptake, the experiments were repeated at a peptide concentration of 2 μM at 37°C and at 0°C. The 2 μM peptide concentration has been proven to be non-toxic under all the conditions used. At 0°C endocytic transport is suppressed [18], so that comparison with the uptake at 37°C allows an estimate of non-endocytic contributions to be made. The results obtained are presented in Figure 2A and are in accord with the data in Table 2, indicating that bias of the results from the 5 μM concentration by cell injury is unlikely.

Additionally, in Figure 2B the peptide fraction which is partially accessible to the membrane-impermeable reagent, diazotized 2-nitroaniline, is shown. Other than for the intact (actually internalized) peptide fraction, the quantities of this membrane-associated fraction suggest a correlation with the number of positive charges as well as with the helix parameters hydrophobicity and hydrophilic face (Table 2; Figure 2B) and, therefore, peptide–membrane lipid interactions are implicated.

As shown in Figure 2 A clear peptide uptake into the cell was found in all cases at 0°C, indicating non-endocytic internalization. The ratio between the uptake at 37° and 0°C differed for the individual peptides (Figure 2A), indicating different contributions to the cellular uptake by the various mechanisms involved, according to the peptides' structure. Except for peptide **VIII** the membrane-associated peptide fractions (accessible to diazotized 2-nitroaniline) showed no significant difference at 37°C and at 0°C (Figure 2B), which again points to interactions of this fraction with membrane lipids. In contrast to the other peptides, the respective modified portion of **VIII** is clearly reduced at 0°C for reasons which are, as yet, unclear (Figure 2B).

DISCUSSION

With the goal of rationalizing the design of cell-permeable peptides, we investigated the influence of helix parameters, molecular size and charge on the internalization of α-helical peptides into endothelial cells. As the parent compound we used the α-helical amphipathic model peptide FLUOS-KLALKLALKA-LKAALKLA-NH_2 (**I**), recently shown by our group to

be extensively taken up by mammalian cells [9,10]. With the exceptions that a molecule size of lower than 16 amino acid residues or a negative net charge both abolished uptake, we found none of the investigated structural parameters to decisively affect the translocation activity of the peptides. Thus our results, in accord with previous studies, imply helical amphipathicity to be the only essential structural requirement for cellular uptake of this type of cell-permeable peptide. The pivotal role of helical amphipathicity for cellular uptake was confirmed by results obtained with two further pairs of amphipathic/non-amphipathic peptides entirely unrelated to **I**. Only the amphipathic counterparts showed an extensive internalization comparable to that of **I**. The observed large tolerance towards structural alterations promises multiple degrees of freedom for optimizing vector peptide structure for delivery of special cargo molecules into various cell types. Moreover the observed lack of correlation between uptake activity and membrane toxicity opens the prospect for improved efficiency/toxicity ratios.

The membrane toxicity of the peptides studied herein was negligible only at concentrations below 2 μM. For such applications as the inhibition of contacts between intracellular signal proteins by complementary short peptides, mostly requiring concentrations higher than 2 μM [19–22], such toxicity may be limiting. This principal shortcoming is attenuated in the case of the presently available peptides by the more than fivefold intracellular enrichment observed previously [10] and again in this study (an internalized peptide amount of 100 pmol/mg protein corresponds to a concentration of 8 μM within the cell, taking into account the ratio of 110 μg protein/10^6 cells and the cell volume of 1.4 pL). For induction of down regulation effects with antisense oligonucleotides, mostly requiring only nanomolar intracellular concentrations [23], on the other hand, the achievable non-toxic peptide concentrations should suffice in nearly all cases.

With respect to the as yet unclear mode of entry into the cell interior our results suggest a lack of correlation between the internalized peptide quantity and helix parameters or number of positive charges. Both types of structural parameter, however, play a pivotal role during interactions of membrane-active peptides with lipid membranes [24–27]. Hence, an interpretation of the mode of uptake by a transient membrane destabilization and concomitant internalization as proposed recently for Ant-P, a natural cell-permeable α-helical amphipathic peptide [4], is not reconcilable with our results. In comparison to the internalized peptide fraction the peptide fraction found to be partially accessible to the membrane-impermeable reagent 2-nitroaniline appears dependent on hydrophobicity, hydrophilic face and number of positive charges and not on temperature. Thus peptide–lipid interactions also play a noticeable role in our case but appear to have no effect upon internalization within the non-toxic concentration range.

As an alternative interpretation we previously proposed peptide–protein interactions to account for translocation across the plasma membrane [10]. In that study we found evidence for the involvement of multiple, energy-dependent and -independent mechanisms in the cellular uptake of **I** and we presumed this to be analogous to interactions of natural leader sequences with protein transport machines [28–30]. The uptake results of the current study particularly concerning the essential role of amphipathicity and the large range of structural tolerance, support the previous notions. Moreover, the observation in the present study of distinct ratios for temperature-sensitive and -insensitive contributions to the cellular uptake for different peptides indicates distinct structural preferences for each of the multiple mechanisms involved. Likewise this observation promises the prospect of creating vector peptides favouring selected mechanisms.

Taken together, the present study reveals extensive cellular uptake of various helical amphipathic model peptides with substantially varying structural parameters. The structural tolerance observed with respect to the translocation activity promises multiple degrees of freedom for designing optimized variants for different applications. Evidence is provided against peptide–lipid interactions as being mainly responsible for cellular uptake and for the involvement of multiple, probably protein-based mechanisms in the translocation of the peptides across plasma membranes.

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