Cell-penetrating Peptides Split into Two Groups Based on Modulation of Intracellular Calcium Concentration

Annely Lorents1, Praveen Kumar Kodavali1, Nikita Oskolkov1, Ülo Langel5, Mattias Hällbrink3, and Margus Pooga1,2

From the 1Institute of Molecular and Cell Biology, University of Tartu, EE51010 Tartu, Estonia, the 2Department of Neurochemistry, Arrenius Laboratories, Stockholm University SE10691 Stockholm, Sweden, and the 3Institute of Technology, University of Tartu, EE50411 Tartu, Estonia

Background: Uptake of various cell-penetrating peptides (CPPs) can be toxic to cells.

Results: Amphipathic CPPs disorder the plasma membrane inducing the influx of calcium ions that in turn can activate recovery mechanisms.

Conclusion: Influx of calcium ions and subsequent toxicity induced by the uptake of CPPs can be averted efficiently.

Significance: Membrane-active CPPs can be exploited as efficient transport vectors.

Cell-penetrating peptides (CPPs) promote the uptake of different cargo molecules, e.g. therapeutic compounds, making the harnessing of CPPs a promising strategy for drug design and delivery. However, the internalization mechanisms of CPPs are still under discussion, and it is not clear how cells compensate the disturbances induced by peptides in the plasma membrane. In this study, we demonstrate that the uptake of various CPPs enhances the intracellular Ca2+ levels in Jurkat and HeLa cells. The elevated Ca2+ concentration in turn triggers plasma membrane blebbing, lysosomal exocytosis, and membrane repair response. Our results indicate that CPPs split into two major classes: (i) amphipathic CPPs that modulate the plasma membrane integrity inducing influx of Ca2+ and activating downstream responses starting from low concentrations; (ii) non-amphipathic CPPs that do not evoke changes at relevant concentrations. Triggering of the membrane repair response may help cells to replace distorted plasma membrane regions and cells can recover from the influx of Ca2+ if its level is not drastically elevated.

The plasma membrane constitutes an essential barrier between the extra- and intracellular environments, and one of its many roles is to protect cells from harmful external influences. For example, mechanical stress or trauma, a common form of cell injury, must be neutralized rapidly for cells to survive. This process is known as the membrane repair response (MRR)3 or “wound healing” and it is initiated by the influx of Ca2+ through the disruption site. Elevated cytosolic Ca2+ levels trigger homotypic (vesicle-vesicle) and exocytotic (vesicle-plasma membrane) fusion events (1–4). Hence, the breach in the plasma membrane is resealed with enlarged vesicles derived from the endocytic compartment, e.g. lysosomes, by a “patching” mechanism (5, 6). Another critical step in the repair mechanism is the disassembly of the cortical filamentous actin, which is required for facilitating the fusion of the plasma membrane and lysosomes (7).

The barrier function of the plasma membrane, on the other hand, is the major obstacle in drug delivery and gene therapy as many otherwise promising bioactive compounds cannot traverse the lipid bilayer. To overcome this obstacle, a class of specific transport vectors called cell-penetrating peptides (CPPs) can be utilized. Nowadays, CPPs are either of natural, chimeric, or artificial origin and share a common ability to deliver otherwise impermeable molecules into cells. Even though their potential as efficient carrier molecules has been demonstrated repeatedly, both in vitro and in vivo (8–12), the mechanism by which they enter cells is still a subject of dispute. For instance, clathrin-mediated endocytosis (13), caveolin-dependent endocytosis (14), and macropinocytosis (15) are all found to play a role in the cell entry of CPPs. Other published data suggest that a rapid non-endocytic transduction mechanism also is involved (16, 17) and demonstrate that CPPs cause disturbances in the plasma membrane and can locally remodel the actin network (15). In addition, the uptake mechanism(s) depend on the experimental conditions, such as the particular CPP used, its concentration, type of cargo and cells, etc (16–18). Currently, a consensus has been reached that CPPs mainly exploit different endocytosis pathways; however, evidence for other functional pathways under certain conditions exists.

We have demonstrated previously that two well known CPPs, a model amphipathic peptide (MAP) and penetratin, induced plasma membrane repair, however, at markedly different concentrations (19). The membrane repair mechanism

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To whom correspondence should be addressed: Inst. of Molecular and Cell Biology, Riia 23, 51010 Tartu, Estonia. Tel.: 372-7-375-049; Fax: 372-7-420-286; E-mail: mpooga@ebc.ee.
1 The abbreviations used are: MRR, membrane repair response; CPP, cell-penetrating peptide; MAP, model amphipathic peptide; TP10, transportan 10; LAMP-2, lysosome-associated membrane protein 2; CLSM, confocal laser scanning microscopy; AM, acetoxyethyl ester; TP, transportan; pVEC, peptide derived from vascular endothelial-cadherin.
Influx of Calcium Ions Induced by Cell-penetrating Peptides

**TABLE 1**

| Names and sequences of cell-penetrating peptides used in this study |
|---------------------------------------------------------------|
| **Peptide** | **Sequence** |
| MAP | 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in 1% nonfat dry milk in PBS for 1 h, followed by incubation with a 1:400 dilution of Alexa Fluor 488-conjugated goat anti-mouse antibody (Invitrogen) in 1% nonfat dry milk in PBS for 30 min at room temperature in the dark. The coverslips were washed and mounted on glass slides with Fluoromount G (Electron Microscopy Sciences). Samples were analyzed with an Olympus FV1000 confocal laser scanning microscope (Olympus) and processed with Adobe Photoshop CS2.

Annexin V Affinity Assay

1 × 10⁶ Jurkat cells per ml were incubated with 1–50 μM peptides or with 250 ng/ml Fas ligand-binding antibody to induce apoptosis in serum-free medium for 30 min at 37 °C. Cells were harvested by centrifugation for 5 min at 200 × g, resuspended in the medium with serum, and were incubated for an additional 24 h. Treated cells were collected, resuspended in binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM CaCl₂, pH 7.4), and incubated with Alexa Fluor 488-conjugated annexin V (1:100 dilution, Invitrogen) for 15 min at room temperature in the dark. After washing with binding buffer, 0.5 μg/ml of DAPI was added to the buffer, and cells were incubated for 5 min. Data from 10,000 cells were recorded with a FACs, and analysis was performed with CellQuest software (Becton-Dickinson) and GraphPad Prism (version 5.0, GraphPad, Inc.). The data represent ± S.E. of at least three independent experiments.

β-Hexosaminidase Efflux Assay

HeLa cells grown in 24-well plates (1 × 10⁵ cells/well) were used for experiments performed in triplicate 1 day after seeding. Cells were washed twice with Hepes-Krebs-Ringer buffer (125 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄·7H₂O, 1 mM CaCl₂·2H₂O, 1.2 mM KH₂PO₄, 25 mM Hepes, 6 mM D-glucose, pH 7.4) and peptides at the indicated concentrations in the same buffer were added. After incubation for 30 min at 37 °C, 10 μl of cell-exposed buffer was transferred into 96-well plates and incubated with 1 mM 4-methylumbelliferyl N-acetyl-β-D-glucosaminide in 0.1 M sodium citrate buffer (pH 4.5) for 1 h at 37 °C. Subsequently, K₂CO₃ buffer (pH 10.5) was added, and β-hexosaminidase activity was determined fluorometrically at 365/445 nm (emission/excitation). Untreated cells were defined as no leakage, and total β-hexosaminidase release by lysing cells with 0.1% Triton X-100 was defined as 100% leakage.

RESULTS

**Amphipathic CPPs Induce Ca²⁺ Influx in Jurkat Cells at Low Concentrations**—To evaluate whether the CPPs induce membrane disturbances and a subsequent influx of Ca²⁺, Jurkat cells were preloaded with a Ca²⁺-sensitive dye Fluo-4-AM and treated with CPPs at different concentrations.

MAP, as the most membrane-active peptide, induced the Ca²⁺ influx into Jurkat cells in a concentration-dependent manner as detected by the increased fluorescence signal after 5 min (Fig. 1A). Incubation of Jurkat cells with 0.5 μM MAP led to a 10% increase in the Ca²⁺ level and 5 μM (the highest used concentration) to a 55% increase. When the incubation was prolonged to 30 min, the fluorescence signals were even higher: 0.5 μM MAP showed a moderate increase that yielded a 20% rise in Ca²⁺ signal, whereas 5 μM MAP elevated Ca²⁺ signal by 85% (Fig. 1A).

Transportan (TP) was less membrane-active, and treatment of Jurkat cells with 1 μM peptide did not induce changes in the intracellular Ca²⁺ concentration either at 5 or 30 min measurement (Fig. 1B). However, starting from 3 μM TP concentration the first effect was observed that caused a slight elevation after 5 min of incubation and 10 μM peptide led to a 40% increase. The TP-induced Ca²⁺ influx was continuous, the 30-min incubation with 3 μM peptide increased the fluorescence signal by 20% and 5 μM TP elevated the Ca²⁺ concentration up to 60% as compared with ionomycin where a plateau seemed to be reached (Fig. 1B). A shorter analog of transportan, TP10, induced a detectable Ca²⁺ influx at a higher concentration than TP; no detectable changes were observed at 1–5 μM concentrations 5 min after addition of the peptide to the medium (Fig. 1C).
Arginine-rich CPPs Do Not Induce Significant Ca\textsuperscript{2+} Influx in Jurkat Cells—In contrast, penetratin and pVEC triggered the Ca\textsuperscript{2+} influx into cells at markedly higher concentrations. 3–10 μM penetratin and pVEC did not induce substantial changes in Ca\textsuperscript{2+} levels; however, their effect was detectable starting from 20 μM concentrations after 5 min of treatment (Fig. 1, D and E). 20 μM penetratin increased the Ca\textsuperscript{2+} level by 20% and pVEC induced a 35% increase at this concentration. Prolonged incubation, surprisingly, did not increase the Ca\textsuperscript{2+} influx and the detected fluorescence signals remained at the comparable levels for 30 min after the addition of these CPPs (Fig. 1, D and E).

The cationic-only peptides, Tat peptide and nona-arginine, did not influence the intracellular Ca\textsuperscript{2+} level even at very high concentrations. The cellular Fluo-4 fluorescence remained at the basal level throughout the measurement in the used concentration range from 5 to 50 μM (Fig. 1, F and G).

Amphipathic CPPs Induce Ca\textsuperscript{2+} Influx and Blebbing of Plasma Membrane in HeLa Cells—As intracellular Ca\textsuperscript{2+} measurements performed using a fluorometer detected the signal from whole cell suspension, it was not possible to distinguish whether the observed changes were a result of substantial Ca\textsuperscript{2+} influx in a subpopulation only or a minor uniform increase in the whole cell population. To analyze the dynamics of Ca\textsuperscript{2+} concentration in single cells as well as on a subcellular level, we continued with experiments in HeLa cells applying confocal laser scanning microscopy (CLSM).

Treatment of cells with ionophore ionomycin led to a significant influx of Ca\textsuperscript{2+} into HeLa cells, and a high diffuse fluorescence signal in the cytoplasm and nucleus reached a maximum in 5 min (Fig. 2B). In addition, some intracellular vesicles were more intensely fluorescing as detected by CLSM, which was more pronounced after a 30-min treatment with ionomycin (data not shown).

MAP-induced increase in the intracellular Ca\textsuperscript{2+} concentration was detectable by CLSM within 5 min in ~20% of cells starting from 1 μM concentration of the peptide (Fig. 2C). An increase of MAP concentration to 3 μM led to a Ca\textsuperscript{2+} influx in a larger population (~30%) (Fig. 2D), and after treatment with 5 μM MAP most of the HeLa cells showed an increased diffuse staining of the cytoplasm and nucleus (Fig. 2E). A vesicular rather than a diffuse staining was present in cells after 30 min of treatment with 5 μM MAP (Fig. 2F), which might be caused by the efflux of the Ca\textsuperscript{2+} indicator from the cytoplasm and nucleus but not from the vesicles. In addition, the incubation of HeLa cells with MAP induced blebbing of the plasma membrane starting from 3 μM concentration of the peptide and a 30-min incubation (data not shown). At a higher concentration of MAP, blebbing of the cells was induced faster and was detectable already after an 8-min incubation with 5 μM MAP (arrows in differential image contrast (DIC) image of Fig. 2F).

Transportan did not induce Ca\textsuperscript{2+} influx or plasma membrane blebbing at 1–3 μM concentration (data not shown). However, the influx of Ca\textsuperscript{2+} was detected starting from 5 μM concentration, which led to an elevated fluorescence signal in the cytoplasm and nucleus in ~30% of HeLa cells after 5 min of incubation (Fig. 2G) and in the vesicles after 30 min of treatment, which was also accompanied by occasional blebbing of the plasma membrane (arrows in Fig. 2H). An increase in the intracellular Ca\textsuperscript{2+} concentration in most of the cells in the population was detected after 5 min of treatment of cells with 10 μM TP, where plasma membrane blebbing also became more evident (Fig. 2I). A 30-min incubation with 10 μM TP led to changes in morphology of HeLa cells (Fig. 2J).

A shorter analog of transportan, TP10, caused detectable membrane perturbations at higher concentrations than transportan. TP10 did not trigger the Ca\textsuperscript{2+} influx at 5 μM concentration within 5 min, and a negligible increase of diffuse signal was detected in ~20–30% of HeLa cells after 30 min (data not shown). Starting from 10 μM concentration, TP10 induced an elevation of the intracellular Ca\textsuperscript{2+} in ~90% of cells within 5 min (Fig. 2K). The initial diffuse Ca\textsuperscript{2+} signal in cells turned more vesicular after 30 min and was accompanied by some blebbing of the plasma membrane (arrows in Fig. 2L).

Secondary Amphipathic CPPs Induce Ca\textsuperscript{2+} Influx at High Concentration but Do Not Evoke Plasma Membrane Blebbing—In analogy with the results obtained in Jurkat cells, incubation of HeLa cells with penetratin and pVEC caused similar effects. An increase in the Ca\textsuperscript{2+} levels and thus a diffuse staining in ~15% of cells was detected starting from 20 μM concentrations within 5 min (supplemental Fig. S1). A longer incubation (30 min) with these peptides did not have an additional effect on the Ca\textsuperscript{2+} levels in cells, and no blebbing of the plasma membrane was detected (data not shown).

Non-amphipathic CPPs Do Not Induce Ca\textsuperscript{2+} Influx or Blebbing of Plasma Membrane in HeLa Cells—CLSM results corroborated that Tat peptide and nona-arginine did not affect the intracellular Ca\textsuperscript{2+} concentration in HeLa cells. Even 30 min after addition of 50 μM peptides, no increase in fluorescence signal was detected (supplemental Fig. S1). As expected, Tat peptide and nona-arginine did not induce detectable blebbing of the plasma membrane.

Ca\textsuperscript{2+} Influx Induced by CPPs Triggers Plasma Membrane Repair Response—To further characterize the effect of CPPs on the integrity of the plasma membrane, we analyzed their potency to trigger the MRR. We have previously demonstrated the ability of MAP and penetratin to induce the MRR (19). MAP peptide triggered the translocation of LAMP-2 to the plasma membrane at low concentrations (even below 1 μM), whereas with penetratin a significant effect was detected starting from 20 μM concentration.

In our study, the plasma MRR was in very good correlation with the cytosolic Ca\textsuperscript{2+} concentration induced by CPPs. The fluorescence microscopy experiments demonstrated that 1 μM TP did not induce a Ca\textsuperscript{2+} influx and did not trigger the MRR (Fig. 3G). On the other hand, raising the concentration to 3 μM or 5 μM induced the appearance of LAMP-2 on the plasma membrane in ~30% or 80% of cells in the observed population, respectively (Fig. 3, H and I). The pVEC peptide, in contrast, stimulated the translocation of LAMP-2 to the cell surface at markedly higher concentrations. At concentrations up to 10 μM, no accumulation of LAMP-2 on the plasma membrane could be detected (Fig. 3E); however, starting from 20 μM concentration, pVEC triggered the MRR.
in \( \sim 20 - 30\% \) of cells in analogy with penetratin (Fig. 3F). In accordance with the Ca\(^{2+}\) influx assays, incubation of HeLa cells with Tat peptide or nona-arginine did not trigger an exposure of the lysosomal lumenal proteins to the plasma membrane even at very high concentrations (up to 50 \( \mu \)M) (Fig. 3, C and D). Even though the membrane-active CPPs potently triggered the MRR, only a small fraction of LAMP-2 antigen was targeted to the plasma membrane and became accessible to the antibodies, as the fixation of cells with methanol led to a considerable increase in the LAMP-2 signal in HeLa cells (Fig. 3A, note the 2-fold difference of the exposure time).

**Coupling of Cargo Protein to CPPs Decreases Membrane Activity of Peptides**—It is known that the presence of a cargo molecule attached to the CPP may alter the uptake mode of the CPP (22) and its cytotoxic effects. Therefore, we assessed the
induction of the MRR by amphipathic CPPs after conjugation with a ~67-kDa cargo protein avidin at 3:1 molar ratio.

MAP-avidin and TP-avidin complexes were both less potent in the induction of the MRR compared with the respective peptides alone. MAP-avidin complexes did not induce the translocation of lysosomes to the plasma membrane at 0.5 μM peptide concentration. Triggering of the MRR was observed in a few cells at 1 μM peptide concentration (data not shown); however, the exposure of LAMP-2 on the plasma membrane was detectable in >50% of cells after incubation with 3 μM MAP-avidin (Fig. 4) and in ~80% of cells after incubation with 5 μM MAP-avidin (Fig. 4). 10 μM MAP-avidin triggered the accumulation of LAMP-2 on the plasma membrane of cells in the whole population and in ~60% of cells, this was also accompanied by the influx of propidium iodide (Fig. 4).

TP-avidin complexes triggered the translocation of LAMP-2 to the surface of cells starting from 3 μM peptide concentration but only in ~15% of cells. 5 μM TP-avidin and 10 μM TP-avidin induced the appearance of the lysosomal protein on the plasma membrane in ~30 and ~70% of the cell population, respectively (data not shown). Avidin itself did not activate the MRR, and therefore, LAMP-2 did not translocate to the plasma membrane (Fig. 4).

Amphipathic CPPs Induce Significant Release of Lysosomal β-Hexosaminidase from HeLa Cells, whereas Non-amphipathic CPPs Do Not — To corroborate the fusion of lysosomes with the plasma membrane and the release of their content, we measured the efflux of lysosomal enzyme β-hexosaminidase into the extracellular medium. Among the studied CPPs, MAP was the most potent in inducing the release of the lysosomal content as shown previously. MAP increased the release of β-hexosaminidase from HeLa cells ~2- to 5-fold at 1 μM or 20 μM concentration, respectively (Fig. 5). TP induced a 2-fold increase starting from 10 μM concentration and reached an ~4-fold increase when 20 μM concentration was used, whereas treatment of cells with TP10 led to an ~3-fold increase of β-hexosaminidase activity at 20 μM concentration. Penetratin and pVEC, on the other hand, caused significant exocytosis of lysosomes only at the highest used concentration (20 μM), promoting efflux of the enzyme 2-fold and 0.5-fold, respectively. In case of Tat pep-

FIGURE 3. Uptake of amphipathic CPPs triggers cellular membrane repair response. HeLa cells were incubated with medium alone (B), 50 μM Tat peptide (C), 50 μM nona-arginine (D), 10 μM pVEC (E), 20 μM pVEC (F), 3 μM penetratin (G), 5 μM penetratin (H), 10 μM penetratin (I), 1 μM TP (J), 3 μM TP (K), or 5 μM TP (L) at 37 °C for 30 min, and cells permeabilized with methanol (A) were used as control. LAMP-2 was visualized in fixed cells with monoclonal LAMP-2 antibody and with Alexa Fluor 488-conjugated anti-mouse antibody. The exposure time for recording A is 2-fold shorter than for other panels. The image has been color-inverted for better visualization of LAMP-2 staining on the plasma membrane (arrows) compared with lysosomal LAMP-2 staining (arrowheads). Scale bar, 20 μm.

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**Figure 6. Viability of Jurkat cells after treatment with CPPs.** Cells were incubated with Fas ligand binding antibody (Fas-Ab) to induce apoptosis or with CPPs at indicated concentrations for 30 min at 37 °C. Viability of cells was recorded by flow cytometry using double staining with annexin V and DAPI (Fig. 6). As expected, MAP was the most toxic peptide inducing apoptosis or necrosis of cells in a concentration-dependent manner. After treatment with 1 μM or 5 μM MAP, the viability of cells decreased to ~82 and ~64%, respectively (Fig. 6 and supplemental Table S1). Transportans were significantly less toxic and after treatment with 10 μM TP or TP10, >80% of Jurkat cells were still viable. 20 μM penetratin altered the viability of cells to some extent inducing apoptotic or necrotic events in ~18% of cells (supplemental Table S1). However, the viability of cells stayed at a comparable level with untreated cells after incubation with 20 μM pVEC, 50 μM Tat peptide, and 50 μM nona-arginine (Fig. 6).

To assess the barrier function of the plasma membrane of HeLa cells, we analyzed the uptake of DAPI to the cells after the challenge with the highest concentrations of TP. After a 30-min incubation with 5 μM TP, only a few cells were permeable to DAPI (supplemental Fig. S2A) and with 10 μM TP, ~20% of cells became permeable to DAPI (supplemental Fig. S2B). Furthermore, we visualized the exposure of LAMP-2 on the plasma membrane of HeLa cells 24 h after treatment with CPPs. The results showed that after incubation with 3 μM MAP or 10 μM TP, LAMP-2 was still detectable in <20% of HeLa cells in the population (data not shown).

**DISCUSSION**

Cell-penetrating peptides mediate the uptake of various impermeable cargo molecules making them powerful tools in biotechnology and hopefully attractive for biomedical applications. Currently, the application of CPPs is growing explosively despite the controversies regarding the internalization mechanism(s). The majority of reports have suggested that CPPs use different endocytic pathways (20, 21), but several studies also have demonstrated a direct penetration through the plasma membrane (16, 17, 23). We have shown recently by transmission electron microscopy that binding of CPPs to the cell surface results in a less organized lipid bilayer, inferring the impairment of the plasma membrane integrity by some CPPs at higher concentrations (24). Moreover, we observed a rather similar effect with a peptide derived from granzyme B (19), which shares analogous characteristics with CPPs in its uptake mode (25). In parallel with the impairment of regularity of the plasma membrane packing, the granzyme B peptide treatment induced Ca^{2+} influx into cells (19).

Ca^{2+} is a key intracellular messenger in a multitude of cellular processes. On the other hand, Ca^{2+} is a potential toxin and its concentration in the cytoplasm of cells is controlled precisely. The low cytosolic Ca^{2+} concentration is maintained by the bidirectional ion transport across the plasma membrane of cells. However, mammalian cells are often exposed to a mechanically challenging environment, and in case of injury, the barrier function of the plasma membrane is breached, and the cell interior is exposed to the high external Ca^{2+} concentration. To protect cells from the uncontrolled influx of external molecules and ions as well as from the efflux of essential cytosolic constituents, a rapid membrane resealing response is activated (1). The MRR involves vesicle-vesicle fusion and exocytosis of lysosomes, which are activated by the influx of Ca^{2+} through the damaged region of the plasma membrane (4, 6).

We demonstrated earlier that in analogy with granzyme B-derived peptides, some widely used CPPs induce the influx of Ca^{2+} into the cytoplasm of mammalian cells and trigger the MRR (19). In the current study, we examined whether all the most widely used CPPs cause membrane disturbances to the extent of allowing a detectable steady influx of Ca^{2+} into cells and induce the downstream responses. However, it should be noted that we have introduced considerable modifications to our intracellular Ca^{2+} measurement methods that have led to a more precise estimation of the Ca^{2+} levels in the cells. Namely, we have employed Ca^{2+} indicator Fluo-4 instead of Fura-2 because the latter exhibits pronounced compartmentalization and binding to proteins. This affects Ca^{2+} sensitivity of Fura-2 because the emission from the dye bound to the proteins or inside compartments does not reflect the cytosolic Ca^{2+} con-
Influx of Calcium Ions Induced by Cell-penetrating Peptides

In accordance with our earlier results, the MAP peptide was the most potent inducer of the Ca\(^{2+}\) influx acting even at submicromolar concentrations in Jurkat (Fig. 1A) and HeLa cells (Fig. 2, C–F). Cellular translocation of TP and TP10 elevated the intracellular Ca\(^{2+}\) concentration starting from low micromolar concentrations (Fig. 1, B and C), indicating that the primary amphipathic CPPs (29) induce a continuous Ca\(^{2+}\) influx even at relatively low concentrations. The secondary amphipathic CPPs (29), penetratin, and pVEC, are less potent in the induction of the influx of Ca\(^{2+}\) (Fig. 1, D and E, and supplemental Fig. S1). The non-amphipathic CPPs, Tat peptide and nona-arginine are unstructured in solution and also less prone to stably associate with membranes. As such, they do not induce the Ca\(^{2+}\) influx into cells even at very high concentrations (Fig. 1, F and G, and supplemental Fig. S1).

Tat peptide was recently shown to form pores and induce the transition of lipid bilayers from the lamellar organization to the cubic and inverted hexagonal phase (30), which should enable influx of Ca\(^{2+}\) ions. However, the stable association of Tat peptide and nona-arginine only took place in the case of artificial vesicles that contain a high proportion of lipids with anionic headgroups. As the latter are not present in the outer leaflet of the plasma membrane, Tat peptide and nona-arginine most probably do not stably associate with the lipids of the plasma membrane to interfere with its packing and, therefore, do not induce the influx of Ca\(^{2+}\) into living cells.

Based on the interaction with the plasma membrane, the induction of the Ca\(^{2+}\) influx, and downstream responses, three subgroups of CPPs can be distinguished. This division is in very good concordance with the classification of CPPs by Ziegler et al. (29) to primary and secondary amphiphatic and non-amphiphatic CPPs. The first ones stably interact with membranes and trigger Ca\(^{2+}\) influx into cells, whereas the latter do not modulate the intracellular Ca\(^{2+}\) levels. However, secondary amphipathic CPPs elevate the intracellular Ca\(^{2+}\) at significantly higher peptide concentrations as compared with primary amphiphatic ones and, therefore, we categorize CPPs into two major groups.

The influx of Ca\(^{2+}\), which accompanies the cellular translocation of CPPs, also triggers different activities in cells, including the MRR; hence, the internal vesicles are transported to the plasma membrane to reseal the damaged region(s) (Fig. 3). This process, for example, can be detected by the exposure of the lysosomal protein LAMP-2 on the cell surface or by the release of the lysosomal content, e.g. \(\beta\)-hexosaminidase, into the extracellular medium. Our results demonstrate that the targeting of lysosomes to the cell periphery and the associated fusion events, as well as the release of \(\beta\)-hexosaminidase, were triggered by the primary amphipathic CPPs (MAP, TP, and TP10) at markedly lower peptide concentrations compared with pVEC and penetratin. However, coupling of a cargo protein, e.g. complexation of biotinylated MAP to avidin, drastically reduced the membrane disturbing and Ca\(^{2+}\) influx inducing activity of the peptide (Figs. 3 and 4). An analogous effect where cargo coupling reduced the toxicity of CPP has been observed with large unilamellar vesicles (31). In concordance with the data from the intracellular Ca\(^{2+}\) measurements, Tat peptide and nona-arginine did not induce the fusion of lysosomes with the plasma membrane (Fig. 3, C and D) and did not release of \(\beta\)-hexosaminidase from the cells (Fig. 5).

Blebbing of the plasma membrane is quite a common cellular process accompanying cytokinesis (32), migration (33), virus uptake (34), and apoptosis (35). In addition, cytoplasmic blebbing of the plasma membrane has been observed during physical and chemical stress and usually is preceded by an elevation of intracellular Ca\(^{2+}\) that has to rise \(>\sim 300\) nM concentration (36). The blebbing of HeLa cells induced by amphipathic CPPs was dependent on both the concentration of used CPP and time (Fig. 2). These results imply that a certain intracellular Ca\(^{2+}\) concentration has to be reached and retained before the depolymerization of the actin cortex takes place. Only then can the local membrane-cytoskeleton interactions be interrupted and, as a result, the blebbing of the plasma membrane occur. In case of pVEC and penetratin, the formation of big stable blebs was not detected during a 30-min incubation even at the highest used concentration (20 \(\mu\)M), suggesting that although the intracellular Ca\(^{2+}\) concentration was increased, its level did not reach the threshold necessary for triggering the blebbing. Tat peptide and nona-arginine did not modulate the intracellular Ca\(^{2+}\) concentrations in Jurkat and HeLa cells in our experiments and, as expected, blebbing was not induced in these cells. Interestingly, recent findings suggest that blebbing may be a defense mechanism against plasma membrane injury; namely, the membrane blebs act as “traps” that block the detrimental influx of extracellular ions and the efflux of cytoplasmic constituents at the bleb “neck” until the damaged plasma membrane regions are repaired (37).

Ca\(^{2+}\) influx induces a random aggregation of endosomes and lysosomal exocytosis (4, 6), and the extensive fusion of enlarged vesicles with the plasma membrane markedly increases the cell surface area. This in turn might activate a compensatory response: endocytosis. Therefore, CPPs that increase the cytosolic Ca\(^{2+}\) concentration may promote their uptake by endocytosis. Consistent with our speculations, compensatory endocytosis has been detected in several cell types in parallel with exocytosis (38, 39). In addition, pore-forming proteins such as the cytolytic protein perforin and the bacterial toxin streptolysin, trigger lysosomal exocytosis in response to the influx of Ca\(^{2+}\) through pores, and in parallel, induce rapid endocytosis (40, 41). Other published data suggest that lysosomal exocytosis that is activated by the influx of Ca\(^{2+}\) through the plasma membrane wounds is necessary for the release of the acid sphingomyelinase from the lysosomes into the extracellular environment to activate endocytosis that contributes to cell recovery (42). According to the hypothesis, acid sphingomyelinase mediates changes in the lipid bilayer that favors inward membrane bending and the consequent formation of endosomes (43, 44).

Significant elevation of the cytoplasmic Ca\(^{2+}\) might become toxic to the cells; however, activation of different damage con-

\[\text{Ca}^{2+}\]
Control mechanisms (e.g. blebbing, plasma membrane repair, pumping of Ca\textsuperscript{2+} out of the cell or into Ca\textsuperscript{2+} stores) might help the cell to restore the intactness of the membrane and restrain the uncontrolled influx of Ca\textsuperscript{2+} ions. Indeed, our results demonstrate that Jurkat and HeLa cells are able to overcome the overload of cytosolic Ca\textsuperscript{2+} to a considerable extent. Studies with the bacterial toxin streptolysin O showed that the plasma membrane repair response functions effectively if the intracellular Ca\textsuperscript{2+} concentration stays between 5–10 \(\mu M\) (45).

In conclusion, during the uptake of membrane-active CPPs, the integrity of the plasma membrane is impaired and the Ca\textsuperscript{2+} concentration in the cytoplasm increases. This, in turn, triggers the translocation and the fusion of lysosomes with the cell surface. This process replaces the damaged regions, allowing cells to overcome the stress caused by CPPs. The extensive lysosomal exocytosis might in turn promote a compensatory response such as the facilitated endocytotic uptake of CPPs or their constructs with cargo. However, it has to be considered that cells survive the disturbances only if the Ca\textsuperscript{2+} concentration in the cytosol does not exceed the toxicity threshold. The non-amphipathic CPPs, on the contrary, do not modulate the Ca\textsuperscript{2+} concentration in cells; therefore, one might speculate that they use different mechanisms for cell-penetration and delivery of cargo molecules as compared with, for example, penetratin.

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