Nanocarrier-Cell Interaction
Rehman, Zia ur

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chapter 4

Protein kinase A inhibition modulates the intracellular routing of gene delivery vehicles in HeLa cells, leading to productive transfection

Zia ur Rehman, Dick Hoekstra and Inge S Zuhorn*

University Medical Center Groningen, University of Groningen, Department of Cell Biology, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

Journal of Controlled Release (2011) 30; 156(1):76-84.
Abstract
Cellular entry of nanoparticles for drug- and gene delivery relies on various endocytic pathways, including clathrin- and caveolae-mediated endocytosis. To improve delivery, i.e., the therapeutic and/or cell biological impact, current efforts are aimed at avoiding processing of the carriers along the degradative clathrin-mediated pathway towards lysosomes, and promoting that along the caveolae-mediated pathway. Here, we demonstrate the effective internalization of branched polyethyleneimine polymers (BPEI), complexed with nucleic acids, by HeLa cells along both pathways. However, transfection efficiency or nuclear ODN delivery primarily occurs via the caveolae-mediated pathway, along which delivery into lysosomes is avoided. Interestingly, inhibition of intracellular protein kinase A (PKA) activity modulates the intracellular trafficking of both poly- and lipoplexes along the clathrin-mediated pathway by impeding trafficking into the late endosomal/lysosomal compartments, thus avoiding degradation. In case of BPEI polyplexes this promotes their transfection efficiency by 2–3 folds. Evidence excludes early endosomes as a major site for BPEI-mediated release/delivery. Rather, we identify a novel compartment, tentatively characterized as a transferrin/rab9/LAMP1 compartment, to which cargo within the clathrin-mediated pathway of endocytosis is rerouted upon inhibition of PKA, and which may act as an alternative and effective site of cargo release in gene delivery. Our findings offer new opportunities for improving gene delivery by non-viral based nanoparticles.

Introduction
Cationic lipids and polymers are promising tools for gene delivery into mammalian cells. When mixed with (negatively charged) DNA, so-called lipoplexes and polyplexes spontaneously assemble, resulting in effective packaging and shielding of the nucleic acid. Cellular entry of these nanoparticles is accomplished by endocytic mechanisms. Following endocytosis the genetic cargo is released into the cell's cytoplasm which is required for subsequent nuclear import. It is generally thought that the cationic lipids within lipoplexes mediate cargo release from an endosomal compartment by local destabilization of the endosomal membrane, whereas polyplexes have been claimed to induce rupture of acidifying endosomes via the generation of an osmotic shock (proton sponge theory). The endocytic pathways that are involved in the internalization of gene delivery vehicles by cells are diverse, and include but are not limited to clathrin-mediated endocytosis (CME), macropinocytosis, and caveolae-mediated endocytosis. Importantly, none of these pathways is mutually exclusive. Given the relatively low poly- or lipoplex-mediated transfection efficiencies compared to those obtained with viral carriers, the question can be raised whether efficiencies by the former could be improved by directing their uptake into a specific endocytic pathway. Thus, entry via caveolae rather than the clathrin-mediated pathway might favor particle processing along a non-acidic non-degradative pathway, avoiding lysosomal digestion, thereby optimizing opportunities for endosomal escape of the genetic cargo and, presumably, transfection efficiency. However, if polyplex-mediated delivery requires acidic conditions for release, the caveolae-mediated pathway should instead be avoided and the clathrin pathway preferred, whereas the opposite may hold for
PKA inhibition modulates the intracellular trafficking of nanocarriers

lipoplex-mediated transfection. Yet, it should be taken into account that crosstalk between different endocytic pathways may occur. In order to target nanoparticles into specific endocytic routes, specific ligands, recognized by distinct cellular receptors may be attached, such as transferrin to target the transferrin receptor, which is internalized via clathrin-mediated endocytosis, or folate to target the GPI-linked folate receptor, which enters the cell via caveolae. However, it has been noted that the conjugation of ligands to cargoes/nanoparticles may alter their cellular processing. For instance, transferrin-liposome conjugates were shown not to travel via the transferrin receptor, while cell penetrating peptides were reported to lose their ability to permeate the plasma membrane when coupled to macromolecules. Nevertheless, in previous work we demonstrated that non-targeted nanoparticles may be processed along distinct cellular entry pathways, depending on their size. Moreover, the involvement of specific receptors, i.e., β1-integrin receptors, in mediating effective transfection by non-targeted lipoplexes in polarized MDCK cells was shown. Since cellular internalization pathways for different gene delivery vehicles vary, it is very likely that differences in parameters such as chemical structure, particle size, and morphology co-determine the interaction of such nanoparticles with (receptors at) the cell surface, and hence their subsequent processing and transfection efficiency.

Interestingly, it is becoming increasingly apparent that early nanoparticle-cell surface interaction may trigger cellular signaling cascades, modulating a variety of kinase activities, which among others have been implicated in viral entry. Evidently, such parameters might also play a key role in nanoparticle trafficking and should therefore be taken into account when devising and applying nanoparticles for entry along specific pathways, in particular in efforts aimed at improving delivery efficiency. Here, we have investigated the influence of a distinct set of kinases on the cellular internalization of lipoplexes and their subsequent transfection efficiency. Our data reveal a regulatory role of protein kinase A (PKA) activity in the trafficking of lipoplexes. Our findings reveal that PKA inhibition strongly promotes transfection efficiency with lipoplexes that relatively poorly penetrate endosomes, such as branched PEI, which appears to be related to a kinase-dependent modulation of intracellular trafficking, i.e., away from lysosomes.

Materials and methods

Reagents, antibodies, and plasmids

Lipofectamine 2000 was purchased from Invitrogen (Invitrogen, CA), and linear polyethylenimine (LPEI) (average MW = 22 kDa) from Polyplus-transfection (Illkirch, France). Branched polyethylenimine (BPEI) (average MW = 25 kDa) and FITC-labeled poly-L-lysine (FITC-PLL) were purchased from Sigma (Zwijndrecht, the Netherlands). Alexa Fluor 555-labeled cholera toxin subunit b (CTxB), Alexa Fluor 568-labeled transferrin, and LysoTracker Red DND-99 were obtained from Invitrogen (Invitrogen, CA). FITC-labeled dextran (FITC-dextran) was purchased from Sigma.
Primary antibodies were obtained from the following sources: rabbit anti-caveolin and mouse anti-clathrin heavy chain were from Becton Dickinson transduction laboratories (Breda, The Netherlands), rabbit anti-EGFR was from Santa Cruz Biotechnology (Santa Cruz, CA), mouse anti-LAMP1 H4A3 was obtained from the Developmental Hybridoma Bank, University of Iowa, mouse anti-beta-tubulin was from Sigma (Zwijndrecht, The Netherlands), and rabbit anti-phospho PKA substrate antibody from Cell Signaling Technology (Beverly, MA, USA). Fluorescently-labeled secondary antibodies were purchased from Jackson (cyanine-labeled, Jackson ImmunoResearch, UK) and Invitrogen (Alexa Fluor-labeled, Invitrogen, CA). Infrared dye-labeled goat anti-rabbit 680 nm secondary antibody was from Li-Cor Biosciences (USA). All other reagents were from Sigma (Zwijndrecht, The Netherlands). Plasmid DNAs were obtained from the following sources: pEGFP-N1 was from Clontech (USA) and pRab9-dsRed was purchased from Addgene (Cambridge, MA USA). Plasmid DNAs were isolated from E-coli using Sigma Aldrich GenEluteTM HP Plasmid Mini/Midiprep kits (Sigma-Aldrich), following the manufacturer's protocol.

**Cells**

HeLa cells were cultured in 25 cm² Costar flasks in Dulbecco's Modified Eagle Medium nutrient mixture F-12 (DMEM/F-12, Gibco, the Netherlands), containing 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine (Gibco, the Netherlands), 100 units/ml penicillin (Invitrogen) and 100 μg/ml streptomycin (Invitrogen) at 37 °C and 5% CO₂. Cell were passaged every third day.

**Endocytic pathway inhibitors and kinase inhibitors/activators**

Cells were treated with endocytic pathway inhibitors for 30 min before complexes (lipoplexes and polyplexes) were added unless stated otherwise. 6 μg/ml chlorpromazine was used to inhibit clathrin mediated endocytosis, 5 μg/ml filipin III or 25 μg/ml nystatin to inhibit caveolar endocytosis, and 40 μM DMA (5-(N,N-dimethyl) amiloride hydrochloride) to inhibit macropinocytosis. Effective concentrations of the inhibitors were determined by measuring their effect on the uptake of markers for specific endocytic pathways by HeLa cells, i.e. transferrin (5 min) for CME, CTxB (30 min) for the caveolar pathway, and dextran (4 kDa; 4 h) for fluid phase endocytosis. The absence of cytotoxicity of the effective concentrations of the endocytic pathway inhibitors towards HeLa cells was verified by an MTT assay.

To inhibit kinase activities the following inhibitors and concentrations were used: 20 μM H89 (Calbiochem) for PKA (cAMP-dependent protein kinase A), 50 μM PD98059 (LC Labs, USA) for p42/44 MAPK, 0.5 μM U0126 (Calbiochem) for mitogen activated protein kinase kinase (MAPK), 10 μM SB203580 (LC labs, USA) for p38 mitogen activated protein (MAP) kinase, 1 μM KN62 (LC labs, USA) for Ca²⁺/calmodulin dependent protein kinase II (CaMKII), 25 μM Y27632 (Calbiochem) for Rho associated kinase (ROCK-I/II), 20 μM LY294002 (VWR international) for phosphatidylinositol 3-kinases (PI-3 kinase), 10 μM roscovitin (LC labs, USA) for cyclin-dependent kinases (CDK kinases), 20 μM PP2
PKA inhibition modulates the intracellular trafficking of nanocarriers

(www.proteinkinase.de) for src kinases, 30 μM genistein (LC labs, USA) for tyrosine kinases, 34 μM tyrphostin (AG490) (Calbiochem) for PTK Janus kinase 2 inhibition. To activate PKA, 100 μM dibuttryryl-cAMP (dbcAMP) (Biolog, Germany), 30 μM forskolin (Sigma), and 1 mM IBMX (3-isobutyl-1-methylxanthine) phosphodiesterase (Sigma) were used unless stated otherwise.

Preparation of lipoplexes and polyplexes and cellular transfection
HeLa cells were plated in 12-wells plates at 1.5 × 10⁵ cells per well. After 24 h lipoplexes composed of Lipofectamine 2000 (Invitrogen) and pEGFP-N1 (Clontech) or fluorescently-labeled oligonucleotides (ODNs) (Biognostik GmbH, Germany), and LPEI- and BPEI-polyplexes at an N/P ratio of 5 and 8, respectively, were prepared according to the manufacturers’ protocols. Cells were washed and 0.5 ml of serum-free medium was added per well. Lipoplexes and polyplexes containing 1 μg of pDNA or 0.1 nmol of ODNs were added per well and incubated for 2 h at 37 °C. Then the transfection medium was replaced for complete cell culture medium, which was refreshed after 24 h, and transfection efficiency was measured after 48 h using FACS-analysis (Elite, Coulter, 10,000 events, λex. 488 nm/λem. 530 nm).

For fluorescence microscopy, BPEI polyplexes were labeled with FITC-PLL. Briefly 3.6 μl of 1 mM FITC-PLL was mixed with 2 μg of plasmid DNA and incubated for 15 min at room temperature. Then BPEI was added and further incubated for 20 min at room temperature to allow complex formation. At this concentration FITC-PLL has no effect on the size, as measured with a Malvern Zetasizer nano-S (Malvern Instruments, Worchestershire, UK), nor the transfection efficiency of BPEI complexes. Alternatively, lipoplexes and polyplexes were prepared using Cy3-labeled pDNA (Mirus, MA).

Internalization of BPEI polyplexes and nuclear delivery of oligonucleotides
24 h before the experiment HeLa cells were plated at 1.5 × 10⁵ cell/well in 12-wells plates. Cells were treated with inhibitors and activators for 30 min prior to the addition of FITC-PLL-labeled BPEI polyplexes and further incubated for 2 h. Cells were immediately prepared for FACS analysis by trypsinizing cells at 37 °C for 5 min and resuspending them in complete medium, containing 0.2% trypan blue solution to quench extracellular fluorescence. FITC-positive cells were detected using a 488 nm wavelength laser line (λex. 488 nm, λem. 530 nm 10,000 events/sample). To determine nuclear delivery of ODNs, cells were incubated for 2 h with BPEI polyplexes containing FITC-labeled ODNs, and investigated with confocal microscopy. Fluorescence images were taken of three independent experiments and of 20 nuclei/sample the fluorescence intensity was measured using ImageJ software.
Chapter 4

Colocalization studies

HeLa cells were plated 24 h before the experiment at 1.5 × 10^5 cells/ml on glass coverslips (12 mm diameter) in a 12 wells plate. After treatment with PKA inhibitor/activator, cells were treated with fluorescently-labeled BPEI polyplexes (2 h), DiI-LDL (2 h), EGF (30 min), and dextran (2 h). Subsequently, cells were washed three times with PBS, fixed for 20 min with 4% p-formaldehyde (PFA) in PBS, quenched with 0.1 M glycine in PBS, permeabilized with 0.2% triton X-100 for 2 min, and blocked with 1% BSA and 0.05% Tween20 in PBS (PBS-T) for 1 min. Subsequently, cells were incubated with primary antibodies for 1 h at 37 °C in a humid chamber. The primary antibodies were diluted in PBS-T. Cells were washed 4 times with PBS and once with PBS-T for 1 min. Next, cells were incubated with fluorescently-labeled secondary antibodies for 30 min at 37 °C in a humid chamber. The Alexa Fluor-labeled antibodies were diluted 1:1000, while cyanine-labeled antibodies were diluted 1:400 in PBS-T. After three times washing with PBS, coverslips were mounted on glass slides using Dako mounting medium (Dako, Carpinteria, USA). Samples were analyzed using a confocal microscope (Leica TCS SP2; Germany) equipped with a 60× oil immersion lens. For colocalization analysis, samples were captured in a sequential manner and analyzed using ImageJ software (NIH). For early endosome labeling, Alexa Fluor 633 labeled transferrin (5 μg/ml) was added to the cells and incubated for 5 min at 37 °C before cells were fixed with 4% PFA. In case of incubation with EGF, cells were serum starved for 6 h prior to the addition of PKA inhibitor/activator, and immunostained for EGFR following EGF incubation. For the determination of colocalization of BPEI polyplexes with rab9, cells were transfected with pRab9-DsRed one day prior to the experiment.

Western blot analysis of phosphorylated PKA substrates

HeLa cells were plated at 1.5 × 10^5 per milliliter in six wells plates. After 24 h, cells were washed three times with ice-cold HBSS, lysed in lysis buffer (1% Triton X-100 in HBSS), containing a protease inhibitor cocktail (Roche) and a phosphatase inhibitor cocktail (10 mM sodium fluoride, 2 mM sodium pyrophosphate decahydrate, 1 mM sodium orthovanadate, 2 mM beta-glycerophosphate) for 10 min, and whole cell lysates were collected using a cell scraper (Corning Incorporated, USA). The protein concentration in the lysate was determined using BCA protein assay reagent (Pierce IL, USA) according to manufacturer's instructions. 10 μg of protein was boiled for 5 min in bromophenol blue sample buffer containing beta-mercaptoethanol, shortly spun, loaded onto 10% SDS–polyacrylamide gels, and subjected to SDS–PAGE. Subsequently, proteins were transferred to a nitrocellulose membrane, and the membrane was blocked with Odyssey blocking buffer (Li-Cor biosciences, USA), incubated with rabbit anti-phospho PKA substrate antibody (1:1000) for 2 h, washed with PBS/Tween 20 (0.5%) three times 5 min, and once with PBS. Membranes were then incubated with infrared dye-labeled goat anti-rabbit 680 nm secondary antibody, washed three times with PBS/Tween 20 (0.5%), washed once with PBS, and directly visualized using ODYSSEY infrared imaging system (LI-COR
Biosciences, USA) according to the manufacturer's instructions. Obtained signals were analyzed using ODYSSEY infrared imaging system software.

**Results**

**Modulation of transfection efficiency by protein kinase A activity**

To examine the potential role of kinase activity in lipo- or polyplex-mediated transfection, we first examined the effect of a panel of widely-used kinase inhibitors on the transfection of HeLa cells with three different transfection agents, i.e. Lipofectamine 2000 (LF2000), linear polyethyleneimine (LPEI), and branched polyethyleneimine (BPEI).

Essentially all of the tested inhibitors were without a significant effect on transfection efficiency (Supplementary Figure 1), except for H89, which among others inhibits protein kinase A (PKA) activity. Intriguingly, H89 treatment enhanced the transfection of HeLa cells approximately two-and-a-half fold when BPEI was used as a transfection agent, while leaving the transfection efficiency with LF2000 and LPEI, in terms of % GFP-positive cells, unaffected (Figure 1a). As shown in Figure 1b, the stimulatory effect of H89 was concentration-dependent, and at optimal conditions, the level of BPEI-mediated transfection efficiency was similar to levels usually obtained for LF2000 lipoplexes and LPEI polyplexes.

**Figure 1:**

The PKA-inhibitor H89 increases the transfection efficiency of BPEI polyplexes. (a) Following treatment with H89 (20 μM) for 30 min, HeLa cells were transfected with lipo- and polyplexes (mean ± SEM, n = 12), setting the relative percentage of BPEI-mediated transfection (expressed as % GFP-positive cells) in the absence of H89 as 100%. (The experimentally measured transfection efficiency in case of BPEI (control) was 17.9 ± 3.1 %.) (b) Following treatment with the indicated concentrations of H89 for 30 min, HeLa cells were transfected with BPEI polyplexes (mean ± SEM, n = 6), setting the relative percentage of BPEI-mediated transfection in the absence of H89 as 100%. (The experimentally measured transfection efficiency in case of BPEI (control) was 13.1 ± 3.8%.)

To obtain further support for a specific role of PKA activity, we took into account that H89 has been described to also affect Rho-kinase activity\textsuperscript{18}. However, as demonstrated in Figure 2a (and Supplementary Figure 1), the Rho kinase inhibitor Y27632 was without...
effect on BPEI-mediated transfection of HeLa cells, which excludes that the observed H89-mediated enhancement in transfection is due to Rho kinase inhibition. To verify therefore the specificity of the effect of H89 as a modulator of PKA activity in promoting BPEI-mediated transfection, we next investigated the effect of the PKA activators dibutyryl cyclic AMP (dbcAMP) and forskolin/isobutyl-1-methylxanthine (F/IBMX). As opposed to the inhibitor H89, both PKA activators, in a concentration-dependent manner, inhibited BPEI-mediated transfection of HeLa cells (Figure 2a and b).

Figure 2:
The increase in transfection efficiency by H89 is due to protein kinase A inhibition. (a) HeLa cells were pretreated with the indicated kinase inhibitors and activators for 30 min. Subsequently, cells were transfected with BPEI polyplexes (mean ± SEM, n = 6). All data were expressed relative to the experimentally measured transfection efficiency of BPEI (control), which was 17.9 ± 3.1%. (b) HeLa cells were pretreated with the indicated concentrations of either forskolin and IBMX (F/IBMX, top panel) or dbcAMP (bottom panel) for 30 min. Subsequently, cells were transfected with BPEI polyplexes (mean ± SEM, n = 6), setting the relative percentage of BPEI-mediated transfection in control cells as 100%. (c) HeLa cells were incubated with BPEI polyplexes, H89 (20 μM), dbcAMP (1 mM), F/IBMX (30 μM/2 mM) and a combination of H89 (20 μM) and forskolin/3-isobutyl-1-methylxanthine (F/IBMX, 30 μM/2 mM) for 2 h. Whole cell lysates were analyzed for the presence of PKA phosphorylated substrates using equal protein loading. Tubulin was used as a loading control.

Moreover, a direct effect of inhibitor (H89) and activators (dbcAMP and F/IBMX) on PKA activity per se, as well as the inertness of BPEI toward modulating PKA activity, could be revealed in an in vitro phosphorylation assay (Figure. 2c). The basal level of PKA phosphorylation activity in HeLa cells was low, as shown by the limited presence of phosphorylated PKA substrates (Figure 2c, control lane), implying that a decrease in the
PKA inhibition modulates the intracellular trafficking of nanocarriers

Phosphorylation of PKA substrates by H89 was therefore not detectable (Figure 2c, lane H89). However, the inhibitory activity of H89 could be confirmed by its ability to preclude the increase in PKA-induced substrate phosphorylation following PKA activation by forskolin/IBMX (Figure 2c, lanes F/IBMX versus F/IBMX + H89). Thus, the data support the notion that the H89-stimulated BPEI-mediated transfection efficiency involves a PKA-dependent step, implying that a diminished PKA activity promotes, whereas an enhancement of its activity inhibits transfection.

PKA inhibition enhances the escape of BPEI-delivered nucleic acids from intracellular compartments

There are several possibilities to explain the promotion of transfection efficiency upon PKA inhibition. An obvious one would be that PKA inhibition promotes the cellular uptake of BPEI polyplexes; alternatively, an enhanced release of plasmid into the cytosol, following initial internalization of the polyplexes, might also promote transfection efficiency. The following experiments were undertaken to discriminate between these possibilities. First, we determined the cellular uptake of BPEI polyplexes in the absence and presence of H89. To this end the interaction of fluorescently-labeled BPEI polyplexes was measured in control cells, and cells treated with H89, dbcAMP or forskolin/IBMX. After 2 h of incubation, the cells were washed and surface-bound fluorescently-labeled polyplexes were quenched with trypan blue, the remaining cell-associated fluorescence reflecting the genuinely internalized pool of BPEI nanoparticles. As shown in Figure 3a, none of these treatments affected the net cellular internalization of the polyplexes. Accordingly, these data would therefore suggest that PKA-inhibition interferes with a step secondary to internalization, presumably at the level of escape from an intracellular endocytic compartment.

To evaluate whether H89 promoted the efficiency of release of BPEI-delivered nucleic acids from intracellular compartments, we monitored the nuclear accumulation of BPEI-mediated delivery of fluorescently-labeled oligonucleotides (ODNs). The release of tagged ODNs from BPEI polyplexes from an intracellular compartment into the cytosol is readily reflected by their rapid transport and subsequent accumulation into the nucleus. As shown in Figure 3b, over a two hour incubation period, both control and dbcAMP-treated cells showed a prominent punctuate distribution pattern of fluorescent polyplexes, presumably reflecting the capture and processing of the polyplexes within intracellular compartments. Occasionally, weakly-labeled fluorescent nuclei were apparent. Interestingly, in H89-treated cells, the number of fluorescently-labeled nuclei was drastically enhanced, relative to control cells (Figure 3b), and equally striking was the increase in fluorescence intensity of the nuclei in H89-treated cells, compared to control and PKA-activated cells (Figure 3c).

Consistently, and in line with the ineffectiveness of H89 treatment on LF2000 and LPEI-mediated transfection (as reported above), no increase in LF2000- or LPEI-mediated ODN delivery was observed, neither in terms of cellular delivery nor in nuclear fluorescence intensity (not shown). These results thus indicate that upon inhibiting PKA activity, the
compartmental release of ODNs, as delivered by BPEI polyplexes, is strongly facilitated, causing their enhanced nuclear accumulation when compared with control and dbcAMP-treated cells. Next, we investigated the nature of the compartments involved in BPEI processing and ensuing nucleic acid release, as modulated by PKA activity.

**Figure 3:**
PKA inhibition results in an enhanced endosomal release of BPEI delivered ODNs. (a) HeLa cells were treated with the PKA inhibitor H89 (20 μM) and activators forskolin/3-isobutyl-1-methylxanthine (F/IBMX, 30 μM/2 mM) and dbcAMP (1 mM) for 30 min, followed by a 2 h incubation with fluorescently-labeled BPEI polyplexes. The extent of polyplex internalization was determined by FACS-analysis (mean ± SEM, n = 6). (b) HeLa cells were treated with H89 (20 μM) and dbcAMP (1 mM), 30 min before BPEI polyplexes were added to the cells. After another 2 h of incubation, the nuclear accumulation of ODNs was visualized in live cells with confocal microscopy (scale bar = 10 μM, n = 4). (c) The amount of nuclear accumulated ODNs was quantified using ImageJ (NIH). Twenty nuclei were randomly selected and the fluorescence intensity per nucleus was measured.

**BPEI polyplexes are internalized via both CME and caveolae, but only uptake via the caveolar pathway leads to productive transfection**
Thus far the data indicate that following an incubation of the cells with BPEI polyplexes in the presence of H89, such a treatment promoted the escape of the nucleic acid cargo into the cytosol from an intracellular compartment, without affecting net cellular uptake. Since intracellular transport of materials is linked to their cellular entry mechanism, while subsequent release of cargo will depend on the pathway of polyplex processing, the entry pathway for BPEI polyplexes was determined in the absence and presence of H89, using pharmacological inhibitors of potential pathways for endocytic entry. Chlorpromazine was used to inhibit clathrin-mediated endocytosis, nystatin and filipin III to inhibit entry via caveolae, while dimethylamiloride (DMA) was used to inhibit macropinocytosis.
PKA inhibition modulates the intracellular trafficking of nanocarriers
determine effective inhibitor concentrations, control experiments were carried out using
appropriate ligands (not shown), as previously described. At these conditions, no toxic
effects of the inhibitors were apparent, as determined by an MTT assay, and both the
cellular uptake of polyplexes and the transfection efficiency were measured.
In Figure 4 it can be seen that none of the pharmacological inhibitors of endocytosis
seemingly influence the net internalization of BPEI polyplexes in HeLa cells. Possibly, the
polyplexes are internalized via multiple endocytotic pathways, inhibition of uptake via one
pathway resulting in a compensatory increase in uptake via another pathway, as previously
reported by others. Intriguingly, whereas chlorpromazine, an effective inhibitor of
nanoparticle internalization along the clathrin-mediated endocytic pathway, did not affect
the transfection efficiency of BPEI polyplexes, the presence of nystatin and filipin III, both
considered to inhibit caveolae-mediated entry, diminished the transfection efficiency by
more than 50%. Together, these data support the notion that in control HeLa cells caveolae-
mediated entry of BPEI polyplexes, rather than clathrin-mediated endocytosis, leads to
productive gene expression. Moreover, since the inhibition of transfection efficiency is not
mirrored by a change of BPEI internalization per se, apparently compensatory entry
mechanisms are operating.
Subsequently, we carried out similar experiments in the presence of H89. Thus, after a
preincubation of the cells with H89, in the presence or absence of CPM, nystatin or FIII,
respectively, BPEI complexes were added and further incubated for 2 h. The levels of
nanoparticle internalization and transfection efficiency were then determined, and the data
are shown in Figure 4. Remarkably, while caveolae-mediated transfection is most
prominent in control cells, the H89-mediated enhancement in transfection was effectively
eliminated following co-treatment of the cells with CPM, i.e., the inhibitor of the clathrin-
mediated endocytic pathway. Consistently, the remaining level of transfection is
comparable to that obtained in the absence of H89, as contributed by the caveolae-mediated
pathway of BPEI entry. Indeed, in agreement with a connection between the stimulatory
effect of H89 and the clathrin-mediated pathway of BPEI internalization was the
observation that neither nystatin nor FIII significantly affected the H89-induced
enhancement in transfection efficiency (Figure 4). At the same time the internalization of
BPEI polyplexes remained the same, implying that H89 did not modulate internalization
along one pathway at the expense of another. Furthermore the potential contribution of
macropinocytosis can be ruled out as dimethylamiloride (DMA), irrespective of the
presence of H89, was essentially without effect on the transfection efficiency of BPEI
polyplexes (Figure 4). Entirely consistent with this observation, when the cells were
pretreated with dynasore, a selective inhibitor of dynamin, which interferes with both
clothrin- and caveolae-mediated endocytosis, the transfection of BPEI polyplexes, either in
the absence or presence of H89, was significantly inhibited (Figure 4).
Effect of PKA inhibition on cellular internalization of BPEI polyplexes via clathrin-mediated endocytosis and via caveolae. HeLa cells, plated one day before the experiment, were treated with chlorpromazine (CPM; 6 μg/ml), nystatin (NYS; 25 μg/ml), filipin III (FIII; 5 μg/ml), or dynasore (80 μM), with or without H89 (20 μM) for 30 min. For determining the extent of internalization, BPEI polyplexes, labeled with FITC-PLL, were incubated with the cells for 2 h, and samples were subsequently analyzed by FACS measurement. The transfection efficiency of the BPEI polyplexes was measured after 48 h (mean ± SEM, n = 10). All data are expressed relative to the internalization and transfection efficiency (both set at 100%) as obtained for untreated cells at otherwise identical conditions. The experimentally measured transfection efficiency in case of control BPEI was 22.5 ± 5.2%. Asterisks indicate significant differences †,‡, #, * (P < 0.05) in control versus H89 + nystatin, control versus H89 + FIII, nystatin versus H89 + nystatin and FIII versus H89 + FIII respectively, as evaluated with Student's t-test.

Together, the data thus strongly suggest that the H89-mediated increase in transfection efficiency, i.e., upon inhibition of PKA activity, must originate from polyplexes that were internalized via clathrin-mediated endocytosis, a pathway that in the absence of H89 does not significantly contribute to productive transfection. To obtain further support for such a scenario, and to investigate at which step along the clathrin-mediated endocytic pathway H89 exerts its potentiating effect on transfection by inhibiting PKA activity, we next examined intracellular BPEI polyplexes processing by fluorescence microscopy.

**Inhibition of PKA precludes BPEI polyplexes from reaching late endosomes and lysosomes thereby promoting transfection efficiency**

First we examined the intracellular processing of BPEI polyplexes in the presence and absence of H89 by determining the colocalization of the polyplexes with known markers for endosomal compartments. As appropriate markers we employed transferrin, rab9 and...
PKA inhibition modulates the intracellular trafficking of nanocarriers

LAMP1 to visualize early endosomes, late endosomes and endolysosomes, respectively. Colocalization of BPEI polyplexes with the endosomal markers transferrin (early endosomes) and rab9 (late endosomes) was determined 2 h after internalization of the polyplexes, while colocalization with the endolysosomal marker LAMP1 was measured after a three hour incubation period. As shown in Figure 5a (left panels), in the absence of H89 (control), BPEI polyplexes colocalized with transferrin, rab9 and LAMP1. In contrast, in the presence of H89, BPEI polyplexes colocalized with transferrin, but not with rab9 or LAMP1 (Figure 5a, middle panels).

Intriguingly, after a three hour incubation period and in the presence of H89, most BPEI complexes had disappeared from the transferrin-containing early endosomes (data not shown) and now resided in compartments that were also devoid of rab9 and LAMP1 (Figure 5a, middle panels). In fact, this localization was maintained for at least 24 h, i.e., after this time interval BPEI polyplexes were found in similar compartments as after 3 h, i.e., negative for transferrin, rab9, and LAMP1 (not shown).

In the presence of dbcAMP (Figure 5a, right panels) the colocalization of BPEI polyplexes with transferrin, rab9, and LAMP1 was similar to the pattern of colocalization in control cells. Accordingly, these data would suggest that upon inhibition of PKA activity internalized BPEI polyplexes, although reaching early endosomes are not transported into late endosomes/lysosomes, as observed in control or PKA-activated cells. Rather, PKA inhibition causes their rerouting from an early transferrin-containing endosomal compartment to a compartment tentatively characterized as being devoid of transferrin, rab9 and LAMP1. Moreover, an effect of H89 in the caveolae-mediated pathway of internalization could be excluded as the colocalization of BPEI complexes with caveolin-1, either in the presence or absence of the PKA inhibitor, was essentially the same in treated and control cells (Supplementary Figure. 2).

In the presence of chlorpromazine, inhibiting clathrin-mediated endocytosis, BPEI polyplexes did not colocalize with LAMP1 (Figure 5b), irrespective of H89 treatment. Assuming that the observed internalized FITC-labeled BPEI complexes thus largely reflect their presence in caveolar compartments, as described in the previous paragraph (see Figure. 4), the data imply that polyplexes that are internalized via caveolae do not reach lysosomes (i.e., within the incubation time interval of 3 h), thus avoiding potential degradation. In contrast, in the presence of nystatin, blocking caveolae-mediated entry, BPEI polyplexes now enter via the pathway of clathrin-mediated endocytosis, a degradative route, and indeed colocalize with LAMP1 (Figure 5c).
PKA inhibition modulates the intracellular trafficking of nanocarriers

Figure 5:
PKA inhibition precludes trafficking of BPEI polyplexes into late endolysosomal compartments. (a) Hela cells, 24 h after seeding, were first treated with H89 (20 μM) and dbcAMP (1 mM) for 30 min; then FITC-PLL-labeled BPEI polyplexes were added, in the presence of inhibitors. Colocalization of the complexes with transferrin and Rab9 was determined after 2 h, and colocalization with LAMP1 after 3 h of incubation (scale bar = 5 μM, n = 4). (b, c) HeLa cells were pre-treated with either chlorpromazine (CPM; 6 μg/ml) or nystatin (NYS; 25 μg/ml), with or without H89 (20 μM) as indicated, for 30 min and then FITC-PLL-labeled BPEI polyplexes were added to the cells. After another 3 h, the cells were fixed and immunostained with anti-LAMP1 antibody. A CY3-labeled secondary antibody was used to visualize LAMP1 (scale bar = 5 μM, n = 3).

Interestingly and consistently, when H89 was included in the incubation mixture, BPEI polyplexes no longer colocalized with LAMP1 (Figure 5c). Accordingly, these observations further strongly support the notion that inhibition of PKA precludes the transport of polyplexes within the clathrin-dependent pathway towards late endosomes/lysosomes. A summary of these findings is schematically presented in Figure 6.

In principle, the observations potentially point to early endosomes and/or the transferrin/rab9/LAMP1-devoid compartments as the most likely sites for nucleic acid release. Clearly, effective release from early endosomes would thus require the presence of H89, since although BPEI accumulates similarly in early endosomes in both control and PKA-stimulated cells, the contribution of the clathrin-mediated pathway towards transfection at these conditions is limited (Figure 4). To simulate these conditions and hence to better define the intracellular site of contents release, the cells were pretreated with the microtubule-disrupting agent nocodazole, which allows membrane flow and nanoparticle transport to early endosomes, but not subsequent transport to late endosomes. Following a subsequent incubation with BPEI polyplexes for 2 h, with or without H89, the transfection efficiency was determined after 48 h.
Figure 6: **Schematic representation of trafficking of BPEI polyplexes in control and PKA-inhibited cells.** Clathrin-mediated endocytosis (CME) and caveola/raft-mediated endocytosis are the major pathways of cellular entry of BPEI polyplexes in HeLa cells. At control conditions or upon treatment with dbcAMP (left part of the scheme), most polyplexes, traveling along the classical endocytic pathway, pass through early and late endosomal compartments, thereby reaching the digestive lysosomes. Particularly the fraction of the polyplexes entering via caveola displays the capacity to release nucleic acid contents, and does not reach the late endolysosomal compartments. Following treatment with the PKA inhibitor H89 (right part of the scheme), trafficking of the polyplexes along the CME pathway is impeded and/or perturbed in that complexes, after passing through early Trf-containing endosomes, primarily localize in compartments that are devoid of Trf, Rab9 and LAMP1. Presumably, cargo release from this compartment results in an enhanced delivery of ODNs into the nucleus and a substantial increase in transfection efficiency. For further details see text. For reasons of simplicity only one EE is indicated. We do not mean to suggest that cargoes within the pathways of CME and caveolar endocytosis necessarily mix at the level of the EE. (CME: clathrin-mediated endocytosis; CCV: clathrin-coated vesicle; EE: early endosome.)

As shown in Figure 7, the uptake of polyplexes by the cells, irrespective of treatment, were similar; however, in the presence of nocodazole, the relative transfection efficiency, irrespective of the presence of H89, was strongly inhibited.
In fact the residual transfection efficiency, which is proportionally stimulated upon treatment of the cells with H89 (i.e., as observed in control cells) likely relates to a cell fraction that was not affected by nocodazole. Accordingly, these data suggest that rerouting of BPEI upon PKA inhibition presumably represents an essential step that leads to the promoting effect of PKA inhibition on the transfection efficiency.

Evidently, the PKA-induced modulation of intracellular routing raised the question to which extent this event was related to the processing of a specific nanoparticle, i.e., BPEI polyplex, or whether it reflected a typical cell biological PKA-regulated feature of cargo that is taken up by the cells via clathrin-mediated endocytosis. Therefore, we next investigated the effect of PKA inhibition, following addition of H89, on the endocytic flow of the other gene delivery vehicles, i.e., Lipofectamine 2000 and LPEI, as well as the unrelated endocytic markers dextran and low density lipoprotein (LDL).

**PKA inhibition precludes transport into late endosomes/lysosomes of cargo that is internalized by clathrin mediated endocytosis**

To better appreciate the PKA-mediated effect on endocytic trafficking, the intracellular flow of LF2000 lipoplexes and LPEI polyplexes was examined by fluorescence microscopy. Similar to BPEI polyplexes, LF2000 lipoplexes and LPEI polyplexes colocalized with LAMP1 after 3 h of incubation in the absence of H89, while in the
presence of H89 their transport into late endosomes/lysosomes was precluded (Supplementary Figure 3a–c). Next, the cellular processing of dextran and LDL was analyzed. Both ligands are internalized by eukaryotic cells along different endocytic pathways, i.e. fluid-phase endocytosis, and clathrin-mediated endocytosis, respectively, but eventually reach the lysosomes. The fluid phase marker dextran was shown to reach lysosomes, both in the absence and presence of H89, as reflected by its colocalization with lysotracker (Supplementary Figure 3d). As anticipated, in control cells LDL is similarly transported, albeit along the clathrin-mediated pathway, to the lysosomes. However, in sharp contrast, in H89-treated cells LDL does not significantly colocalize with LAMP1 (Supplementary Figure 3e). As a control, dbcAMP treatment of the cells revealed the usual processing of both dextran and LDL towards the lysosomes (Supplementary Figure 3d,e). Moreover, virtually identical results as observed for LDL transport were obtained in case of processing of EGF along the clathrin-mediated pathway (not shown). Importantly, like in the case of BPEI internalization, the presence of H89 did not affect the internalization of either ligand (not shown). Hence these data imply that upon inhibiting PKA activity endocytic transport of gene delivery vehicles as well as common ligands to late endosomes/lysosomes is precluded. Moreover, this inhibitory effect is particularly related to an interference with relatively early steps in the clathrin-mediated pathway of endocytosis, i.e., transport from early to late endosomes.

Discussion

To improve lipo- and polyplex-mediated delivery of nucleic acids into eukaryotic cells for therapeutic and cell biological purposes, insight into regulatory mechanisms of nanoparticles internalization and subsequent processing leading to transfection, is imperative. Thus, parameters like the chemical nature and particle size of applied vehicles may govern the nature of the endocytic pathway of entry (clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, etc.)14,22-24. Here, we have investigated the involvement of protein kinases, being part of the regulating endocytic machinery, as a governing co-factor in determining internalization per se and/or the kinetics of particle processing17,25,26, thereby potentially modulating cellular transfection. The present work reveals that of the kinases examined thus far (Supplementary Figure 1), inhibition of protein kinase A activity is of particular interest as it perturbs trafficking of gene delivery vehicles along the clathrin mediated pathway of endocytosis (CME), precluding their delivery to late endosomal/lysosomal compartments. As a result, BPEI mediated-transfection efficiency increases approximately 2.5 fold. PKA modulation does not affect BPEI internalization per se and affects the endocytic processing of LDL and EGF similarly as observed for polyplexes trafficking, emphasizing a general and typical effect of PKA inhibition on relative early trafficking steps in the pathway of CME. Moreover, whereas in control cells BPEI polyplexes are internalized via both CME and caveolae (Figure 4), primarily the latter pathway contributes to transfection efficiency. Accordingly, these observations support the notion, as summarized in Figure 6, that impediment of particle delivery into the late endolysosomal track of the clathrin-mediated pathway may strongly
promote transfection efficiency of complexes that are otherwise ineffective in this pathway. Although care should be taken as to the precise interpretation of the effect of pharmacological inhibitors in defining intracellular pathways, our data, in conjunction with the detailed colocalization studies of the lipo/polplexes with markers of the clathrin- and caveolae-mediated pathways, convincingly support the conclusion of the clathrin-mediated pathway as the primary target of H89 (Figure 4, Figure 5 and Figure 2). Apparently, in contrast to BPEI, LPEI polplexes and LF2000 lipoplexes do not benefit from an altered endosomal processing as they efficiently mediate endosomal escape of genetic cargo well before their uptake into digestive lysosomes\textsuperscript{27-29}. Hence, rather than by targeting gene carriers into the caveolar pathway, thereby avoiding lysosomal delivery (in some but not all cell types), transfection efficiency of relative poorly delivering complexes may particularly benefit from modulating their endolysosomal trafficking, as accomplished by inhibiting PKA activity as demonstrated in the present work. Our data indicate that as a result of this inhibition, trafficking of the BPEI nanoparticles does not proceed into the degradative pathway with lysosomes as their final destination, but rather leads to their delivery into a compartment that we tentatively define as a transferrin/rab9/LAMP1-devoid compartment (Figure 6). Since release of plasmids from early endosomes, as mimicked by nocodazole treatment, is not promoted in the presence of H89, the data support the transferrin/rab9/LAMP1-devoid compartment to act as the major site from which plasmids and ODNs are released by an as yet unknown mechanism (Figures 3 and 7). In a more general context, these data imply that the kinetics of poly- and lipoplex-mediated delivery of nucleic acids into late endolysosomal compartments represent the limiting step in overall delivery efficiency. The underlying mechanism as to how PKA exerts its action in the process of BPEI-mediated gene delivery remains to be determined. Our data show that in H89-treated HeLa cells, BPEI polplexes transiently reside in early (transferrin-containing) endocytic compartments, and at later time points localize in transferrin/rab9/Lamp1-devoid vesicles (Figure 5 and Figure 7). Usually, the maturation of early endosomes into late endosomes is regulated by the loss of rab5 and concomitant recruitment of rab7. Furthermore, in cells treated with H89 an increase in the size of both endolysosomes (LAMP1) and in particular lysosomes (Supplementary Figure 3, dextran) was often observed with a concomitant decrease in the number of these organelles. These observations might hint to an impediment of (vesicular) transport upon inhibiting PKA activity, since the effectivity of rabs and rab effector proteins mediating these processes depends on their state of phosphorylation\textsuperscript{30,31}. Finally, the elucidation of cellular signaling pathways as modulators of gene delivery efficiency, will be highly instrumental in further broadening our understanding of the interaction between lipo/polplexes and cells at the molecular level, and may thus contribute to the design of protocols that further improves gene delivery efficiency. Since such pathways might be affected by the lipo/polplexes themselves\textsuperscript{16}, the present findings may not only be relevant to, for example, ex vivo (therapeutic) protocols, but rather, may bear relevance to in vivo studies as well. Clearly, this merits further investigations.
Supplementary Figures

Figure S1

Influence of kinase inhibitors on the transfection efficiency of LF2000 lipoplexes, and LPEI and BPEI polyplexes. HeLa cells, plated one day before transfection, were treated with the indicated kinase inhibitors, 30 min before lipo- and polyplexes were added. Subsequently, the cells were incubated for another 2 h with lipo- and polyplexes in the presence of the inhibitors. Transfection efficiency was measured after 48 h. Concentrations of the applied inhibitors, as well as their specificity, are indicated in Materials and methods (mean ± SEM, n = 6).
PKA inhibition modulates the intracellular trafficking of nanocarriers

**Figure S2.**
PKA inhibition does not affect the colocalization of BPEI polyplexes with caveolin-1, following internalization along the caveolae-mediated pathway. HeLa cells, plated one day before the experiment, were pretreated with H89 (20 μM) for 30 min. BPEI polyplexes containing Cy3-labeled plasmid DNA (red) were added and further incubated for 2 h. Subsequently, the cells were fixed and immunostained for caveolin-1 (green). Scale bar = 5 μM. (See Materials and methods.)
Figure S3:
PKA inhibition precludes the trafficking of LF2000 lipoplexes, LPEI polyplexes and LDL, but not dextran, into LAMP1-positive compartments. (a–c) HeLa cells pretreated with H89 (20 μM) for 30 min, were incubated with BPEI polyplexes, LPEI polyplexes and LF2000 lipoplexes, containing Cy3-labeled plasmid DNA, for 3 h. Subsequently, cells were fixed and immunostained for LAMP-1. (d, e) HeLa cells, pretreated with H89 (20 μM) and dbcAMP (1 mM) for 30 min, were incubated with FITC labeled dextran (4 kDa, 4 μg/ml) for 3½ h and further incubated with lysotracker red (100 nM) for 30 min. Live cells were directly observed by confocal microscopy. For LDL tracking, similarly treated cells were incubated with Dil-labeled LDL (15 μg/ml) for 2 h before the cells were fixed and immunostained for LAMP1 (scale bar = 5 μM, n = 3). LDL: low density lipoprotein.
PKA inhibition modulates the intracellular trafficking of nanocarriers

References


