Chloride Accumulation and Swelling in Endosomes Enhances DNA Transfer by Polyamine-DNA Polyplexes

Received for publication, August 6, 2003, and in revised form, August 22, 2003
Published, JBC Papers in Press, August 27, 2003, DOI 10.1074/jbc.M308643200

N. D. Sonawane‡, Francis C. Szoka, Jr.§, and A. S. Verkman‡¶
From the ‡Departments of Medicine and Physiology, Cardiovascular Research Institute, and §Department of Biopharmaceutical Sciences and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143

The “proton sponge hypothesis” postulates enhanced transgene delivery by cationic polymer-DNA complexes (polyplexes) containing H+ buffering polyamines by enhanced endosomal Cl− accumulation and osmotic swelling/lysis. To test this hypothesis, we measured endosomal Cl− concentration, pH, and volume after internalization of polyplexes composed of plasmid DNA and polylysine (POL), a non-buffering polyamine, or the strongly buffering polyamines polyethylenimine (PEI) or polyamidoamine (PAM). [Cl−]pH were measured by ratio imaging of fluorescently labeled polyplexes containing Cl− or pH indicators. [Cl−]pH increased from 41 to 80 mM over 60 min in endosomes-contained POL-polyplexes, whereas pH decreased from 6.8 to 5.3. Endosomal Cl− accumulation was enhanced (115 mM at 60 min) and acidification was slowed (pH 5.9 at 60 min) for PEI and PAM-polyplexes. Relative endosome volume increased 20% over 75 min for POL-polyplexes versus 140% for PEI-polyplexes. Endosome lysis was seen at >45 min for PEI but not POL-containing endosomes, and PEI-containing endosomes showed increased osmotic fragility in vitro. The slowed endosomal acidification and enhanced Cl− accumulation and swelling/lysis were accounted for by the greater H+ buffering capacity of endosomes containing PEI or PAM versus POL (>80 mM vs 46 H+/pH unit). Our results provide direct support for the proton sponge hypothesis and thus a rational basis for the design of improved non-viral vectors for gene delivery.

Although gene delivery using non-viral vectors offers potential advantages over virus-based delivery systems, the relatively low transfection efficiency of non-viral vectors has been their major limitation for in vivo applications (1–4). The archetypal non-viral gene delivery system is the cationic polycation-DNA complex (polyplex), in which plasmid DNA and a cationic carrier are condensed into a tight complex suitable for cellular internalization by endocytosis (5, 6). Transgene delivery to the nucleus is thought to require escape of the polyplex from endosomes, DNA/polymer dissociation, cytoplasmic DNA diffusion, and nuclear uptake (5, 7, 8). The low efficiency of polyplex escape from endosomes is thought to be an important determinant of the overall efficiency of non-viral gene transfer.

Polyamines are useful polycatonic macromolecules for non-viral gene transfer because of their high density of positive charges, ease of synthesis, and efficient polyplex formation (8–10). Cationic polyamines with fixed, non-titratable charges such as polylysine (POL) are substantially less efficient at gene transfer than polyamines with titratable amines such as polyamidoamine (PAM) (11) and polyethylenimine (PEI) (12, 13). The lower efficiency is not caused by differences in morphology of the complexes or cell association (14). To explain this observation it has been postulated without direct evidence that the high H+ buffer capacity of polyamines containing titratable amines results in endosomal Cl− accumulation during acidification with presumed osmotic endosome swelling and enhanced polyplex escape (11, 15). This “proton sponge hypothesis” if correct, would have important consequences in defining the barriers to non-viral gene delivery and in the design of improved non-viral vectors.

Here, we test the proton sponge hypothesis by comparing the kinetics of acidification, Cl− accumulation, and swelling in endosomes containing polyplexes composed of DNA and fluorescent probe-labeled polyamines (POL, PEI, or PAM). We previously developed long-wavelength, Cl−-sensing fluorescent probes for the measurement of [Cl−]pH in cellular endosomal and Golgi compartments and showed that inward H+ pumping by the bafilomycin-sensitive vacuolar ATPase was coupled quantitatively to Cl− entry in endosomes produced by fluid-phase (16) or receptor-mediated (17) endocytosis. We find here that “macropinocytic” endosomes containing DNA polyplexes with titratable amines have remarkably increased buffer capacity, reduced acidification, increased Cl− accumulation, and increased swelling/lysis compared with endosomes containing polyplexes with fixed charges, providing direct evidence in support of the proton sponge hypothesis.

* This work was supported by National Institutes of Health Grants EB00415, HL59198, DK53124, HL73856, and EY13574 and Cystic Fibrosis Foundation Grants R613 (to A. S. V.) and DK46052 and EB00415, HL59198, DK35124, HL73856, and EY13574 and Cystic Fibrosis Foundation Grants R613 (to F. C. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: Cardiovascular Research Institute, 1246 Health Sciences East Tower, Box 0521, University of California, San Francisco, CA 94143-0521. Tel.: 415-476-8530; Fax: 415-663-3847; E-mail: verkman@tsa.ucsf.edu.
‡ The abbreviations used are: polyplex, cationic polycation-DNA complex; POL, polylysine; PAM, polyamidoamine; PEI, polyethylenimine; PBS, phosphate-buffered saline; TMR, tetramethylrhodamine; TMR-POL, TMR-labeled polylysine; TMR-PAM, TMR-labeled PAM; TMR-PEI, TMR-labeled PEI; BAC, 10,10-bis[j-carboxypropyl]9.9’-biacridinium dinitrate; BAC-dextran-S-S-2Py, BAC-dextran carrying dithian-2-pyridyl linker; FITC, fluorescein isothiocyanate; FITC-PAM-TMR, PAM labeled with FITC and TMR; FITC-PEI-TMR, PEI labeled with FITC and TMR; FITC-POL-TMR, POL labeled with FITC and TMR; N/P, amine-to-phosphate; GFP, green fluorescent protein; CHO, Chinese hamster ovary; BAC-dextran-PAM-TMR, tetramethylrhodamine-labeled polyamidoamine conjugated with BAC-labeled dextran; BAC-dextran-PEI-TMR, tetramethylrhodamine-labeled polyethylenimine conjugated with BAC-labeled dextran; BAC-dextran-POL-TMR, tetramethylrhodamine-labeled polylysine conjugated with BAC-labeled dextran.
**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Chinese hamster ovary (CHO)-K1 cells (American Type Culture Collection, Manassas, VA) were grown in Ham’s F12K medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured on 18-mm diameter round coverglasses at 37 °C in 95% air/5% CO₂ and used just prior to confluence.

**Fluorescently Labeled Polyamine Conjugates for [Cl−] Measurements**—The polymers POL (Aldrich, 25 kDa), PAM (43 kDa, 6th generation fractured dendrimer) (18), and PEI (Aldrich, 25 kDa) were purified and characterized as described previously (14). An aqueous solution of polyamine (POL, PAM, or PEI) (5 µmol in 5 ml of PBS) was stirred slowly with tetramethylrhodamine succinimidyl ester (15 µmol, from Me₂SO stock) at room temperature for 1 h (Fig. 1A). Unreacted dye was removed by gel filtration (Sephadex G25, PBS). Using molar absorbance data, TMR/polyamine mol ratios were 3.3:1 (TMR-POL), TMR/polyamidoamine mol ratios were 2.8:1 (TMR-PAM), and TMR/polyethylenimine mol ratios were 4.1:1 (TMR-PEI). Equal volumes of each TMR-polyamine (5 µmol in degassed PBS) and iminothiolane (13 µmol in PBS containing 5 mM EDTA, pH 8) were incubated for 1 h at room temperature in the dark under N₂. Unreacted iminothiolane was removed by gel filtration. Sulfhydryl (SH) group/ligand mol labeling ratios were 0.96:1 (for TMR-POL), 0.93:1 (for TMR-PAM), and 0.95:1 (for TMR-PEI). TMR-polyamine-SH (5 µmol) was then incubated with BAC-dextran-S-S-2Py (2 µmol in 4 ml of PBS containing 5 mM EDTA, pH 8), synthesized as described in Ref. 16 for 18 h at room temperature under N₂. Unreacted TMR-polyamine-SH and BAC-dextran-S-S-2Py were removed by gel filtration, and the product was lyophilized and stored at −20 °C.

Molecular masses of the conjugates (before and after cell internalization) were determined by column chromatography (Sephacryl 300HR). Eluted fractions were assayed for TMR and BAC fluorescence. In fluorescence-quenching studies, microaliquots of NaCl (1 µ stock) were added to 3 ml of fluorescent ligand (10 µM, pH 7.4). Stern-Volmer constants (Ksv) were calculated from the slope of F/F₀ 1 versus [Cl−] plots (F₀/F₁ − 1 = Ksv [Cl−]), where F₀ is BAC fluorescence in absence and F₁ is the presence of Cl−.

**Fluorescently Labeled Polyamines for pH Measurements**—TMR-POL, TMR-PAM, and TMR-PEI (5 µmol) prepared as above were gently stirred for 1 h with fluorescein isothiocyanate (35 µmol from Me₂SO stock) in 10 ml of aqueous NaHCO₃ (pH 8). Reaction products (FITC-POL-TMR, FITC-PAM-TMR, and FITC-PEI-TMR) were purified by gel filtration chromatography. Thin layer chromatography showed no free dye contamination. FITC/polymer molar labeling ratios were 5.5, 6.0, and 6.8 for POL, PAM, and PEI, respectively.

**Polyamine-DNA Polyplexes**—DNA complexes containing Cl− or pH-sensing fluorophores (or non-fluorescent polyamines) were prepared by mixing plasmid DNA (pCDNA3 encoding GFP, 3.8 kb; 80 µg/ml in 10 mM HEPES, 10 mM NaCl, 10% glucose) and polyamine (in distilled water) at specified primary amine/phosphate (N/P) ratios (2.2, 5.5, and 6.0 for PEI, PAM, and POL, respectively). Polyplexes were dispersed in PBS for endosomal uptake. For measurements of transfection efficiency, CHO cells were incubated with polyplexes at different N/P in 8-well plates for 30 min in serum-free medium and then for 36 h in serum-containing medium. GFP fluorescence was quantified by a fluorescence plate reader.

**Endosome Labeling, [Cl−], and pH Measurements**—Cells were incubated in serum-free medium for 15 min at 37 °C and then pulse-labeled by incubation with DNA polyplex (50 µg/ml) in PBS for 5 min at 4 °C. Coverslips were washed twice at 4 °C with PBS containing dextran sulfate (10 kDa, 1 mg/ml) and washed once in PBS, then transferred to a pre-cooled perfusion chamber. Sets of BAC and TMR images (for Cl−) or FITC and TMR images (for pH) were acquired at specified times after perfusion with PBS at 37 °C. In some experiments, the perfusate contained NH₄Cl (5–40 mM) or chloroquine (100 µM). For in vivo calibration of TMR/FITC fluorescence ratio versus [Cl−], perfusate and endosomal [Cl−] were equalized by the incubation of cells for 15–20 min at 37 °C in 120 mM KCl/KNO₃ (0–120 mM Cl−), 20 mM NaCl/NaNO₃, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES (pH 7.4) containing the ionophores nigericin, valinomycin, carbonyl cyanide p-chlorophenylhydrazone, monensin (all 10 mM), and bafilomycin (200 mM). For calibrations of TMR/FITC fluorescence ratio versus pH, cells were incubated with high

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**FIG. 1. Synthesis and characterization of fluorescently labeled Cl−-sensing polyamines.** A, structures of the polyamines POL, PAM (second generation), and PEI. PAM and PEI contain tritatable amines at physiological pH, whereas POL does not. B, left, TMR-labeled polyamines were conjugated to BAC-labeled dextran by a disulfide linker to generate ratioable Cl−-sensing polyamines (see “Experimental Procedures”; right, agarose gel electrophoresis with ethidium bromide staining of plasmid DNA (lane 1) and polyplexes consisting of plasmid DNA and BAC-dextran-PAM-TMR (lane 2) or FITC-PAM-TMR (lane 3). C, optimization of polyamine/DNA (N/P) ratio for CHO cell transfection. Transfection efficiency was quantified by GFP fluorescence at 36 h.
K⁺ solutions containing nigericin, valinomycin, and bafilomycin (pH adjusted to 4--8). 

**Endosomal Buffer Capacity**—Buffer capacity (β) was determined from the rapid increase in endosomal pH in response to the addition of 5–40 mM NH₄Cl to the per fusate: $\beta = (\Delta \text{pH}) / (\Delta \text{pH} + \Delta \text{pH}_{\text{final}})$, where $\Delta \text{pH}$ is the pH increase just after NH₄Cl addition, $\Delta \text{pH}_{\text{final}}$ is the pH increase after addition of NH₄Cl, and $\Delta \text{pH}_{\text{endosomal}}$ is endosomal pH after the addition of NH₄Cl. 

**Per fusate** [NH₄Cl] was chosen to increase endosome pH from ~6.1 to 6.7.

**Fluorescence Microscopy**—Cells were imaged using a Leitz upright epifluorescence microscope equipped with Nipkow-wheel confocal attachment and a 14-bit cooled (–30 °C) charge-coupled device camera (19). Fluorescence was collected using a 100× oil immersion objective (Nikon Plan Apo, numerical aperture 1.4), a custom BAC filter set (excitation = 470 ± 5 nm, dichroic = 505 nm, emission = 535 ± 20 nm), and standard TMR and FITC filter sets. Image analysis was done using custom software to compute area-integrated background-subtracted pixel intensities (16). Endosome volumes were determined from computed image areas of individual endosomes assuming spherical shape.

**In Vitro Endosome Osmotic Fragility**—At 30 or 45 min after internalization of FITC/TMR-labeled polyplex and 37 °C chase, cells were homogenized in 110 mM K gluconate, 10 mM NaCl, 1.5 mM CaCl₂, and MgCl₂, 1.5 mM Hepes (290 mosm, pH 6). After low speed centrifugation (5000 × g for 10 min), the homogenate supernatant containing polyplex-labeled endosomes was added to buffers of various osmolalities to create an osmotic stress. The fraction of solution-accessible polyplex was determined by cuvette fluorimetry from the immediate *versus* delayed increase in FITC fluorescence after the addition of NaOH (from 1 M stock) to increase pH to ~8.0.

**RESULTS**

**Characterization of Fluorescent Polyplexes**—The polyamines PEI and PAM contain titratable secondary and tertiary amines in the physiological pH range, whereas POL does not (Fig. 1A). PEI, POL, and PAM were labeled with TMR and conjugated 1:1 with BAC-dextran using a disulfide linker (Fig. 1B). BAC fluorescence is green and Cl⁻-sensitive, whereas TMR fluorescence is red and Cl⁻-insensitive. Gel electrophoresis indicated the absence of uncomplexed DNA (representative gel shown in Fig. 1B, right). However, at the charge ratios used to form the complexes, there was an excess of fluorescent polymers observed migrating toward the cathode (Fig. 1B right, *upper fluorescent material*), as is usually present in transfection mixtures. Gel filtration chromatography of polyamines before and after cellular internalization of Cl⁻ indicators showed single peaks at apparent molecular masses of 90, 71, and 73 kDa for PAM, PEI, and POL, respectively (not shown). The BAC and TMR fluorescence signals co-migrated, and no fluorescence was seen at lower molecular sizes, indicating stability of the fluorescent polyamine conjugates in *vivo*.

Fluorescence spectra of BAC-dextran-polyamine-TMR polyplexes showed excitation maxima at 365 and 434 nm and emission maximum at 505 nm for BAC; for TMR, excitation maximum was at 556 nm and emission maximum was at 577 nm. BAC fluorescence in the polyplexes was quenched by Cl⁻ by a collisional mechanism with a Stern-Volmer constant of 36 M⁻¹, whereas TMR fluorescence was not Cl⁻-sensitive. Thus, BAC fluorescence quenching was not affected by polyamine conjugation and DNA complexation.

Transfection efficiencies of the polyamine-DNA polyplexes were measured to establish optimal N/P for subsequent experiments. Cells were incubated for 30 min at 37 °C with polyplexes containing unconjugated or fluorescently labeled polyamines. Transfection efficiency, measured as cellular GFP fluorescence at 36 h, was ~5 times higher for N/P-optimized PAM versus POL polyplexes (Fig. 1C). Polyplex transfection efficiencies and optimal N/P were similar for unconjugated versus fluorescent polyamines and the relative transfection efficiencies are comparable with published results (12, 14).

**Cl⁻ Concentration and pH in Polyplex-containing Endosomes**—Fig. 2A shows fluorescence micrographs of cells labeled with polyplexes containing BAC-dextran-PAM-TMR (top) and BAC-dextran-POL-TMR (bottom). The cell surface was stained after incubation for 5 min at 4 °C (micrographs labeled 0–1 min), at which time the green BAC fluorescence was relatively dim because of the high extracellular [Cl⁻]. Polyplexes were rapidly internalized upon warming to 37 °C. Endosomes labeled with polyplexes containing PAM and PEI, but not POL, became substantially larger over time.

Calibrations of R/G (TMR/BAC fluorescence ratio) *versus* [Cl⁻] were done by incubating cells with polyplex-labeled endosomes with a high K⁺ buffer containing ionophores and bafilomycin. Fig. 2B, left shows similar R/G *versus* [Cl⁻] for

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**Fig. 2.** CHO cell labeling with fluorescent Cl⁻ and pH-sensing polyplexes. A, confocal micrographs showing BAC (green) and TMR (red) fluorescence after cell labeling for 5 min at 4 °C with polyplexes (50 μg/ml) containing plasmid DNA and BAC-dextran-PAM-TMR (top) or BAC-dextran-POL-TMR (bottom). Micrographs taken before (0–1 min) and at indicated times after 37 °C chase. Bar, 10 μm. B, left, in situ calibration of R/G versus pH for endosomes labeled with FITC-PAM-TMR polyplex; right, pseudocolored ratio image showing pH in endosomes labeled with FITC-PAM-TMR polyplex after a 45-min chase.
BAC-dextran-PAM-TMR in aqueous solution (black circles) and in cells (white circles), with a Stern-Volmer quenching constant of 37 m⁻¹. Similar quenching constants of 36–38 m⁻¹ were determined for polyplexes containing BAC-dextran-POL-TMR and BAC-dextran-PEI-TMR. The R/G versus [Cl⁻] calibration permitted determination of [Cl⁻] in individual endosomes by ratio image analysis as shown in the pseudocolored image in Fig. 2B, right, which was computed for images taken at 45 min after BAC-dextran-PAM-TMR labeling.

A calibration of R/G (TMR/FITC fluorescence ratio) versus pH for the pH-sensing polyamine FITC-PAM-TMR was done in cells using high K⁺ buffer containing ionophores and bafilomycin (Fig. 2C, left). R/G versus pH was similar in cells and solution (pKᵣ ~ 6.3). Similar results were obtained for FITC-POL-TMR and FITC-PEI-TMR polyplexes. Fig. 2C, right shows pH in individual FITC-PAM-TMR-labeled endosomes at 45 min after labeling.

After pulse-labeling at 4 °C and rapid warming to 37 °C, averaged [Cl⁻] in endosomes containing PAM polyplexes increased from 40 to 115 mM during the 60-min chase period and then decreased to 82 mM at 75 min (Fig. 3A). As discussed below, the late decrease in average endosomal [Cl⁻] probably results from lysis/pre-lysis of a subset of endosomes. Similar [Cl⁻] kinetics were measured in endosomes containing PEI polyplexes. Endosomes containing POL polyplexes accumulated significantly less Cl⁻ (41–80 mM at 60–75 min). Endosomal acidification was measured in parallel experiments (Fig. 3B). Averaged pH in endosomes containing PAM and PEI polyplexes decreased slowly from 7.2 to 5.9 over 60 min, increasing to 6.5 at 75 min. Acidification of endosomes containing POL polyplexes was relatively rapid, with pH decreasing to 5.9 in only 15 min. Bafilomycin blocked Cl⁻ accumulation and acidification in endosomes containing each of the polyplexes (not shown).

We postulated that the increased Cl⁻ accumulation and slowed acidification in endosomes containing PAM or PEI versus POL polyplexes was caused by their greater H⁺ buffer capacity (β). Using the NH₄⁺ pulse method, β (in the pH range ~6.1–6.7) was 99 and 91 mM/pH units for endosomes containing PAM and PEI polyplexes, respectively, which was much greater than that of 46 mM/pH units for endosomes containing POL polyplexes (Fig. 3C). The 2-fold increased buffer capacity in endosomes containing PAM or PEI versus POL polyplexes accounts for the increased Cl⁻ accumulation (Δ[Cl⁻] ~ 80 versus 40 mM over 60 min) and slowed acidification (ΔpH ~ 0.5 versus 1 unit over 15 min).

Swelling and Lysis of Polyplex-containing Endosomes—The greater volume of polyplex-containing (macropinocytic) endosomes compared with endosomes formed by fluid-phase or receptor-mediated mechanisms permitted estimation of their volume by light microscopy. Fig. 4A, left shows that the averaged volume of fluorescent endosomes containing PAM polyplexes increased significantly with time after internalization, with a smaller increase for POL polyplexes. The weak base chloroquine increased volume and Cl⁻ accumulation (and inhibited acidification) in endosomes containing POL polyplexes (Fig. 4A, left and right), providing further evidence linking Cl⁻ accumulation and swelling.

To determine whether endosome swelling is associated with permeabilization/lysis, the kinetics of pH in individual endosomes was measured, reasoning that endosome permeabilization or lysis would result in endosome disappearance or alkalization (to dissipate endosome-cytoplasm pH gradient). Fig. 4B left, shows alkalization in 15–20% of endosomes containing PAM polyplexes at 60–75 min after labeling. Also, some PAM polyplex-containing endosomes disappeared as early as 30 min after labeling, probably as a consequence of lysis and polyamine diffusion (Fig. 4C). In contrast, under the same conditions, no POL polyplex-containing endosomes disappeared or strongly alkalized (Fig. 4B, right).

These data predict that swollen endosomes containing PAM or PEI polyplexes should be more sensitive to osmotic lysis than endosomes containing POL polyplexes. Cell homogenates containing labeled endosomes, prepared at 45 min after cellular internalization of fluorescent polyplexes, were subjected to osmotic challenge, and the fraction of solution-exposed polyamine was deduced from the immediate versus delayed fluorescence increase after NaOH addition (Fig. 4D, inset). Fig. 4D shows remarkably greater permeabilization/lysis in endosomes containing PAM versus POL polyplexes after osmotic challenge, as predicted.

**DISCUSSION**

The experiments here were designed to test the proton sponge hypothesis, which postulates that enhanced transgene delivery by polyplexes containing titratable amines is caused by increased endosomal Cl⁻ accumulation and consequent swelling/lysis. Cl⁻ accumulation was found to be remarkably greater in endosomes containing PEI and PAM versus POL polyplexes. Acidification was correspondingly reduced, swelling was increased, and buffer capacity was greater. Permeabilization and lysis were seen at 30–75 min in PAM and PEI but not POL polyplex-containing endosomes, and osmotic fragility was greater. Finally, POL polyplex-containing endosomes could be made to resemble PAM polyplex-containing endosomes by inclusion in the bathing solution of chloroquine, a weak base that accumulates in acidifying compartments. We conclude that increased polyplex buffer capacity results in increased endosomal Cl⁻ entry because of H⁺/Cl⁻ charge coupling, promoting osmotic swelling and endosome leak/lysis.
These results provide direct evidence in support of the proton sponge hypothesis.

Our fluorescence labeling strategy involved covalent coupling of Cl\(^{-}\) and pH-sensitive chromophores to polyamines for subsequent condensation with plasmid DNA. Because of quenching of the BAC chromophore upon direct conjugation to polyamines, the ratioable Cl\(^{-}\) sensor required coupling of BAC-labeled dextran with TMR-labeled polyamine. Control studies demonstrated stability of fluorescent polyplexes in cells, similar in vitro and in vivo Cl\(^{-}\) and pH sensitivities, absence of un conjugated polyamines, and comparable transfection efficiencies of fluorescent and unlabeled polyplexes.

Endosomes containing DNA polyplexes are remarkably greater in size than endosomes internalizing relatively small fluid-phase markers such as fluorescent dextran, or high affinity ligands such as transferrin and macroglubulin (6, 20). Internalization of large DNA polyplexes into macropinoctytic endosomes requires surface sialic acid (21) and thus appears to be a fundamentally different process than conventional fluid-phase or receptor-mediated endocytosis. Polyplex gene transfer protocols in cell cultures usually employ a charge excess of the cationic polymer such that non-complexed polymer also associates with cells. We found that, as for polyplexes, free polymer was efficiently internalized, and that endosomes containing free polymer had similar acidification kinetics and chloride accumulation as endosomes containing DNA polyplexes (not shown). It is not known whether the composition and membrane transport functions of polyplex-containing endosomes are different from those of endosomes generated by fluid-phase or receptor-mediated mechanisms. However, our results show that like their smaller counterparts, polyplex-containing macropinoctytic endosomes acidify utilizing a vascular-type H\(^{+}\) ATPase and electrogenic Cl\(^{-}\) shunt mechanism.

Our findings confirm previous conjectures of how PAM and PEI polyplexes mediate gene transfer in cultured cells (11, 12). Kichler et al. (13) showed that treatment of cells exposed to PEI polyplexes with bafilomycin A1 resulted in a 7–74-fold decrease in reporter gene expression. They concluded that the transfection efficiency of PEI is partially because of proton capture during endosomal acidification and that gene transfer occurs within 4 h of polyplex exposure. However, the role of endosomal acidification and the proton sponge hypothesis have been questioned. Godbey et al. (22) suggested that gene delivery does not proceed by means of the endosomal compartment, and Bieler et al. (23) suggested that gene transfer occurs from the lysosomal compartment and not the endosome. The temperature-jump protocol employed in the measurements here allowed us to examine in a synchronized manner early events that occurred within 75 min of exposure to the polyplexes. These early events are consistent with the time course of gene transfer, because gene expression is observed within 4 h after cell exposure to DNA polyplexes and peaks at ~24 h after exposure (13). Substantial Cl\(^{-}\) transport and acidification occur in the first 60 min of uptake and are concurrent with endosomal enlargement. The abrupt disappearance of fluorescence from PAM/PEI-containing but not POL-containing endosomes suggests that the endosome has ruptured, resulting in polyplex release into the cytoplasm. Furthermore, because DNA microinjected into the cytoplasm does not diffuse from the site of injection (24, 25), the disappearance of fluorescence also implies that the fluorescent polymer has dissociated from the DNA and diffused throughout the cell, making it unlikely that a polymer-DNA complex is transported from the site of release into the nucleus (26).

Our results provide the first direct confirmation that PAM/PEI buffers endosomal pH, increases chloride content, and produces endosomes that are more sensitive to osmotic stress. The fact that the fluorescence abruptly disappears has an additional implication for the design of non-viral vectors for efficient transgene delivery, suggesting the need to incorporate components into the polyplex to promote the transfer of the released DNA into the nucleus.

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