A fluorescent probe of polyamine transport accumulates into intracellular acidic vesicles via a two-step mechanism

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Running title: Vesicular Polyamine Uptake in Mammalian Cells
SUMMARY

Mammalian polyamine carriers have not yet been molecularly identified. The fluoroprobe Spd-C2-BODIPY faithfully reports polyamine transport and accumulates almost exclusively in polyamine-sequestering vesicles (PSVs). Polyamines might thus be imported first by a plasma membrane carrier and then sequestered into pre-existing PSVs (model A), or be directly captured by polyamine receptors undergoing endocytosis (model B). Spd-C2-BODIPY uptake was unaffected in receptor-mediated endocytosis-deficient Chinese hamster ovary (CHO) cell mutants. PSVs strongly colocalized with acidic vesicles of the late endocytic compartment and the trans Golgi. Virtually perfect colocalization between PSVs and acidic vesicles was found in CHO mutants that are blocked either in the late endosome/lysosome fusion process or in the maturation of multivesicular bodies. Prior inhibition of the V-ATPase dramatically decreased total Spd-C2-BODIPY accumulation while increasing cytosolic fluorescence. Conversely, cells pre-loaded with the probe slowly released it from PSVs upon V-ATPase inhibition. The present data thus support model A, and indicate that polyamine accumulation is primarily driven by the activity of a vesicular H⁺:polyamine carrier.
INTRODUCTION

Polyamines (putrescine, spermidine and spermine) are ubiquitous organic polycations that interact with anionic biomolecules (e.g. DNA, RNA, proteins and phospholipids) and are required in several cellular processes (1,2). Perhaps due to their pleiotropic action and their intrinsic toxicity (3,4), the biosynthesis, degradation and transport of these compounds are tightly regulated. Whereas the molecular biology of polyamine metabolism is known extensively (2), the identity of the mammalian polyamine transporter(s) is as yet unknown. The importance of polyamine uptake in mammalian tissues is illustrated by the dramatic actions exerted by exogenous polyamines on polyamine homeostasis. For instance, antizymes are critical regulators of polyamine pools due to their ability to inhibit both polyamine transport and ornithine decarboxylase, the rate-limiting enzyme for polyamine biosynthesis, and to target the latter for degradation (5). Quite unusually, antizyme depends on a +1 translational frameshift that narrowly responds to changes in polyamine levels. It thus constitutes an elegant chemostat for polyamine homeostasis, especially in the face of large fluctuations of extracellular polyamines (5). Furthermore, spermidine/spermine N⁴-acetyltransferase is induced by exogenous polyamines under situations where the total polyamine pool needs to be rapidly decreased (6).

Concentrations of internalized polyamines required for inducing antizyme or spermidine/spermine N⁴-acetyltransferase are disproportionately small as compared with the size of the endogenous pool (1). This suggests that internalized polyamines transit through a compartment from which the bulk of endogenous polyamines are excluded due to their retention in a form that has little access to cytosolic enzymes (1,7). In fungi, polyamines might be physically sequestered, at least in part, in vacuoles (8). Furthermore, ≥ 95% of spermidine and spermine has been postulated to exist as electrostatic complexes with nucleic acids,
phospholipids and ATP, based on in vitro determinations of the dissociation constants of polyamines in such complexes (9). However, there is currently no reliable methodology to directly assess the pool size of free vs. bound (or sequestered) polyamines in intact cells. Attempts to determine the intracellular distribution of polyamines have relied on invasive techniques such as immunohistochemistry (10–12) or subcellular fractionation (13). While some have reported the presence of polyamines in both cytoplasm and nucleus (12,13), others have found an almost exclusively cytoplasmic distribution (10,11). One problem inherent to such techniques is the redistribution of polyamines that can rapidly follow fixation or membrane disruption, due to their high positive charge (1).

Lack of molecular information about the mammalian polyamine carriers has hampered our understanding of the intracellular fate of internalized polyamines. The introduction of novel fluorescent probes such as N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl), N’-(S-[spermidine-{N⁴-ethyl}]-thioacetyl)ethylenediamine (Spd-C₂-BODIPY)¹ (14) or N⁴(1-[N-methyl]anthramidopropyl)spermidine (15) that behave like natural substrates toward the polyamine transporter(s) has helped to explore the virtually unknown details of intracellular polyamine trafficking. Interestingly, both probes accumulate mostly into intracellular vesicles (polyamine-sequestering vesicles, PSVs) and are excluded from both the cytosol and the nucleus, at least in interphase cells (14,15). Spd-C₂-BODIPY distribution partly overlaps with that of recycling endosomes (14), suggesting that one route of entry for polyamines is receptor-mediated endocytosis, as postulated for Fe⁢^³⁺ uptake (16) (Fig. 1, model B). Belting and coll. have proposed an analogous view whereby polyamines first bind glypican-1 at the exofacial side of the plasma membrane, and the complex is then internalized via caveolation and delivery of the polyamine to the cytosol via an unknown mechanism (17). However, the possibility that sequestration of polyamines into PSVs is a secondary event that follows their
initial influx via a "classical" plasma membrane transporter (Fig. 1, model A) could not be ruled out.

Using both genetic and biochemical approaches, we now provide strong evidence that polyamine transport is most likely initiated by a plasma membrane carrier and is rapidly followed by sequestration of the substrate into PSVs via an active mechanism that requires an outwardly directed H⁺ gradient, in accordance with model A. Vesicular sequestration is the rate-limiting step in the uptake of the polyamine probe. Our data further support the notion that polyamine uptake into PSVs can occur at various stages of the endocytosis pathway, but is especially active in the late endocytic compartment.
EXPERIMENTAL PROCEDURES

Reagents—Spd-C2-BODIPY was synthesized as previously described (14) and stock solutions were prepared at 1 mM in Me₂SO. The final Me₂SO concentration used in all experiments was 0.1% (v/v). Bovine calf serum was from Wisent Laboratories (St. Bruno, QC, Canada). Minimum essential medium with alpha modification (α-MEM) was purchased from Gibco BRL/Life Technologies (Burlington, QC, Canada). BODIPY® FL iodoacetamide, BODIPY® FL-transferrin, Texas Red-transferrin, BODIPY® TR ceramide, fluorescein-labeled dextran (anionic, \( M_r = 10,000 \)) and LysoTracker® Red (LTR) were from Molecular Probes (Eugene, OR). Bafilomycin A₁ was from Calbiochem (Darmstadt, Germany). Other biochemicals and cell culture reagents were obtained from Sigma.

Cell Lines and Tissue Culture—End1 Chinese hamster ovary (CHO) cell mutants (B3853) and their parental cell line WTB were generously provided by Dr. April Robbins (National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD). LEX1 and LEX2 CHO mutants and their cognate parental cells K1-WT were kindly provided by Dr. Masato Ohashi (Dept. of Molecular Physiology, National Institute for Physiological Sciences, Okazaki, Japan). Polyamine transport-deficient CHO mutants (CHO-MG) and their parental cell line (CHO-TOR) were a generous gift from Dr. Wayne Flintoff (University of Western Ontario, London, Ont., Canada). All cell lines were routinely grown in α-MEM supplemented with 10% bovine calf serum, 100 units of penicillin per ml, 100 µg of streptomycin per ml, 2 mM L-glutamine at pH 7.4 and 37°C (except for the End1 mutants which were grown at the permissive temperature of 34°C) in a H₂O-saturated, 5% CO₂ atmosphere.
Determination of Spd-C₂-BODIPY Transport in Endocytosis-Defective CHO cells by Flow Cytometry— End1 cells and their parental cell line were plated in 24-well culture plates and grown for 3-4 d. Cells were transferred to 40°C for 4 h to allow expression of the defective endocytosis phenotype. Cells were then incubated with 1 µM Spd-C₂-BODIPY for 1 h at 34°C in serum-free, supplemented α-MEM (i.e. α-MEM containing 100 units of penicillin per ml, 100 µg of streptomycin per ml and 2 mM L-glutamine), and then for an additional hour at 40°C. Parallel cell cultures were incubated with BODIPY FL-transferrin (5 µg/ml). Cells were washed three times with ice-cold PBS containing 1 mM spermidine, and then twice with ice-cold PBS. They were next collected after trypsinization, resuspended in ice-cold α-MEM and immediately processed for FACS analysis using an Epics Profile II cytofluorometer (Beckman Coulter Canada, Mississauga, ON, Canada) with an Ar laser tuned at 488 nm, using the FL1 photomultiplier (bandwidth pass: 525 = 30 nm).

Colocalization of Spd-C₂-BODIPY with Acidic Vesicles— Cells were plated on coverslips coated with UV-sterilized collagen 3 to 4 d prior to the experiment and were grown to ~ 80% confluence. Cells were then exposed for 1 h at 37°C to 1 µM Spd-C₂-BODIPY in serum-free, supplemented α-MEM. LTR (50 nM) was subsequently added to the cells for a further 15-min period. The latter conditions are optimal for labeling and for minimizing interference with intravesicular pH (18). Cells were then washed three times with ice-cold PBS containing 1 mM spermidine and twice with ice-cold PBS. Coverslips were then mounted on microslides and cells were observed by confocal laser scanning microscopy.
Staining the Golgi Complex in Living CHO-TOR Cells with BODIPY-Labelled Sphingolipids—To stain the Golgi in intact cells, BODIPY TR ceramide was added as a complex with BSA that was prepared as described (19). Cells grown on glass coverslips were rinsed three times with PBS. The cells were then incubated for 30 min at 4°C with 5 µM of the BODIPY TR ceramide/BSA solution. The samples were rinsed several times with ice-cold α-MEM and incubated in fresh medium containing 1 µM Spd-C2-BODIPY for a further 30-min period at 37°C, before being prepared for confocal laser scanning microscopy as above.

Effects of Bafilomycin A1 and Monensin on Spd-C2-BODIPY Distribution in CHO-TOR Cells—Cells grown on coverslips as above were pre-incubated with either 0.1 µM bafilomycin A1, 7 µM monensin or vehicle (Me2SO) for 1 h at 37°C. Cells were then exposed to 1 µM Spd-C2-BODIPY for 1 h in serum-free, supplemented α-MEM. LTR (50 nM) was added to the cells for an extra 15 min. In other experiments, cells were first labeled with Spd-C2-BODIPY and LTR as described above, and unincorporated probe was then washed out before incubating cells with 0.1 µM bafilomycin A1 for the indicated time interval before confocal laser scanning microscopy. The effect of bafilomycin A1 or monensin on fluid-phase endocytosis and on lysosomal integrity was monitored essentially as described (16). Briefly, cells were labeled for 1 h with 1 mg/ml of lysine-fixable, fluorescein-labeled dextran, rinsed with serum-free, supplemented α-MEM, and then incubated for 1 h with label-free medium, followed by a 2-h incubation ± 0.1 µM bafilomycin A1, and were then immediately processed for confocal laser scanning microscopy.

Confocal Laser Scanning Microscopy—Confocal laser scanning microscopy was performed with a BX-61 microscope equipped with the Fluoview SV500 imaging software (Olympus...
America Inc, Melville, NY), using 100X Plan-Apo oil immersion objective, (NA=1.35) and a 2X numerical zoom. Spd-C$_2$-BODIPY and LTR (or BODIPY TR ceramide) were excited sequentially at 488 nm using an Ar laser and at 543 nm using a Kr/Ar laser (set at 30-50% and 55 % of maximum power, respectively). Lasers were from the Melles Griot Laser Group, (Carlsbad, CA). Fluorescence emission from Spd-C$_2$-BODIPY and LTR (or BODIPY-TR ceramide) were recorded by photomultipliers preset respectively for EGFP (green pseudocolor) and TRITC (red pseudocolor) fluorescent dyes with the Fluoview SV500 imaging software . Thirteen 1-µm confocal z-series were acquired for each observation area and corrected by two Kalman low-speed scans. Acquired z-series images were exported in 24-bit TIFF format. For illustration purposes, the red, green and blue channels were subsequently separated from the original picture with Adobe Photoshop 7.0 software (Adobe Systems Inc., Seattle, WA).

**Image Analysis**— Colocalization analysis was performed using the Color Comparison Javascript plugin developed by William O'Connell (Dept. of Radiology, UCSF) with Image J software, i.e. the Java version of NIH Image developed by Wayne Rasband (Research Services Branch, National Institute of Mental Health, Bethesda, MD) at [http://rsb.info.nih.gov/ij](http://rsb.info.nih.gov/ij). Pearson's sample correlation factor ($R_c$) and the percentage of colocalization $p$ were thereby determined using three sets of representative red-green pictures from confocal laser scanning microscopy observations (20). $R_c$ takes into account the similarity of shapes between images, whereas the $p$ value for a color channel reports the intensity pattern. For instance, the $p$ value represented by red labeling was calculated as

$$ p = \frac{100 \times \text{green intensity}}{\text{green intensity} + \text{red intensity}} \quad (\text{Eq. 1}) $$

The mean and standard deviation of $R_c$ and of $p$ were subsequently determined.
RESULTS

Spd-C₂-BODIPY Uptake Does not Require Receptor-Mediated Endocytosis in CHO Cells—

Previous observations had indicated that within 1 h, CHO cells accumulate Spd-C₂-BODIPY mostly into discrete intracellular vesicles or PSVs (14). Since a subpopulation of the PSVs could be labeled with Texas Red-transferrin, a marker of recycling endosomes, this suggested that receptor-mediated endocytosis was, at least in part, an integral part of the polyamine uptake mechanism. To address that hypothesis, we used End1 CHO mutants that are strongly defective in receptor-mediated endocytosis and exhibit pleiotropic defects at the non-permissive temperature (40°C) (21). The latter include defective ATP-dependent acidification that mainly affects early endosomes and the TGN, with late endosomes and lysosomes being largely spared (21,22).

The uptake of BODIPY FL transferrin was higher in wild-type cells at 40°C than 34°C (Fig. 2A) as expected from the temperature dependence of endocytosis (23). At the permissive temperature (34°C), net uptake of BODIPY FL transferrin in End1 cells and wild-type cells was similar, but was strongly reduced in End1 cells upon transfer to 40°C, as expected from the known phenotype of these mutants (21). On the other hand, no difference was observed in the uptake of Spd-C₂-BODIPY between wild-type and End1 cells at either 34°C or 40°C (Fig. 2B). Thus, receptor-mediated endocytosis is clearly not the main pathway used for Spd-C₂-BODIPY uptake, and strongly argues against model B (cf. Fig. 1) as the major mechanism of polyamine accumulation.

PSVs Are Largely Represented by Strongly Acidic Vesicular Compartments—Because the perinuclear distribution and the heterogeneous size of PSVs are lysosomal characteristics, PSV
localization was compared with that of lysosomes and other strongly acidic compartments (e.g. late endosomes) using the acidotropic fluoroprobe LTR. As shown in Fig. 3, colocalization between LTR-stained vesicles (in red) and PSVs (in green) was 73 ± 3 % ($R_r=0.80 \pm 0.01$; $n=3$), indicating substantial overlap (yellow coarse-grained pattern). By comparison, colocalization between Spd-C$_2$-BODIPY and Texas Red-transferrin was 39 ± 16 % ($R_r=0.45 \pm 0.01$; $n=3$) (Fig. 3, lower left panel), indicating that Spd-C$_2$-BODIPY accumulation is quantitatively more important within the late endocytic pathway than in the recycling compartment.

Spd-C$_2$-BODIPY Is Partially Sequestered in the Trans Region of the Golgi— The TGN typically maintains a slightly acidic internal pH (~5.9) (24). Moreover, a number of organelle transporters, such as the P-type copper-transporting ATPases (25) and the zinc carrier ZnT7 (26) are expressed in the Golgi, including the TGN. Furthermore, amphipathic weak amines such as anthracyclines preferentially accumulate in the TGN and Golgi-derived vesicles (27).

Given the complex trafficking between the Golgi, the plasma membrane and various endocytic vesicles (28), we assessed whether the Golgi might participate in the accumulation of Spd-C$_2$-BODIPY. Cells were pre-loaded at 4°C with BODIPY TR ceramide, and then incubated with the polyamine probe upon transfer to 37°C. This temperature switch causes the selective accumulation of BODIPY-labeled ceramides into the Golgi (19). BODIPY TR ceramide stained juxtanuclear vesicles of various sizes as well as sac-like structures surrounding one pole of the nucleus (Fig. 4, middle column), as previously reported (29). Interestingly, although colocalization between BODIPY TR ceramide and Spd-C$_2$-BODIPY staining was only partial, it was mainly found in the extended saccular elements of the Golgi (Fig. 4, right column, arrows).

The latter results indicate a connection between the Golgi and the pathway of PSV biogenesis. However, the limited degree of overlap between Spd-C$_2$-BODIPY and BODIPY TR ceramide...
staining indicates that the Golgi is a minor site for polyamine accumulation and might correspond to a point of convergence with the late endocytic pathway (cf. "Discussion").

**Intravesicular Accumulation of Spd-C2-BODIPY Can Be Induced at an Early Step of the Late Endosome/Lysosome Degradative Pathway**— It was unclear whether PSVs correspond to lysosomes only since LTR is not specific for the latter organelles but also labels other acidic compartments such as late endosomes (18). Since attempts at fixing Spd-C2-BODIPY in situ for the immunocytochemical localization of specific organelle markers were unsuccessful, we used an indirect approach to determine which organelle subpopulation(s) of the late endocytic pathway is actually represented by PSVs.

LEX1 and LEX2 CHO mutants are defective in two different steps along the late endocytic pathway, namely in the fusion process between late endosomes and lysosomes, and in the maturation of multivesicular bodies (MVBs), respectively (30,31). The LEX2 mutation thus lies at an earlier step in the late endocytic pathway than the LEX1 lesion. To better assess the site(s) of Spd-C2-BODIPY accumulation among the various acidic organelles, we thus compared the pattern of PSVs and LTR staining observed in these two mutant cell lines and in wild-type CHO cells. In LEX1 cells, vesicles labeled by LTR had a larger average size and were less numerous than in control cells, and were characteristically distributed in a fusiform, perinuclear fashion, with frequent major aggregation near one pole of the nucleus (Fig. 5, center panel). These features confirm previous reports, and may correspond to the aggregation of fusion intermediates between late endosomes and lysosomes accumulating in these mutants, with a characteristic arrangement around the microtubule organizing centers (30). Remarkably, the LEX1 mutation did not prevent Spd-C2-BODIPY accumulation, and there was in fact an almost perfect colocalization of PSVs with LTR-positive organelles in LEX1 cells. Acidic organelles detected
in LEX2 cells were typically spherical and even larger than those found in LEX1 cells, and exhibited a perinuclear distribution but without the fusiform alignment typically observed in LEX1 mutants (Fig. 5, right panel). The larger, round acidic compartments found to accumulate in LEX2 cells are likely maturing MVBs, which are much scarcer in wild-type cells, and are a diagnostic feature of these mutants (31). Again, PSVs colocalized to a greater extent with acidic vesicular compartments in LEX2 cells than in control cells.

The marked enrichment in PSVs caused by either the LEX1 or LEX 2 mutation clearly suggests that accumulation of the polyamine probe can occur at an early step in the late endocytic pathway. Moreover, vesicular sequestering of Spd-C2-BODIPY represents a major activity of MVBs, late endosomes and lysosomes.

Both Accumulation and Sequestration of Spd-C2-BODIPY into PSVs Require an Outward Proton Gradient— We next assessed the model whereby polyamines are first transported via a 'classical' plasma membrane carrier, and then rapidly sequestered into acidic PSVs (Fig. 1, model A). We tested the assumption that dissipating the H+ gradient across the PSV membrane using either bafilomycin A1, a specific and potent inhibitor of the V-ATPase or monensin, a polyether ionophore catalyzing K+ (or Na+):H+ exchange, prior to loading cells with Spd-C2-BODIPY, would inhibit probe accumulation if its sequestration is tightly coupled to H+ efflux from the PSV. Indeed, pretreatment with bafilomycin A1 virtually abolished intravesicular acidification, while drastically decreasing intracellular accumulation of the spermidine probe (Fig. 6A, right panel). In fact, flow cytometry showed that total intracellular accumulation of Spd-C2-BODIPY was reduced by 97% by a 1-h preincubation with bafilomycin A1. Quite strikingly, the low intracellular staining with Spd-C2-BODIPY still present in bafilomycin-treated cells was clearly cytoplasmic and diffuse, in marked contrast with the vesicular distribution of the probe found in
control cells (Fig. 6A, left panel). Similar results were obtained with monensin (Fig. 6B). A 2-h incubation of CHO cells with bafilomycin A₁ did not disrupt endocytosis nor detectably affect the structural integrity of lysosomes, as shown by its lack of effect on fluorescein-conjugated dextran accumulation (data not shown).

These results strongly suggest that V-ATPase activity and an outwardly directed H⁺ gradient are required for the intracellular sequestration of Spd-C₂-BODIPY into PSVs. Importantly, although the initial uptake of Spd-C₂-BODIPY into the cytoplasm does not absolutely require V-ATPase activity, total cellular capacity for accumulation of the probe is largely regulated at the level of its H⁺-dependent sequestration into PSVs.

One well-established mechanism that accounts for the preferential sequestering of certain amines into acidic vesicles is simple diffusion of their free base form across the vesicular membrane followed by protonation of the amines in the lower pH environment and trapping of the resulting cationic form (32,33). However, the importance of such 'amine trapping' is negligible for polyamines such as spermidine or spermine (34) because the high pKₐs of their amino groups reduce the fraction of the molecules represented by the free base form to a negligible value at physiological pH (35). Nevertheless, Spd-C₂-BODIPY, with its large hydrophobic side chain, presents structural similarities with acidotropic mono- or oligoamines, such as LTR (cf. Fig. 1B), N-(3-[(2,4-dinitrophenyl)amino]propyl)-N-(3-aminopropyl)methylamine (36), or antimalarial quinolines (37).

Pulse-chase experiments were thus performed to assess the contribution of a lysosomotropic effect in the sequestration of Spd-C₂-BODIPY into PSVs. By loading CHO cells first with Spd-C₂-BODIPY and LTR and then treating labeled cells with bafilomycin A₁ in a polyamine-free medium, we could assess the dependence of probe sequestration on the ΔpH. As shown in Fig.
sequestration of both LTR and Spd-C2-BODIPY into PSVs was stable in the absence of extracellular probe and the vesicular labeling pattern and intensity was preserved with either amine for at least 90 min. As expected from a classical lysosomotropic amine, dissipating the H\(^+\) gradient rapidly abolished any detectable labeling with LTR within 30 min (Fig. 7, right). In sharp contrast, pre-labeling of PSVs with Spd-C2-BODIPY was much more resistant to vesicular alkalinization, although it progressively decreased over the 90-min chase period from a vesicular to a mostly diffuse, less intense cytosolic staining.

Thus, retention of Spd-C2-BODIPY into PSVs clearly depends on an intact ΔpH, but its rate of exit from both PSVs and from the cytoplasm is much slower than that observed for a lysosomotropic agent such as LTR, clearly indicating that Spd-C2-BODIPY accumulation into PSVs does not merely result from an acidotropic effect. The slow but steady release of Spd-C2-BODIPY from PSVs upon V-ATPase inhibition further suggests that intravesicular storage of the probe is an energy- and H\(^+\)-dependent process that counteracts a pathway for polyamine export from the PSVs.

**Lack of Polyamine Uptake Activity in CHO-MG Mutants Is Not Due to Defective Vesicular Acidification**—Since polyamine transport and sequestration into PSVs are strongly dependent on V-ATPase activity, the lack of polyamine transport activity found in CHO-MG cell mutants (38) might be explained by a major defect in the acidification of that compartment. We thus compared the ability of LTR to label vesicles in polyamine transport mutants and parental cells. As shown in Fig. 8, whereas we confirmed the inability of CHO-MG polyamine transport mutants to accumulate Spd-C2-BODIPY (14), there was no difference between parental and mutant cells in either the pattern or intensity of LTR staining. Clearly, the genetic lesion...
responsible for the polyamine transport defect in CHO-MG cells involves a component other than the \(H^+\) gradient required for PSV loading.
DISCUSSION

This report provides clear evidence that the primary step in the uptake of a polyamine probe does not involve binding to a plasma membrane receptor followed by endocytosis of the resulting complex, one of the two models previously proposed (14,17). On the other hand, PSVs are largely represented by the most acidic compartments of the cell. Since LTR behaves as an acidotropic amine, it labels lysosomes as well as other organelles such as late endosomes, but not early endosomes (39). Our current inability to properly fix Spd-C2-BODIPY for immunocytochemistry precludes a definitive identification of the nature of PSVs. Nevertheless, many of the larger LTR-positive vesicles that accumulate and co-localize exactly with Spd-C2-BODIPY in LEX1 and LEX2 mutants exhibited morphological features and a distribution fully consistent with their identity as late endosome/lysosome fusion intermediates (30) and maturing MVBs (31), respectively. Since both intermediates lie on the late endocytic pathway leading to the formation of late endosomes and mature lysosomes from MVBs (40), PSVs likely include all of the latter vesicle types.

The distribution of PSVs overlaps limited regions of the Golgi, and especially structures that likely correspond to the TGN. Polyamine accumulation inside the Golgi had not been previously reported, but is especially interesting in the light of reports that polyamine depletion markedly affects that structure (41). A preferential accumulation of Spd-C2-BODIPY in the TGN may reflect a targeted expression of the putative vesicular polyamine transporter. Alternatively, such compartmentalization might result from sorting and recycling of that carrier by the TGN from vesicles arising from the late endocytic compartment (42) or could be a mere consequence of the $\Delta pH$ dependence of vesicular polyamine transport, since the intracisternal pH of the TGN is $\sim 5.9$ (as compared with a luminal pH of 6.2-6.6 in the cis to medial regions of the Golgi) (43).
The remarkable degree of colocalization of PSVs with vesicles of the late endocytic compartment and the TGN suggests two possible mechanisms for the vesicular uptake of polyamines. First, the close association of Spd-C2-BODIPY labeling with the most acidic organelles might simply follow from a passive lysosomotropic effect, or "amine trapping" (32,33) by virtue of its properties as a triamine bearing a substantial hydrophobic moiety. According to that view, the uncharged form of Spd-C2-BODIPY would passively diffuse through the plasma membrane and then across the membrane of acidic vesicles, where it would be more or less sequestered after undergoing protonation as a function of the intravesicular pH. The amphipathic character of lysosomotropic amines tends to increase the diffusion coefficient of cell membranes toward the free base form of these compounds (44), as verified here in the case of LTR, which diffuses in and out of acidic vesicles and the cytosol within a few min.

However, it is clear that Spd-C2-BODIPY transport does not involve simple "amine trapping" since the probe cannot be accumulated in polyamine transport-deficient mutant despite normal labeling of acidic vesicles by LTR in these cells. Comparison between the structural features of LTR and Spd-C2-BODIPY (Fig. 1, B) clearly indicates that it is the \( N^4 \)-thioethylspermidine moiety of Spd-C2-BODIPY that must hinder its free diffusion across lipid bilayers. Thus, under physiological conditions, Spd-C2-BODIPY behaves very much like a polycation such as putrescine, spermidine and spermine, and does not undergo detectable acidotropic amine trapping by virtue of the low abundance of its uncharged form, and also presumably due to a low permeability coefficient to the latter molecular species.

The refractoriness of polyamine transport mutants to Spd-C2-BODIPY uptake, together with the effect of abolishing the \( \Delta \)pH across vesicular membranes on its sequestration, lead to a second, alternative model that can account for most features of Spd-C2-BODIPY accumulation.
This model (Fig. 9) predicts two consecutive transport steps for polyamine accumulation, in a fashion somewhat similar to monoamine uptake in neuronal cells. In the latter case, neurotransmitter amines released in the synaptic cleft, are (i) first transported across the plasma membrane by a reuptake symporter, and then (ii) concentrated into synaptic vesicles by H\(^{+}\)-coupled, vesicular monoamine transporters (VMATs) that function as 2H\(^{+}\)/monoamine antiporters belonging to the major facilitator superfamily (45,46). The free energy required for sequestering by VMATs is provided by the outwardly directed H\(^{+}\) gradient sustained by the activity of V-ATPases inserted in the vesicle membranes (47).

A similar paradigm can be derived for polyamine transport and compartmentalization. That the first step of polyamine uptake involves a "classical" plasma membrane carrier or channel rather than receptor-mediated endocytosis is supported by the behavior of End1 mutants toward Spd-C\(_{2}\)-BODIPY. Such a plasma membrane carrier does not require a H\(^{+}\) potential since V-ATPase inhibition blocked the vesicular sequestration of Spd-C\(_{2}\)-BODIPY while causing its cytosolic accumulation. Because the massive vesicular accumulation of the polyamine probe greatly lowers its steady-state cytosolic concentration, a plausible assumption is that the plasma membrane step of polyamine transport may well be a downhill process. This challenges the widely accepted view that polyamine uptake proceeds against an outward concentration gradient, but is in accordance with the hypothesis that the bulk of intracellular polyamines is thermodynamically inactive (9). In fact, most available evidence indicates that polyamine uptake is a Na\(^{+}\)-independent mechanism energized by an electronegative plasma membrane potential (48,49). Thus, polyamine uptake might be an electrogenic diffusion process facilitated by a channel or by a uniporter such as those belonging to the SLC22A family of organic cation transporters which includes polyspecific carriers that accept polyamines as substrates (50). The present model does by no mean exclude that heparan sulfates could facilitate the recruitment and
aggregation of polyamines at the proximity of their cognate plasma membrane carriers (Fig. 9) (17).

The present data strongly suggest that vesicular uptake of polyamines occurs secondarily to their initial uptake across the plasma membrane, and that it requires a H⁺ gradient as a free energy source (Fig. 9). The extreme steepness of the vesicle-to-cytosol Spd-C₂-BODIPY concentration gradient is suggested by the only very faint fluorescence detectable at any time in the cytosol under normal conditions (14), and likely reflects the high efficiency of the putative H⁺:polyamine antiporter that is likely involved in the massive polyamine accumulation into PSVs. In fact, experiments with bafilomycin A₁ and monensin lead to the important conclusion that the putative vesicular carrier catalyzes the major fraction of the total polyamine influx measured in intact cells. This is fully consistent with the notion that polyamine transport is under tight negative feedback regulation by antizymes that are induced at the translational level by increases in unsequestered polyamine levels (5). Thus, one potential function of PSVs might be to drive the uptake reaction by removing cytosolic polyamines, thereby preventing antizyme induction until some critical threshold of intracellular accumulation is reached.

The strong colocalization of PSVs with the most acidic compartments of the cell might reflect the requirement for a steep H⁺ gradient for polyamine accumulation, inasmuch as up to 4 positive charges (e.g. spermine) would be involved in polyamine capture by PSVs. Based on the stringent dependence of polyamine uptake by PSVs on the ΔpH, it is tempting to speculate that the vesicular polyamine transporter(s) might be structurally related to VMATs (46). Mammalian H⁺-dependent, non-acidotropic uptake activities of unknown biochemical identity have been described for the sequestering of various organic cations in lysosomes and other acidic vesicles (37,51). Interestingly, the presence of a H⁺ potential-dependent polyamine uptake activity similar
to that mediated by VMATs has been reported in isolated synaptic vesicles (52). This activity might be related or identical to that observed in non-neuronal cells as in this study.

The presence of PSVs among several types of vesicles belonging to the late endocytic pathway might reflect true heterogeneity among the hypothetical vesicular polyamine antiporters. Such heterogeneity indeed exists for subcellular carriers, such as the ZnT zinc facilitators, which include several related isoforms that are expressed in restricted subsets of endomembranes (26). Alternatively, it could be due to functional coupling between the H⁺:polyamine antiporters residing in those vesicles and the progressive decrease in pH observed along the pathway leading from MVBs (or sorting endosomes) to lysosomes (24). Such coupling has been reported to account for the tight association between the H⁺-dependent metal symporter DMT1 and transferrin receptors in the recycling compartment (53).

The present data also support the existence of an efflux system for exporting polyamines from PSVs back to the cytosol. As the polyamine probe was shown to be virtually impermeant to the plasma membrane, diffusion of net Spd-C₂-BODIPY out of PSVs is also very unlikely, and a transporter protein should be required for its extrusion. This vesicular polyamine exporter might be a uniporter or channel, although one cannot rule out that it is identical with the putative H⁺:polyamine antiporter catalyzing the transport reaction in the reverse direction.

In summary, the present data favor a model of polyamine uptake that proceeds in two tightly connected steps, namely [i] the initial transport of the substrate across the plasma membrane into the cytosol via a classical transporter that requires an electronegative membrane potential, rapidly followed by [ii] its sequestration in the PSVs in the late endocytic compartment, through a mechanism that likely involves H⁺ exchange since it requires the pH gradient sustained by V-ATPase activity. Vesicular polyamine accumulation is reversible, but polyamine export from
PSVs is a slow process and becomes only apparent following dissipation of the H\(^+\) gradient across the vesicular membrane.

The function(s) of PSVs could well be consistent with known but poorly understood features of polyamine homeostasis (5,7). One role of PSVs might be to provide cells with a readily available reservoir of polyamines for rapid cellular responses such as the gating of various ion channels (54), which would preclude induction of \textit{de novo} biosynthesis due to their short time frame and cyclical nature. PSVs might thus be envisioned as a segregation mechanism to coordinate the cytosolic level of polyamines with their cellular activity, by analogy with the role of the endoplasmic reticulum in the reversible mobilization of Ca\(^2\) (55). The efflux pathway would thus be activated for releasing sequestered polyamines from the PSVs according to cellular needs, through mechanisms that remain to be characterized but that likely involve the antizymes. A further role for PSVs might be related to the known cytotoxicity of excessive polyamine levels (3,4). Spermine and spermidine are typically present at millimolar levels in mammalian cells if assumed to be uniformly distributed throughout the cell volume (9). Polyamine concentrations of such magnitude in the cytosol would be expected to induce apoptosis (3,4,6).

The physical barrier afforded by endomembranes, together with the regulatory abilities that would be conferred by a vesicular storage system provide a novel paradigm to account for the apparent lack of thermodynamic activity of the bulk of intracellular polyamines (7,9). Clearly, vesicular accumulation as a mechanism for removing polyamines from the cytosol offers several mechanistic advantages over the alternative view that polyamines are passively sequestered through passive binding to nucleic acids and ATP (9). A model of compartmentalization of polyamines into endomembranes could help to explain the paradoxical co-existence of high endogenous polyamine levels with low antizyme titers and high ornithine decarboxylase enzyme activity in rapidly proliferating cells (5).
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REFERENCES


FOOTNOTES

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1The abbreviations used are: Spd-C_2-BODIPY®, N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl), N′-(S-[spermidine-\{N^4-ethyl\}]-thioacetyl)ethylenediamine; PSV, polyamine-sequestering vesicles; α-MEM, minimum essential medium with alpha modification; LTR, LysoTracker Red; CHO cells, Chinese hamster ovary cells; TGN, trans-Golgi network; MVBs, multivesicular bodies; V-ATPase, vacuolar H⁺-ATPase; VMAT, vesicular monoamine transporter.
FIGURE LEGENDS

Figure 1. Two possible models for intravesicular polyamine accumulation. In Model A (left), the polyamine (dark sphere) enters into the cytosol via a plasma membrane transporter, and most molecules are then sequestered into pre-existing intracellular vesicles (or polyamine sequestering vesicles) via a second, vesicular transporter (two-step capture), e.g. via a H⁺:polyamine antiporter similar to the VMATs found in synaptic vesicles (45). In Model B (right), the polyamine first binds to a putative plasma membrane receptor that undergoes endocytosis as a polyamine-receptor complex bound to an endosome (one-step capture). Acidification of the endosome by insertion of V-ATPases would favor dissociation of the latter complex, and promote export of the polyamine toward the cytosol, e.g. via a H⁺:polyamine symporter similar to the DMT1 metal transporter (16).

Figure 2. The uptake of Spd-C₂-BODIPY does not require receptor-mediated endocytosis. WTB (wild-type) and End1 (B3853) cells were preincubated at the permissive (34°C) or non-permissive temperature (40°C) for 4 h, and BODIPY FL-transferrin (5 µg/ml) (A) or Spd-C₂-BODIPY (1 µM) (B) was then added. Cells were collected after a 1-hour incubation with the respective probe, and mean fluorescence intensity was then determined by flow cytometry as described in 'Experimental Procedures'.

Figure 3. PSVs are largely composed of strongly acidic compartments. CHO cells grown on glass coverslips were incubated for 1 h with 1 µM Spd-C₂-BODIPY and for an extra 15-min period with 50 nM LTR. Spd-C₂-BODIPY fluorescence is shown in green (upper left panel) and
LTR fluorescence in red (upper middle panel). The superimposition of both fluorescence emission signals is shown on the upper right panel. The lower panel shows the colocalization analysis for simultaneous labeling with transferrin-Texas Red and Spd-C$_2$-BODIPY on the left scatter plot, and LysoTracker Red and Spd-C$_2$-BODIPY on the right plot. Rr is the value for the Pearson's correlation factor. Other details are provided in 'Material and Methods'.

Figure 4. **PSVs include trans regions of the Golgi complex.** CHO cells grown on glass coverslips were rinsed in HBSS/Hepes and then incubated for 30 min at 4°C with 5 μM of BODIPY TR ceramide–BSA in HBSS/Hepes. Cells were rinsed several times with ice-cold medium and incubated in fresh medium containing 1 μM Spd-C$_2$-BODIPY at 37°C for a further 30 min before confocal laser scanning microscopy. The Spd-C$_2$-BODIPY fluorescence is shown in green (left panels) and the BODIPY TR ceramide fluorescence in red (middle panels). The superimposition of both fluorescence emissions is shown on the right panels for each field observed. Each row represents a different representative field.

Figure 5. **The LEX1 and LEX2 mutations cause the accumulation of active PSVs: PSVs are components of the late endocytic compartment.** Wild type CHO (A), LEX1 (B) and LEX2 (C) mutant cells were incubated at 37°C for these experiments and labeled with Spd-C$_2$-BODIPY and LTR as described in Fig. 3. Spd-C$_2$-BODIPY and LTR fluorescence is shown in green (left columns of panels) and red (middle columns of panels), respectively. The superimposition of both fluorescence emissions is shown on the right columns of panels for each field observed and each cell line.
Figure 6. **Spd-C₂-BODIPY accumulation into PSVs requires a proton gradient.** Cells were pre-incubated for 1 h with either 0.1 μM bafilomycin A₁ (B) or 7 μM monensin (D), whereas cells were treated in parallel with vehicle only (0.1% Me₂SO, v/v) (A, C). One μM of Spd-C₂-BODIPY and was then added to the cells in the presence of either inhibitor, followed by 50 nM LTR (cf. Fig. 3). Spd-C₂-BODIPY and LTR fluorescence is shown in green (left columns of panels) and red (middle columns of panels), respectively. The superimposition of both fluorescence emissions is shown on the right columns of panels for each field observed. A zoomed picture is shown below each set of images to better assess the effect of the inhibitors on Spd-C₂-BODIPY compartmentalization.

Figure 7. **Long-term retention of the polyamine probe within PSVs requires a proton gradient.** Cells were pre-labeled for 1 h with 1 μM Spd-C₂-BODIPY followed by a 15-min incubation with LTR. The fluorescent probes were then removed and cell cultures were incubated for 0, 30, 60 and 90 min with 0.1 μM bafilomycin A₁ (B) or vehicle only (0.1% Me₂SO, v/v) (A). Spd-C₂-BODIPY and LTR fluorescence is shown in green (left columns of panels) and red (middle columns of panels), respectively. The superimposition of both fluorescence emissions is shown on the right columns of panels for each field observed.

Figure 8. **CHO-MG mutants defective in polyamine transport exhibit a normal pattern of intracellular acidic vesicles.** CHO-TOR and the polyamine transport mutants derived from these cells (CHO-MG) were labeled in parallel for 30 min with 1 μM Spd-C₂-BODIPY followed by a 15-min incubation with 50 nM LTR. Spd-C₂-BODIPY and LTR fluorescence is shown in
green (left rows of panels) and red (middle rows of panels), respectively. The superimposition of both fluorescence emissions is shown on the right row of panels for each field observed.

Figure 9. **General model for the two-step internalization of polyamines in mammalian cells**  Spd-C₂-BODIPY (and presumably natural polyamines) enters the cytosolic compartment via a membrane potential-dependent plasma membrane transporter or channel before being transported into PSVs. Glypican-1 may assist the initial binding of polyamines to the latter transporter. Polyamine internalization into these vesicles requires the protonmotive force maintained by the V-ATPase, and could be mediated by a polyamine/H⁺ antiporter similar to the VMATs of synaptic vesicles. PSVs coincide with acidic vesicles (MVBs, late endosomes, lysosomes, etc.) found in the late endocytic compartment. They may originate from the TGN and enter the intracellular vesicle trafficking pathway at the level of the sorting endosome, and/or be recycled back to the TGN along the late endocytic pathway. An efflux pathway is also present that allows the release of the sequestered polyamine back to the cytosol; this release is a relatively slow process as compared to polyamine sequestering and is observed upon alkalization of the PSV. Diamine exporters such as those described by Gerner and coll. (56) are also shown to illustrate the last arm of polyamine trafficking.
EQUATIONS

Eq. 1

\[ p = \frac{100 \times \text{(green intensity)}}{\text{(green intensity} + \text{red intensity})} \]
Figure 2

A  
BODIPY FL-transferrin UPTAKE

- WTB (Wild-Type)
- B3853 (End1)

![Bar chart comparing BODIPY FL-transferrin uptake at 34°C (Permissive Temperature) and 40°C (Non-Permissive Temperature) for WTB and B3853.]

B  
SPD-C₂-BODIPY UPTAKE

- WTB (Wild-Type)
- B3853 (End1)

![Bar chart comparing SPD-C₂-BODIPY uptake at 34°C (Permissive Temperature) and 40°C (Non-Permissive Temperature) for WTB and B3853.]

Figure 4

Golgi complex labeling

SPD-C₂-BODIPY  BODIPY TR Ceramide  Merge

10 μm
Figure 5

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Scale bar: 10 μm
Figure 8

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A fluorescent probe of polyamine transport accumulates into intracellular acidic vesicles via a two-step mechanism
Denis Soulet, Bruno Gagnon, Serge Rivest, Marie Audette and Richard Poulin

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