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Drug Induced Exocytosis of Glycogen from Cultured Pompe Skin Fibroblasts

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Key words

Pompe disease, exocytosis, glycogen, lysosomal storage disorder.

Author Contributions Statement

DB, JH, MF and PM conceived and supervised the study; CT, PJM, MF and DB designed experiments; CT performed experiments; CT, DB, JH, MF and PM analysed data; CT and DB wrote the manuscript; DB, JH, MF and PM made manuscript revisions.

Abbreviations

ERT	enzyme replacement therapy
TFEB	transcription factor EB
MPS	mucopolysaccharidosis
S-1-P	sphingosine-1-phosphate
LPC	lysophosphatidylcholine
PC	phosphatidylcholine
PMA	phorbol 12-myristate
LDH	lactate dehydrogenase
AA	arachidonic acid
EPA	eicosapentaenoic acid
LAMP-1	lysosomal associated membrane protein-1

Abstract

Pompe disease involves an α -glucosidase deficiency, which causes glycogen accumulation in the autolysosomes of patient cells. During cell division ~80% of stored glycogen was exocytosed from Pompe fibroblasts and this may be adapted as a strategy to relocate stored glycogen to a site where it can be degraded. Calcimycin induced exocytosis released 12% of the stored glycogen within two hours, but 80% of glycogen granules were too large to transit the exocytic pore. Drug induced exocytosis has potential for removing some glycogen storage from Pompe cells, but may need to be combined with enzyme replacement to effectively reduce glycogen load.

Introduction

Pompe disease is a progressive form of muscular dystrophy caused by a deficiency in the lysosomal enzyme α -glucosidase, which leads to glycogen accumulation in the autolysosomes of affected cells [1, 2]. Currently there is no effective cure for this lysosomal storage disorder, but clinical approval has been obtained for enzyme replacement therapy (ERT), which reduces the amount of glycogen in a number of affected tissues, including skeletal muscle and heart [3]; significantly reducing the morbidity and prolonging patient survival [4]. However, ERT is ineffective in some patients [3] and has a limited capacity to access glycogen storage in some important sites of pathology, including type II skeletal muscle [1, 5]. The inability of ERT to access glycogen stored in autolysosome compartments [6] warrants the development of novel or adjunct therapeutic options capable of more effectively treating the disease.

If glycogen was relocated from the autolysosome storage compartments of affected cells, into circulation, the excess glycogen could be degraded by other amylases; and this could be developed as an effective adjunct treatment strategy. Up to 80% of the stored glycogen can be exocytosed from cultured Pompe skin fibroblasts, within two hours, under certain culture conditions [7], suggesting that this therapeutic strategy is feasible. However, the culture conditions used to exocytose this large amount of glycogen corresponded to rapid cell division [7], and tissues that have high amounts of glycogen storage in Pompe patients like heart and muscle are terminally differentiated and undergo minimal cell division. The challenge is therefore to find a drug that is capable of exocytosing the glycogen without the need for extensive cell division.

Exocytosis of stored substrate from Pompe and other lysosomal storage disorder cells has been achieved with compounds capable of modulating the exocytic process or by over-expressing proteins involving the exocytic machinery. Kidney cells derived from metachromatic leucodystrophy patients treated with ionomycin demonstrated increased sulphatide release compared to untreated controls, with a concomitant 5-fold increase in β -hexosaminidase release [8]. In another study, the transcription factor EB (TFEB), a regulator of exocytosis, was over-expressed in cells derived from Pompe, multiple sulphatase deficiency, MPS IIIA and neuronal ceroidlipofuscinoses, leading to increased lysosomal exocytosis; as measured by extracellular lysosomal enzyme release and increased cell surface LAMP-1 staining [9]. Increased exocytosis led to reduced intracellular storage, ranging from 20% (MPS IIIA) to 60% (multiple sulphatase deficiency), compared to control cells. Other studies involving TFEB over-expression showed reduced glycogen storage in cultured muscle cells [10, 11] and Pompe mouse muscle [10]. It was hypothesized that glycogen exocytosis could be induced with compounds previously shown to increase exocytosis [12, 13].

Compounds that induce exocytosis in cultured cells include Ca^{2+} -dependent [14] or Ca^{2+} -independent [15] mechanisms. Ca^{2+} -dependent exocytosis releases soluble enzyme content from lysosomes [16] operating by increasing the cytosolic concentration of Ca^{2+} [13] by recruiting extracellular Ca^{2+} [14], liberating Ca^{2+} from organelle stores [17] or by increasing cytosolic cAMP concentration [18, 19]. Ca^{2+} -independent exocytosis is up-regulated in cultured cells in response to the induction of endocytosis [20], which can be achieved by increasing protein uptake at the cell surface [20, 21, 22] or by altering the specific lipid composition of the cell surface plasma membrane [15, 23]. Exposure to these compounds in culture is thought to lead to their incorporation into vesicular membranes, thereby changing membrane curvature to a more suitable configuration for vesicle-plasma membrane fusion [15], but may also alter membrane fluidity, ion channel permeability and membrane-bound enzyme activity [23].

The first aim here was to determine the amount of exocytosis and glycogen release from Pompe skin fibroblasts treated with modulators of both Ca^{2+} -dependent and Ca^{2+} -independent exocytosis. The second aim was to determine the size and amount of glycogen that can be released from Pompe cells, when treated with the most effective modulator of exocytosis identified from this set of compounds.

Materials and Methods

Cell culture

De-identified Pompe and MPS I skin fibroblasts were isolated from skin biopsies referred to the National Referral Laboratory for Lysosomal, Peroxisomal and Related Genetic Disorders (Women's and Children's Hospital, Adelaide, Australia) and used in accordance with Women's and Children's Human Research Ethics Committee approval 668/4/2009. Unaffected skin fibroblasts were derived from skin biopsies from apparently healthy volunteers. Each cell line had a similar growth rate and was sub-cultured less than nine times.

Cytoplasmic glycogen depletion

Cultured cells were washed twice with 12 mL of PBS, pH 7.2 (Sigma, St. Louis, USA), and cultured in FBS-free Basal Eagle medium (Sigma, St. Louis, USA) for 24 hours at 37°C. After discarding this culture medium, the cell monolayer was washed with 12 mL of PBS (three times) and cultured in glucose-free Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, USA) for 24 hours at 37°C.

Cell viability measurement

Cell viability was evaluated by 0.4% (v/v) trypan blue (Thermo Fisher Scientific, Waltham, USA) uptake and lactate dehydrogenase (TOX-7 kit; Sigma, St. Louis, USA) release as described previously [7]. Data were not collected from fibroblast cultures with <90% trypan blue exclusion and >5 µg/mg of total cell protein LDH release.

Immune-fluorescence

Cell surface and intracellular immune-fluorescence was performed on cultured skin fibroblasts as described previously [7].

Glycogen quantification

The mass spectrometry based glycogen assay has been described previously for the quantification of glycogen in culture medium (100 µL analysed per sample) and skin fibroblast extracts (0.1 µg cell protein made up to 10 µL in water analyzed per sample) [7].

β-Hexosaminidase and α-L-iduronidase activity

β-Hexosaminidase and α-L-iduronidase fluorometric assays were as described previously [24, 25].

Exocytic pore size determination assay

Pompe and unaffected cells were cultured to confluence in 6-well plates containing 1 mL/well DMEM, with 10% (v/v) FBS. Texas red-labeled flurospheres at 2.4 and 20 nm in diameter (Invitrogen, Carlsbad, USA) and Texas-red-labeled dextran beads at 40 and 100 nm (Sigma, St. Louis, USA) were added at 20 µg/mL to 1 mL/well fresh culture medium and incubated for 4 hr at 37°C, allowing beads to be endocytosed into the endosome-lysosome network. Non-internalised beads were removed using three sequential washes with PBS, pH 7.2. Cells were treated with 1 µM calcimycin for 2 hrs at 37°C. Exocytosed beads present in the culture medium were removed and cells were rinsed as before. Internalised beads were visualised with a Leica SP5 Spectral Scanning Confocal Microscope.

Glycogen extraction from cultured skin fibroblasts

The glycogen extraction method was adapted from Parker *et al.*, 2007 [26]. Two 75 cm² flasks of Pompe and unaffected cells were cultured to 3 weeks post-confluence and harvested. Cells were re-suspended in 1 mL of 0.25 M sucrose buffer, pH 7, containing 1 mM EDTA and 10 mM Hepes (4°C) and passed through a 23G needle to dissociate the cells. Cells were lysed by hypobaric shock ($\times$ 6) and centrifuged at 400 *g* to remove cell debris, but without breaking the intracellular vesicles. To lyse the intracellular vesicles present in the supernatant, 5 µL of lysis buffer, pH 7, containing 0.1% (v/v) NP-40 in 0.25 M sucrose buffer, was added to the supernatant and incubated for 10 mins at 4°C, before being layered above a step-wise sucrose gradient (25% (top), 50% and 75% (bottom; v/v)) and centrifuged at 300,000 *g* for 2 hrs at 4°C. Glycogen was decanted from the interface of each sucrose layer and the pellet (resuspended in 100 µL of Tris buffer). Glycogen samples were stored at -20°C until analysis.

Size evaluation of glycogen

The size of the glycogen granules extracted from cultured skin fibroblasts was determined using a method adapted from Parker *et al.*, 2007 [26]. Each grid was examined with a Philips CM100 SIS MegaviewII Image Capture Transmission Electron Microscope. The diameter of >600 individual glycogen granules was recorded per sample.

Electron microscopy of cultured skin fibroblasts

Pompe and unaffected cells were cultured to 3 weeks post-confluence and harvested. Cell pellets were fixed by immersion in 2% (v/v) paraformaldehyde/2.5% (v/v) glutaraldehyde, buffered in 0.2

M cacodylate, for 4 hrs at 4°C [27], washed three times in 0.1 M cacodylate (5 mins/wash) and then post-fixed in 2% (v/v) aqueous Osmium tetroxide/0.2 M cacodylate for 2 hrs at 4°C. After three further washes in 0.1 M cacodylate, cells were dehydrated by successive incubations with increased concentrations of ethanol (35, 50, 70, 95 and 100% (v/v)) and then propylene oxide for 15 mins at 20°C. Cells were embedded by incubation in a 1:1 ratio of propylene oxide/epoxy embedding medium for 12 hrs at 4°C, 100% epoxy embedding medium for 3 hrs at 20°C, then cured for 12 hrs at 60°C. Ultra-thin sections (70 nm) were cut, transferred to mesh collodion grids, stained with uranyl acetate for 2 hrs, then lead citrate for 5 mins at 20°C. Each grid was examined with a Philips CM100 SIS MegaviewII Image Capture Transmission Electron Microscope.

Statistical analysis

Differences between two independent groups of data with a normal distribution were determined by the student T-test, and the significance defined by a P value of <0.05. To compare three or more groups of data, analysis of variance (ANOVA) was utilised, with P-values adjusted by the Holm's Stepdown Bonferroni procedure. Statistical analysis was performed in consultation with Nancy Briggs (Data Management and Analysis Centre, Discipline of Public Health, University of Adelaide).

Results

Toxicity assessment of compounds on Pompe skin fibroblasts

Confluent Pompe cells were depleted of cytoplasmic glycogen (i.e. to contain predominantly vesicular glycogen) and treated with compounds to determine the threshold dose for toxicity, with the selected range based on previously published exocytosis studies (Table 1). Table 2 (Ca^{2+} -dependent modulators) and Table 3 (Ca^{2+} -independent modulators) show the concentration range for each compound that was evaluated. Cell viability did not change significantly in Pompe cells treated for two hours with S-1-P (0-80 μM), forskolin (0-200 μM), glucagon (0-100 μM), LPC (0-100 μM), PC (0-500 μM), PMA (0-1 μM) and α -L-iduronidase (0-1 $\mu\text{g/mL}$), compared to the untreated controls (>90% trypan blue exclusion and <5 $\mu\text{g/mg}$ LDH release). Pompe cells treated with high concentrations of the calcium ionophores, calcimycin (2 to 3 μM) and ionomycin (0.5 to 3 μM) and the fatty acids, AA (0.1 μM) and EPA (100 μM), showed a reduction in cell viability compared to untreated controls. However, at lower concentrations no significant differences were observed in cell viability compared to untreated controls. Non-toxic doses of each compound were evaluated for exocytosis and glycogen release, with the results for only the most effective concentrations of these compounds being presented (i.e. largest difference to untreated controls).

Drug induced glycogen and β -hexosaminidase release from cultured Pompe skin fibroblasts

Pompe cells treated with the cytosolic Ca^{2+} modulator calcimycin (1 μM) showed a significant increase in glycogen (3-fold; Fig. 1A) and β -hexosaminidase (2.2-fold; Fig. 1B) release into the culture medium compared to untreated controls ($P < 0.005$). However, other modulators of intracellular Ca^{2+} , including S-1-P, forskolin and PMA, displayed no difference in glycogen and β -hexosaminidase release compared to untreated controls. While there was no change in the amount of glycogen released from glucagon-treated Pompe cells (100 μM), there was a 2-fold increase in the amount of β -hexosaminidase released compared to untreated controls ($P = 0.002$).

The Ca^{2+} -independent modulators of exocytosis, LPC and α -L-iduronidase, resulted in increased β -hexosaminidase (1.8 and 1.5-fold respectively) and glycogen (1.8 and 1.4 -fold respectively) release from Pompe cells ($P < 0.005$). EPA-treated Pompe cells also showed increased release of β -hexosaminidase and glycogen (1.4-fold), but this was not significant due to the variability of the release, when compared to untreated controls. PC also did not result in a significant change in β -hexosaminidase or glycogen release in Pompe cells, when compared to untreated controls. In contrast, arachidonic acid showed a significant reduction in β -hexosaminidase and glycogen release compared to untreated controls

($P < 0.05$). Fig. 2 shows that $>90\%$ of the α -L-iduronidase in the culture medium was internalised into Pompe cells after a 1 hr incubation.

Calcimycin treatment induced exocytosis and glycogen release from cultured skin fibroblasts

The cytosolic Ca^{2+} modulator calcimycin induced the largest amount of glycogen release from Pompe cells and was examined further in a two hour time course on Pompe, MPS I and unaffected skin fibroblasts; to determine the amount of β -Hexosaminidase (nmol/min released into the culture medium per mg of total cell protein) and glycogen (μg released into the culture medium per mg of total cell protein) release, compared to cell surface LAMP-1 staining (alternative marker of exocytosis). A 2-fold increase in β -hexosaminidase release in calcimycin-treated cells was observed compared to untreated controls ($P < 0.005$; Fig. 3A, C, E). Calcimycin-treated Pompe and MPS I cells released more β -hexosaminidase (4 and 10 nmol/min/mg, respectively) after 2 hrs of culture than calcimycin-treated unaffected cells (2 nmol/min/mg). However, when this data was expressed as a percentage of total cellular β -hexosaminidase, a similar proportion of β -hexosaminidase was released in all cell lines (4% of total; $P < 0.005$; Fig. 3B, D, F).

There was no apparent difference between the number, the fluorescence intensity or the cell surface distribution of LAMP-1 punctae in untreated Pompe, MPS I and unaffected cells (Fig. 4A, B, C). Calcimycin-treated cells displayed a 2-fold increase in the fluorescence intensity of cell surface LAMP-1 punctae compared to untreated controls ($P < 0.005$; Fig. 4D, E, F). There was no apparent difference in the response to calcimycin for Pompe, MPS I and unaffected cells. In cells permeabilised by fixation, the intracellular LAMP-1 staining pattern was more extensive than from the cell surface staining of the same cells, with evidence of larger vesicular structures and DAPI nuclear staining (Fig. 4G, H, I).

The amount of vesicular glycogen in cytoplasmic glycogen-depleted cells was higher in Pompe (105 μg /mg of total cell protein) compared to MPS I (40 μg /mg of total cell protein) and unaffected cells (20 μg /mg of total cell protein). There was a significant increase in glycogen release from calcimycin-treated Pompe (2.7-fold), MPS I (3.1-fold) and unaffected cells (3.9-fold), compared to the untreated controls ($P < 0.005$; Fig. 5A, C, E). While Pompe cells released the most glycogen into the culture medium following two hours of calcimycin treatment, when expressed as a percentage of total cellular glycogen released, this was relatively low compared to MPS I and unaffected cells ($P < 0.005$; Fig. 5B, D, F). The glycogen release from cultured cells was not due to cell death, as there was no difference in the amount of LDH released into the culture medium or the exclusion of trypan blue ($>90\%$ trypan blue exclusion and $<5 \mu\text{g}$ /mg LDH release) following 1 μM calcimycin treatment.

There was no detectable difference in the diameter of calcimycin-induced exocytic pores in Pompe and unaffected skin fibroblasts

There was a significant increase in the release of 2.4 nm beads ($\geq 30\%$ increase) and 20 nm beads ($\geq 24\%$ increase) from calcimycin-treated Pompe and unaffected cells, compared to non-calcimycin-treated controls ($P < 0.005$; Fig. 6A, B). However, both 40 nm and 100 nm beads were not released from either Pompe or unaffected cells in response to calcimycin treatment. The uptake of beads and the calcimycin treatment did not increase cell permeability, as measured by the amount of LDH released into the culture medium ($< 5 \mu\text{g}/\text{mg}$).

Vesicular glycogen granules were larger in Pompe than unaffected skin fibroblasts

Glycogen granules were visualized as globular structures by transmission electron microscopy (Fig. 7) and this was confirmed by amylase digestion. Glycogen granules isolated from non-glycogen-depleted Pompe and unaffected cells had a similar size distribution (Table 4). The diameter of these granules ranged between 5 to 110 nm, but with a skewed distribution and a peak between 30 to 50 nm (Fig. 8). In contrast, the glycogen granules isolated from cytoplasmic glycogen-depleted Pompe cells were significantly larger than those from cytoplasmic glycogen-depleted unaffected cells ($P < 0.05$). In the unaffected cells 47% of the glycogen granules were smaller than 40 nm, while in Pompe cells only 33% were smaller than 40 nm (Fig. 9). Glycogen granules were visualized within vesicles of Pompe and unaffected cells, by transmission electron microscopy (Fig. 10), and in Pompe cells confirmed the presence of both intact glycogen granules and partially degraded material (Fig. 10C).

Discussion

Calcimycin is an ionophore that triggers Ca^{2+} ion influx into the cytosol of cells, and this compound increased the exocytosis of β -hexosaminidase and detection of LAMP-1 on the surface of Pompe, unaffected and MPS I skin fibroblasts. Calcimycin treatment of cultured basophils released ~22% of β -hexosaminidase into the culture medium [12], and for mast cells released ~15% of the β -hexosaminidase [13], which was 3-fold higher than observed here for fibroblasts. This difference in β -hexosaminidase exocytosis may be explained by basophils and mast cells acting as specialist immune secretory cells, which can easily trigger vesicular cargo release due to enhanced susceptibility to fluctuations in the cytosolic Ca^{2+} concentration, when compared to fibroblasts [16]. In addition, exocytosis in response to calcimycin treatment depends on the number of lysosomes that are trafficked to be in close proximity to the cell surface, and this ranges from 0% in HeLa cells to 24% in murine embryonic fibroblasts [16]. Despite the variability in exocytosis between different cell types, calcimycin treatment increased the amount of glycogen exocytosis from Pompe affected fibroblasts, indicating that drug induced exocytosis may be able to redirect some of the stored glycogen.

While calcimycin was the most effective compound for the induction of glycogen exocytosis in Pompe skin fibroblasts, the percentage of total glycogen released from calcimycin-treated Pompe cells (13% of total) was less than that released from calcimycin-treated MPS I (23% of total) and unaffected cells (27% of total). This suggested that there might be impaired glycogen exocytosis from Pompe skin fibroblasts, and this could be due to a defect in the exocytic machinery. However, calcimycin induced a similar increase in cell surface LAMP-1 staining and β -hexosaminidase release for Pompe, MPSI and unaffected fibroblasts, indicating that the mechanism of exocytosis *per se* was not impaired. The lysosomal hydrolysis of heparan sulphate is impaired in MPS I cells, but this also appeared to have some effect on the amount of glycogen released from these cells. Pompe disease involves a direct catalytic defect in glycogen hydrolysis and this resulted in large undegraded/partially degraded glycogen granules in the autolysosomes of Pompe cells, which might have limited the process of exocytosis. Consequently we investigated both the calcimycin-induced exocytic pore size generated in Pompe cells and the size of the vesicular glycogen granules in Pompe cells.

The diameter of the exocytic pore induced by cavicapture controls the amount of vesicular material that can be released by cells [28]. Here, the capacity to release fluorescent beads of a known size from the phago-lysosomal compartments of Pompe and unaffected skin fibroblasts was defined, and was used to infer the size of the exocytic pores formed. Both Pompe and unaffected cells appeared to have a calcimycin-induced exocytic pore diameter >20 nm but <40 nm, similar to that previously reported for fibroblasts (30 nm) [29]. This indicated that Pompe and unaffected cells generated similar pore sizes in

response to calcimycin treatment, but that the particle release size would be restricted to less than 40 nm by the limited pore opening due to the cavicapture mechanism.

Cytosolic glycogen granules in healthy cells range from 10 nm to 80 nm in diameter [26, 30], but the size of vesicular glycogen granules has not previously been defined. The mean diameter of glycogen granules from cytoplasmic glycogen-depleted Pompe cells was significantly greater than those from glycogen-depleted unaffected cells ($P < 0.05$). In unaffected cells 47% of the glycogen granules were smaller than 40 nm and potentially able to be released by calcimycin treatment whereas only 33% of the glycogen granules in Pompe cells were smaller than 40 nm. Interestingly, the glycogen granule size of non-depleted Pompe cells (~ 40% of the glycogen in the cytosol) and unaffected cells (~ 90% of the glycogen in the cytosol) was similar, indicating that the size difference was only evident in autolysosomes (vesicular glycogen) and directly attributed to the impaired α -glucosidase degradation process in Pompe cell autolysosomes. The increased diameter of vesicular glycogen granules in Pompe cells provided a plausible explanation for the reduced calcimycin-induced exocytosis of glycogen in Pompe compared to unaffected cells. The increased amount of glycogen within Pompe autolysosomes may also limit the amount of glycogen granules that can be exocytosed during a specific cavicapture event, but this remains to be defined.

Glucagon can modulate Ca^{2+} -dependent exocytosis, but unlike calcimycin, this relies on a cAMP-dependent pathway [31 dyachok]. As both glucagon and calcimycin induce Ca^{2+} -dependent exocytosis it was initially expected that these compounds would release the same or similar amounts of vesicular contents from Pompe skin fibroblasts. However, Pompe cells treated with glucagon showed increased β -hexosaminidase release, but no change in glycogen release. This may be explained by differences in specific concentrations of cytosolic Ca^{2+} induced by the two compounds. In addition, β -hexosaminidase is present in lysosomes [32], whilst vesicular glycogen is present in autophagic compartments and autolysosomes [33]. Consequently the higher cytosolic concentration of Ca^{2+} resulting from calcimycin treatment may induce the exocytosis of both autolysosomes and lysosomes, whereas the comparatively lower concentration of cytosolic Ca^{2+} associated with glucagon treatment may only exocytose lysosomes containing β -hexosaminidase. While this observation provided interesting insight into different mechanisms of exocytosis, it excluded the use of glucagon as a potential drug to induce the exocytosis of glycogen in Pompe patients.

The maintenance of the cell surface membrane area and curvature have been linked to Ca^{2+} -independent mechanisms of exocytosis [15, 20] and this identified a group of compounds that might also be used to exocytose glycogen from Pompe cells. LPC, arachidonic acid, EPA, and α -L-iduronidase each induce Ca^{2+} -independent exocytosis and induced glycogen and β -hexosaminidase release from Pompe

cells. A high concentration of LPC in vesicular membranes of chromaffin cells has been reported to give the vesicle an inverted cone shape, which facilitates vesicle formation and exocytosis [15]. Alteration in the curvature of the vesicular lipid bi-layer was not expected by the sequestration of PC, and consequently no change was observed in either glycogen or β -hexosaminidase release. Arachidonic acid treatment reduced β -hexosaminidase release, which has been previously reported in chromaffin cells. Arachidonic acid alters the curvature of the membrane in the opposite configuration to that observed for LPC, contributing to decreased membrane fusion and reduced exocytosis [15]. While the compounds that were expected to induce inverted cone shape membrane curvature induced glycogen exocytosis from Pompe cells the efficacy of this process appeared to be less than that observed for calcimycin induced Ca^{2+} -dependent exocytosis. A combination of lipids that modifies membrane curvature, together with Ca^{2+} -independent mechanisms may be used to induce effective exocytosis of glycogen from Pompe cells.

The endocytosis of protein results in a reduction in plasma membrane at the cell surface, inducing a corresponding Ca^{2+} -independent exocytic event necessitated by the cellular requirement to maintain the overall surface area of the cell [20]. Here, Pompe skin fibroblasts treated with α -L-iduronidase, a protein internalized by receptor-mediated endocytosis [34] and used as ERT for MPS I, showed a rapid intracellular uptake of this protein, with close to 100% removed from the culture medium after 2 hrs. In response, α -L-iduronidase treatment led to the induction of both glycogen and β -hexosaminidase release. Inducing endocytosis to alter the cell plasma membrane surface area is therefore a strategy to induce glycogen exocytosis in Pompe cells. Based on these observations, it is interesting to note that exocytic induction may be an additional benefit of ERT for Pompe and other lysosomal diseases.

In conclusion, glycogen release was induced from Pompe skin fibroblasts by modulators of both Ca^{2+} -dependent and Ca^{2+} -independent exocytosis. Calcimycin was the most effective compound, but a major proportion of the vesicular glycogen granules were too large to pass through the calcimycin-induced exocytic pores. To overcome this limitation, enzyme replacement therapy might be used in combination with drug induced exocytosis to reduce the size of the glycogen granules in Pompe cells and improve the efficacy of exocytosis. Alternatively, a combination of compounds able to stimulate both Ca^{2+} -dependent and Ca^{2+} -independent exocytosis may improve glycogen release. These combination therapies might provide an effective therapeutic strategy for Pompe patients.

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Tables and Figures

Table 1: Compounds for the stimulation of glycogen exocytosis in Pompe skin fibroblasts

Compound	Concentration Range (μM) ^a	Proposed Mechanism of Exocytic Induction
Calcimycin	0-10	Ionophore; Ca ²⁺ -dependent exocytosis [16]
Ionomycin	0-3	Ionophore; Ca ²⁺ -dependent exocytosis [14]
S-1-P	0-80	Ca ²⁺ -dependent exocytosis [17]
Forskolin	0-200	Modulator of cAMP; Ca ²⁺ -dependent exocytosis [35, 36]
Glucagon	0-100	Modulator of cAMP; Ca ²⁺ -dependent exocytosis [31]
PMA	0-1	Modulator of cAMP; Ca ²⁺ -dependent exocytosis [37]
EPA	0-500	Vesicle membrane curvature; Ca ²⁺ -independent exocytosis [23]
LPC	0-100	Vesicle membrane curvature; Ca ²⁺ -independent exocytosis [15]
AA	0-10	Vesicle membrane curvature; Ca ²⁺ -independent exocytosis [38]
α-L-iduronidase	0-1 μg/mL	Endocytosis; Ca ²⁺ -independent exocytosis [20, 39]

^aConcentration range selected from the literature.

Table 2: The viability of Pompe skin fibroblasts following treatment with modulators of Ca²⁺-dependent exocytosis

Compound	Concentration (μ M)	Trypan blue exclusion (% of viable cells) ^b	LDH release (μ g/mg) ^b	P value ^a
Calcimycin	0	92.3 $\pm$ 2.3	3.2 $\pm$ 1.1	-
	1	93.5 $\pm$ 0.5	3.2 $\pm$ 0.8	0.974
	2	90.7 $\pm$ 2.4	6.5 $\pm$ 1.7	0.048
	3	88.6 $\pm$ 2.7	7.7 $\pm$ 1.3	0.02
Ionomycin	0	94.4 $\pm$ 2.2	3.2 $\pm$ 0.9	-
	1	Cell death	Cell death	-
	2	Cell death	Cell death	-
	3	Cell death	Cell death	-
S-1-P	0	95.7 $\pm$ 2.7	2.7 $\pm$ 0.5	-
	0.1	91.7 $\pm$ 2	2.6 $\pm$ 0.8	0.863
	1	92.3 $\pm$ 2.4	2.4 $\pm$ 0.9	0.64
	10	91.7 $\pm$ 1.3	2.5 $\pm$ 0.3	0.584
Forskolin	0	95.6 $\pm$ 1.8	2.5 $\pm$ 0.3	-
	10	91.8 $\pm$ 2	2.6 $\pm$ 0.6	0.809
	50	93.3 $\pm$ 1.1	2.7 $\pm$ 0.9	0.733
	200	94 $\pm$ 0.8	2.8 $\pm$ 0.3	0.311
Glucagon	0	93.4 $\pm$ 1.8	2.6 $\pm$ 0.6	-
	100	92.1 $\pm$ 0.9	2.5 $\pm$ 0.8	0.933
PMA	0	92.3 $\pm$ 3.2	2.9 $\pm$ 0.6	-
	0.03	93.7 $\pm$ 1.5	2.8 $\pm$ 0.5	0.835
	0.3	92 $\pm$ 2.6	2.6 $\pm$ 0.4	0.511
	1	93.3 $\pm$ 1.5	2.8 $\pm$ 0.7	0.86

^aP-value calculated with the student's t-test.

^bResults are presented as mean $\pm$ one standard deviation (n = 3).

Table 3: The viability of Pompe skin fibroblasts following treatment with Ca²⁺-independent modulators of exocytosis

Compound	Concentration (μ M)	Trypan blue exclusion (% of viable cells) ^b	LDH release (μ g/mg)	P value ^a
AA	0	91.3 $\pm$ 2.5	3.3 $\pm$ 0.6	-
	0.002	91.2 $\pm$ 3.5	3.2 $\pm$ 1	0.833
	0.01	91.3 $\pm$ 2.1	3.3 $\pm$ 0.3	1
	0.1	77.7 $\pm$ 2.1	12.7 $\pm$ 2.2	0.002
EPA	0	90.5 $\pm$ 0.7	2.9 $\pm$ 0.8	-
	20	92.5 $\pm$ 1.6	3.1 $\pm$ 0.8	0.775
	50	93.1 $\pm$ 2.1	3.2 $\pm$ 0.6	0.631
	100	75.3 $\pm$ 4.7	13.3 $\pm$ 2.1	0.001
LPC	0	92.5 $\pm$ 0.5	3.2 $\pm$ 0.6	-
	1	91.6 $\pm$ 0.5	3.0 $\pm$ 0.8	0.893
	2	93.6 $\pm$ 2.1	3.3 $\pm$ 0.4	0.848
	10	91.8 $\pm$ 0.7	3.5 $\pm$ 0.3	0.764
PC	0	94.1 $\pm$ 1.8	2.4 $\pm$ 0.7	-
	1	91.9 $\pm$ 1.1	2.5 $\pm$ 0.5	0.726
	5	94.1 $\pm$ 1.9	2.9 $\pm$ 0.7	0.353
	10	93.3 $\pm$ 2.6	2.2 $\pm$ 0.7	0.932
Idua ^c	0	94.1 $\pm$ 1.8	3.4 $\pm$ 0.7	-
	9 ng/mL	92.3 $\pm$ 2.4	3.3 $\pm$ 0.3	0.831
	72 ng/mL	93.1 $\pm$ 2.1	3.6 $\pm$ 0.5	0.708
	144 ng/mL	93.4 $\pm$ 1.8	3.5 $\pm$ 0.6	0.815
	1 μ g/mL	93.5 $\pm$ 0.5	3.3 $\pm$ 0.5	0.85

^aP-value calculated with the student's t-test.

^bResults are presented as mean $\pm$ one standard deviation (n = 3).

^c α -L-Iduronidase

Table 4: The mean diameter of glycogen extracted from skin fibroblasts

Glycogen source	n	Mean diameter (nm)	Standard deviation	95% confidence	P-value
Pompe	1328	46.5	16.9	43.1 – 50.9	P = 0.63 ^b
Unaffected	1415	47.7	18.5	44.3 – 51.3	
Depleted Pompe ^a	1630	50.8	21.1	47.3 – 55.1	P < 0.05 ^c
Depleted unaffected ^a	610	46.1	18.2	41.8 – 49.6	

^aCells depleted of cytosolic glycogen (contain predominantly vesicular glycogen).

^bThe statistical difference in glycogen size between Pompe and unaffected skin fibroblasts that were non-depleted of cytosolic glycogen (student's t-test).

^cThe statistical difference in glycogen size between Pompe and unaffected skin fibroblasts that were depleted of cytosolic glycogen (student's t-test).

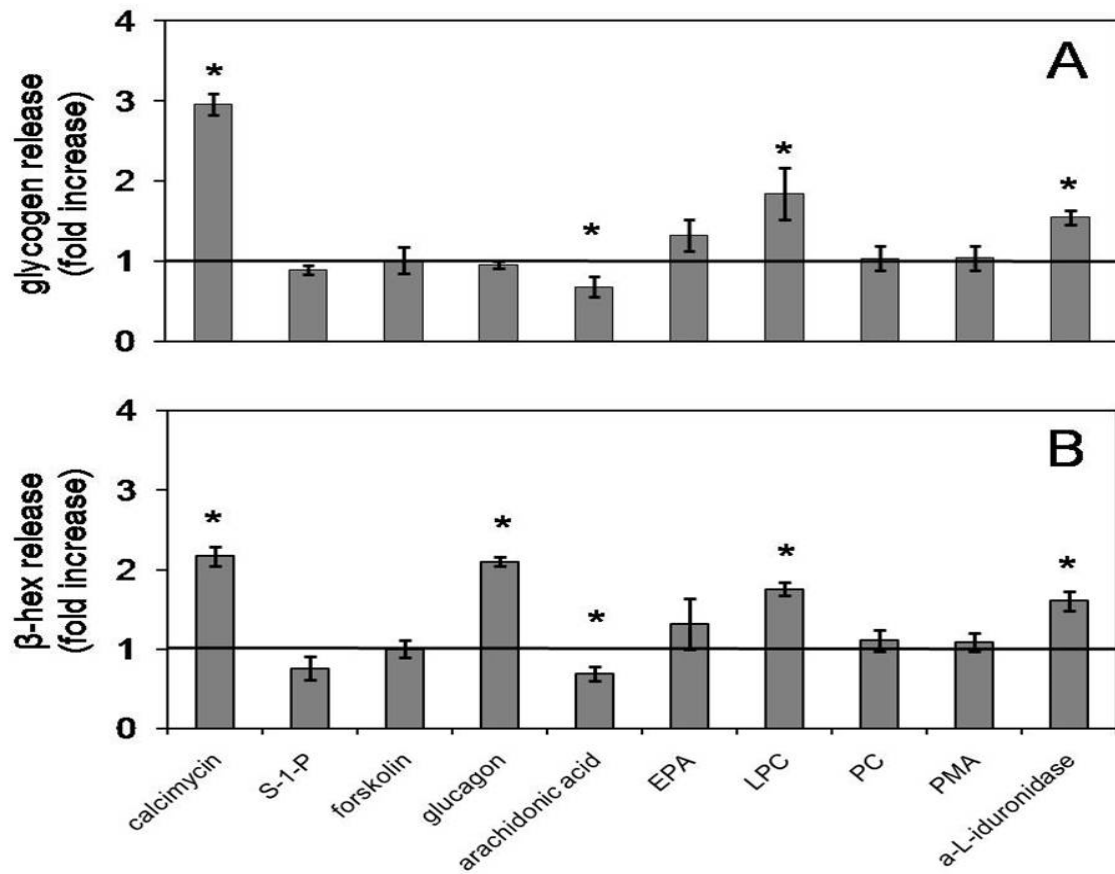


Figure 1: The effect of compounds on exocytosis in Pompe skin fibroblasts. The release of glycogen (A), and β -hexosaminidase (β -hex; B) was measured in Pompe cells depleted of cytoplasmic glycogen. Cells were treated with 1 μ M of calcimycin, 10 μ M of S-1-P, 200 μ M of forskolin, 100 μ M of glucagon, 0.1 μ M of arachidonic acid, 50 μ M of EPA, 2 μ M of LPC, 1 μ M of PC (negative control), 1 μ M of PMA and 1 μ g/mL of α -L-iduronidase for 2 hrs at 37°C. Results are expressed as fold increase in the extracellular release of β -hex or glycogen (percentage of total released into the culture medium), when compared to untreated controls (mean $\pm$ SD; n = 3). *Significance, when compared to untreated controls (P < 0.05).

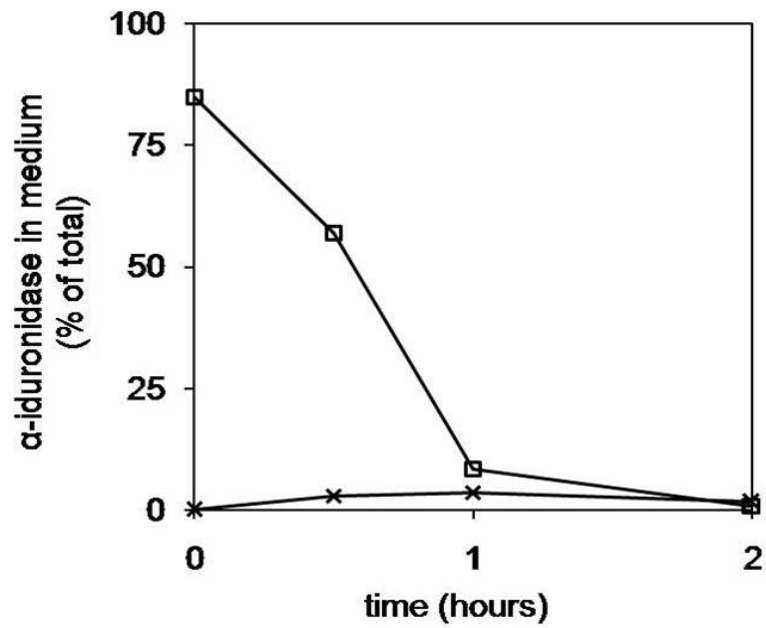


Figure 2: The uptake of α -L-iduronidase into cultured Pompe skin fibroblasts. Glycogen-depleted Pompe cells were cultured with α -L-iduronidase for 2 hrs at 37°C. Cells were treated with 1 μ g/mL of α -L-iduronidase ($\square$) or were untreated ($\times$). Results are expressed as the total percentage of α -L-iduronidase in the culture medium of Pompe cells (mean $\pm$ SD; n = 3).

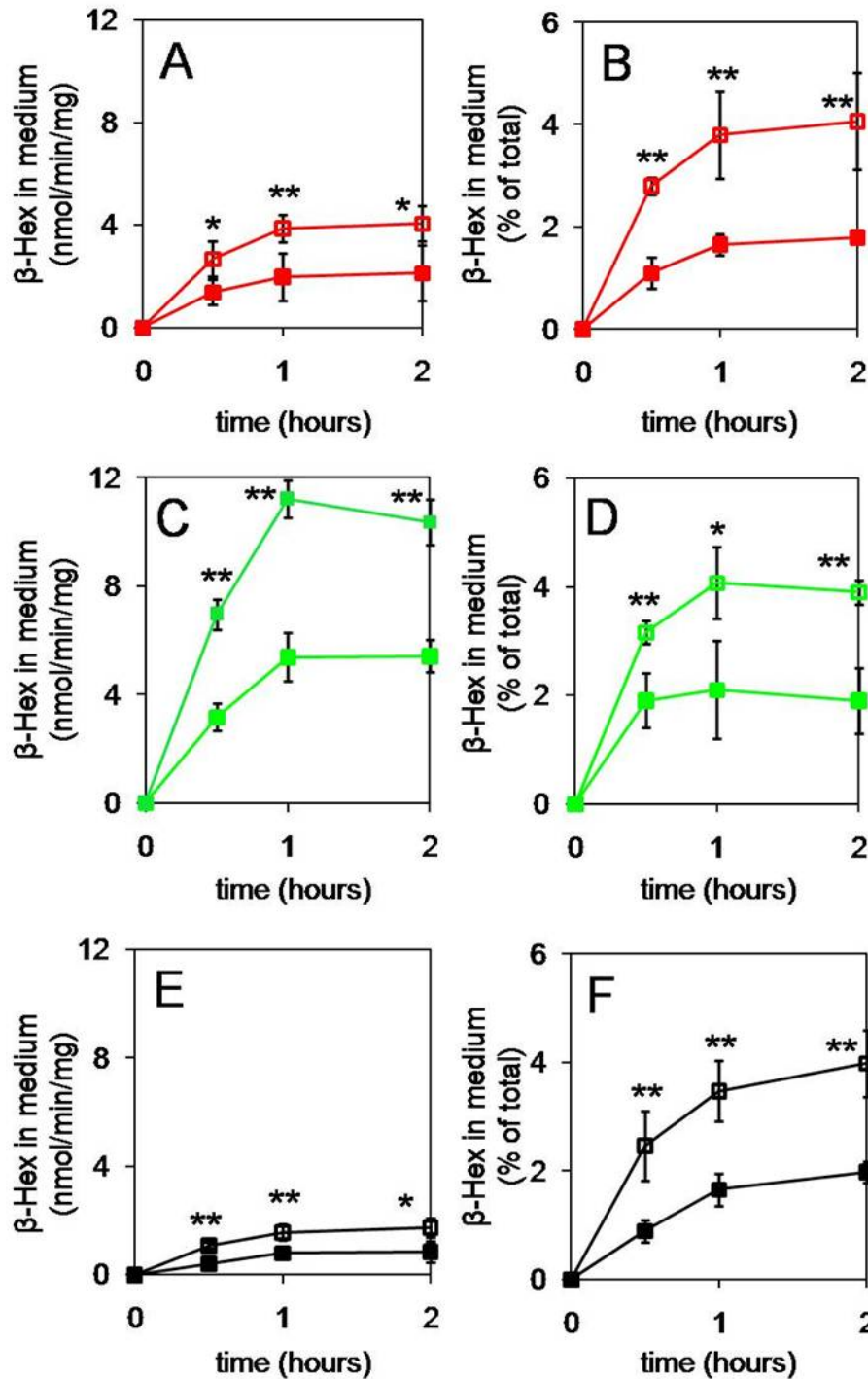


Figure 3: The effect of calcimycin on the release of β -hexosaminidase from skin fibroblasts. Glycogen-depleted Pompe (red), MPS I (green) and unaffected (black) cells were treated with 1 μ M of calcimycin for 2 hrs at 37°C. Results are expressed as nmol/min of β -hexosaminidase per mg of total cell protein released into the culture medium (β -hex; A, C and E) or as the percentage of total β -hex (B, D and F) in the culture medium (mean $\pm$ SD; n = 3). Calcimycin treated (□). Untreated (■). *Significance P < 0.05 when compared to untreated controls. **Significance P < 0.005 when compared to untreated controls.

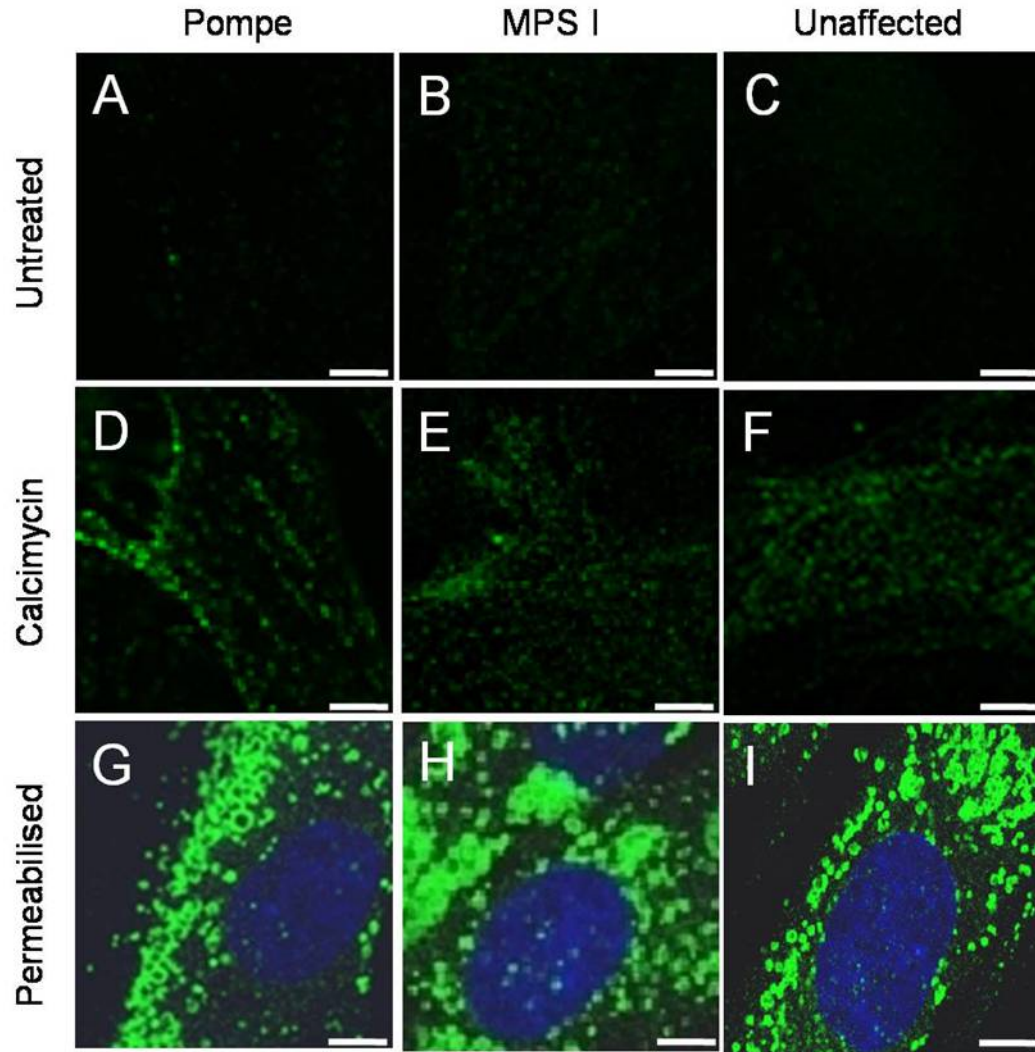


Figure 4: The effect of calcimycin on LAMP-1 staining of skin fibroblasts. Cell surface LAMP-1 fluorescence was evaluated in non-permeabilised untreated Pompe (A), MPS I (B) and unaffected (C) cells, and Pompe (D), MPS I (E) and unaffected (F) cells treated with 1 μ M of calcimycin for 10 mins at 37°C. Intracellular LAMP-1 fluorescence was also measured in Pompe cells (G), MPS I (H) and unaffected (I) cells that were permeabilised by fixation. Cells were glycogen-depleted prior to experimentation. Images are an overlay of LAMP-1 fluorescence (green) and DAPI nuclear stain (blue). Each image is representative of greater than 20 images with each experiment performed in triplicate. Size bar equals 5 μ m.

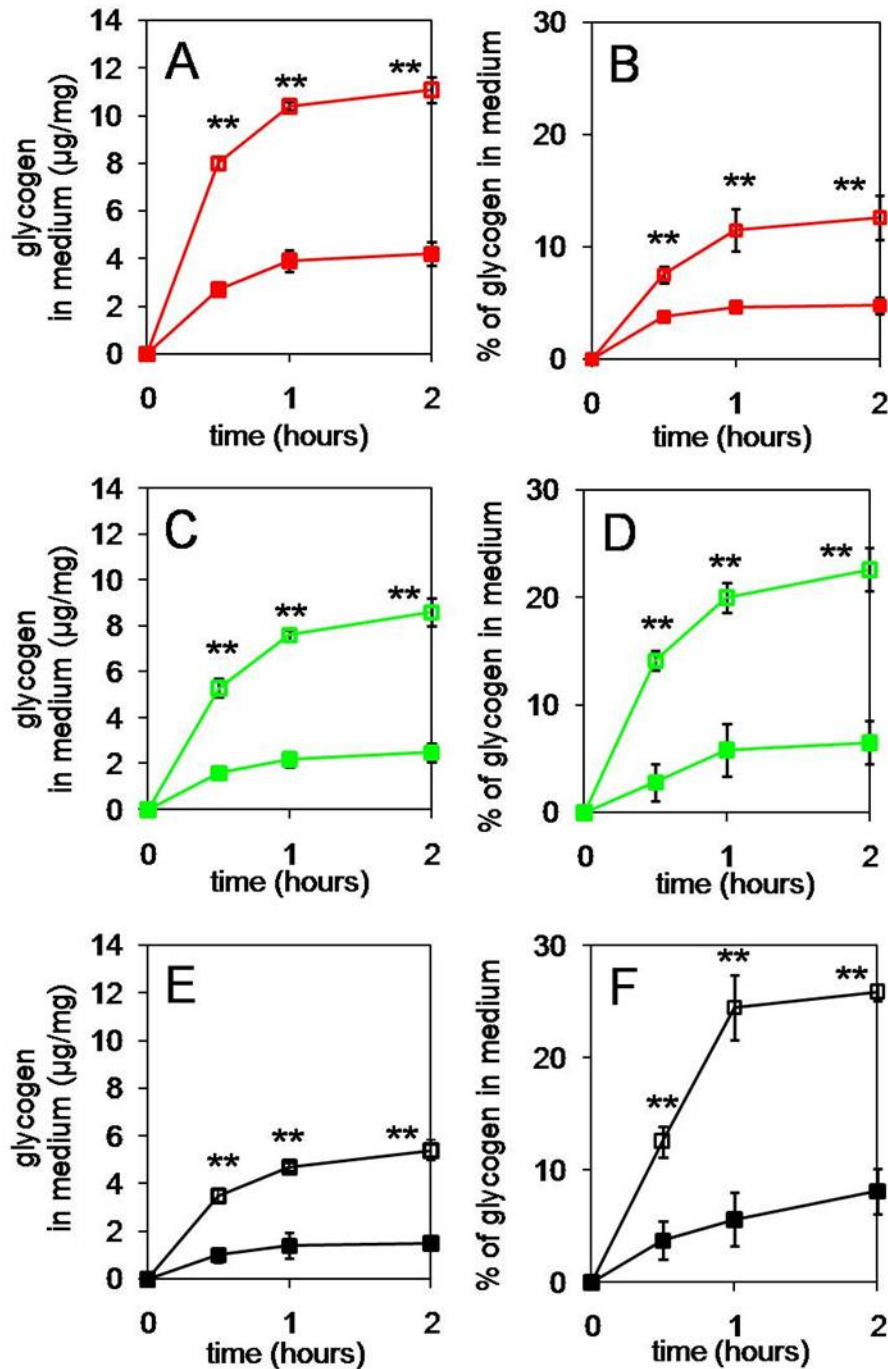


Figure 5: The effect of calcimycin on the release of glycogen from skin fibroblasts. Glycogen-depleted Pompe (red), MPS I (green) and unaffected (black) cells were treated with 1 μ M of calcimycin for 2 hrs at 37°C. Results are expressed as μ g of glycogen per mg of total cell protein in the culture medium (A, C and E) or as the percentage of total cell glycogen released into the culture medium (B, D and F; mean $\pm$ SD; n = 3). All samples were measured in triplicate and the whole experiment was performed three times. Calcimycin-treated ($\square$). Untreated ($\blacksquare$). *Significance P < 0.05 when compared to untreated controls. **Significance P < 0.005 when compared to untreated controls.

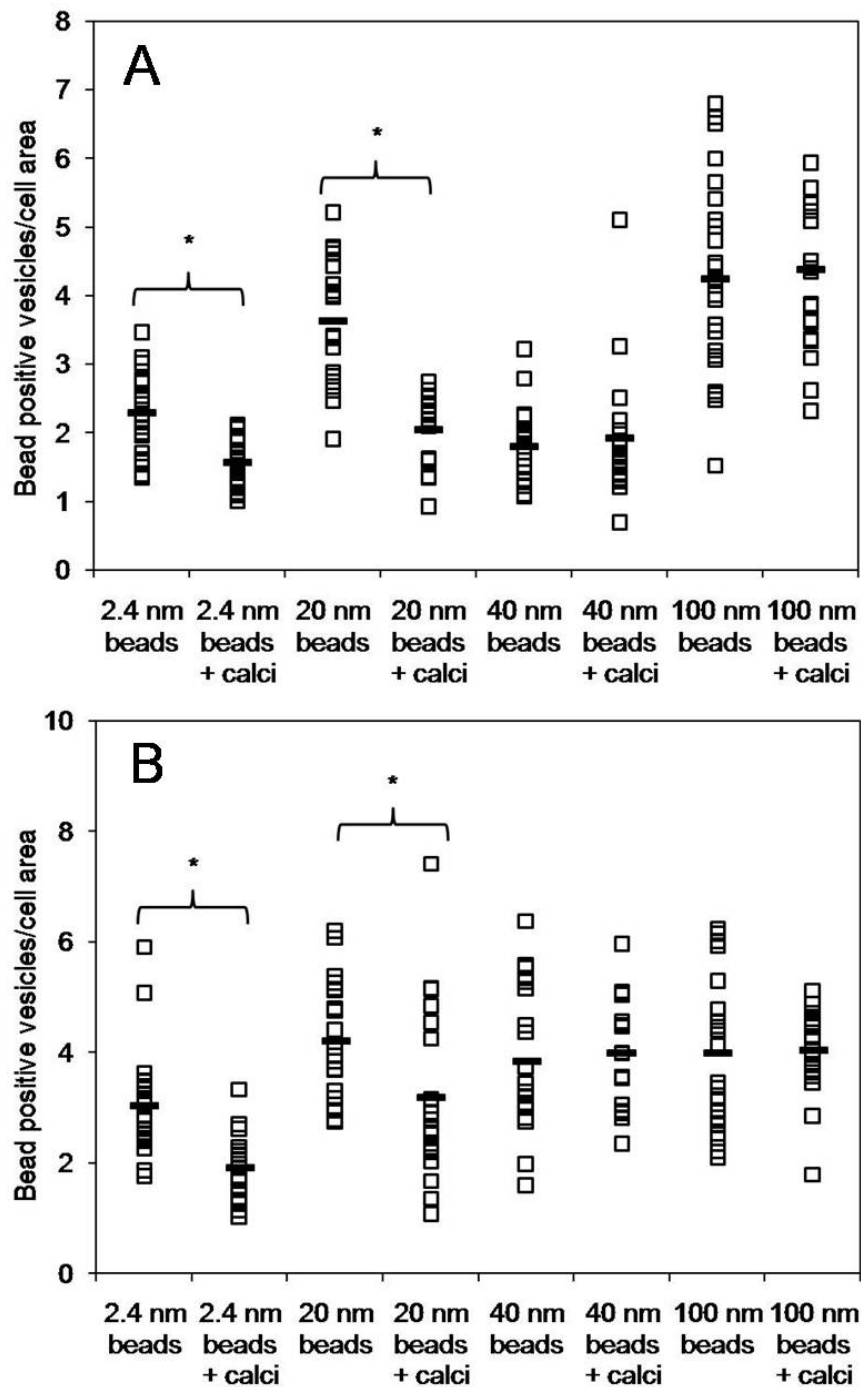


Figure 6: The release of fluorescent beads from calcimycin-treated skin fibroblasts. Pompe (A) and unaffected (B) cells were incubated with fluorescent beads (2.4, 20, 40 and 100 nm in diameter) to allow internalization into the endosome-lysosome network. Cells were calcimycin-treated and the amount of bead exocytosis was measured and then compared to untreated controls. Results are presented as the number of bead-positive vesicles per cell area (i.e. indicates the relative number of beads that could not

be released from the cell). Greater than 20 cells were evaluated per treatment group. *Significance $P < 0.05$.

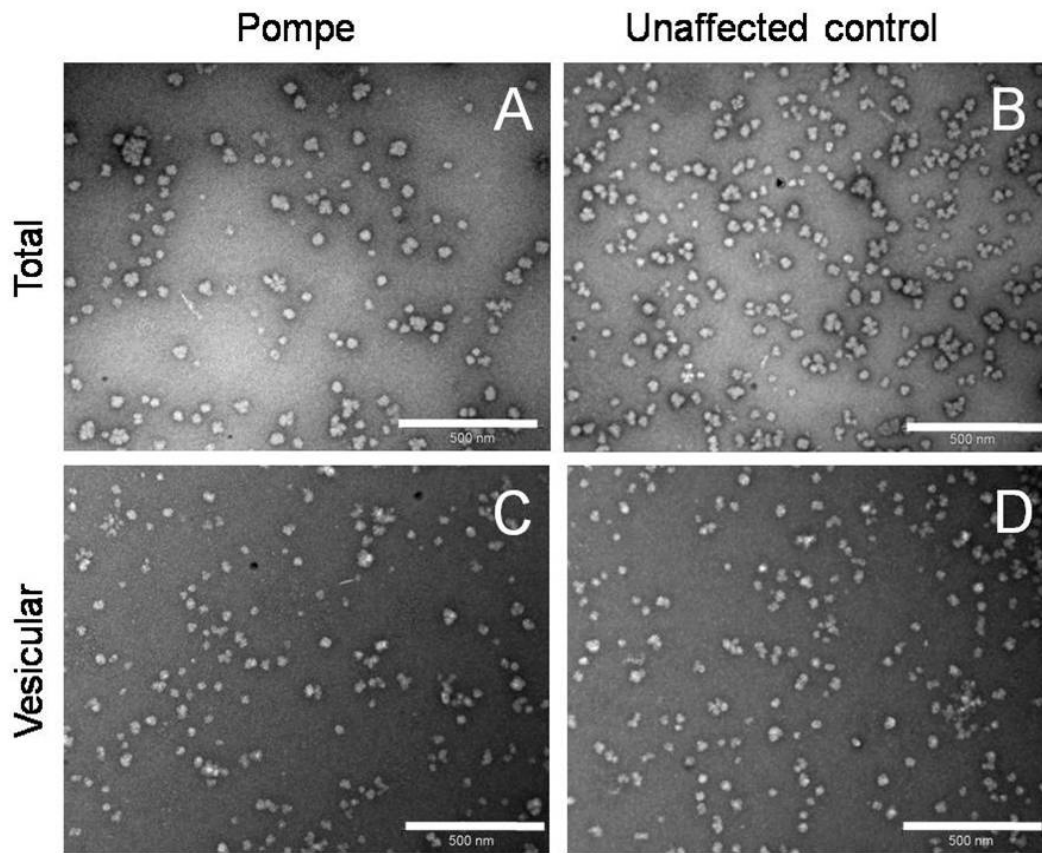


Figure 7: Electron microscopic visualization of purified glycogen granules from skin fibroblasts. Transmission electron microscopy images were captured of glycogen purified from non-glycogen-depleted (containing vesicular and cytosolic glycogen) Pompe (A) and unaffected (B) cells. Glycogen granules purified from depleted (containing predominantly vesicular glycogen) Pompe and unaffected cells are shown in panels (C) and (D). All images are representative for each cell line or culture condition (>40 images were evaluated for each group). Size bar equals 500 nm.

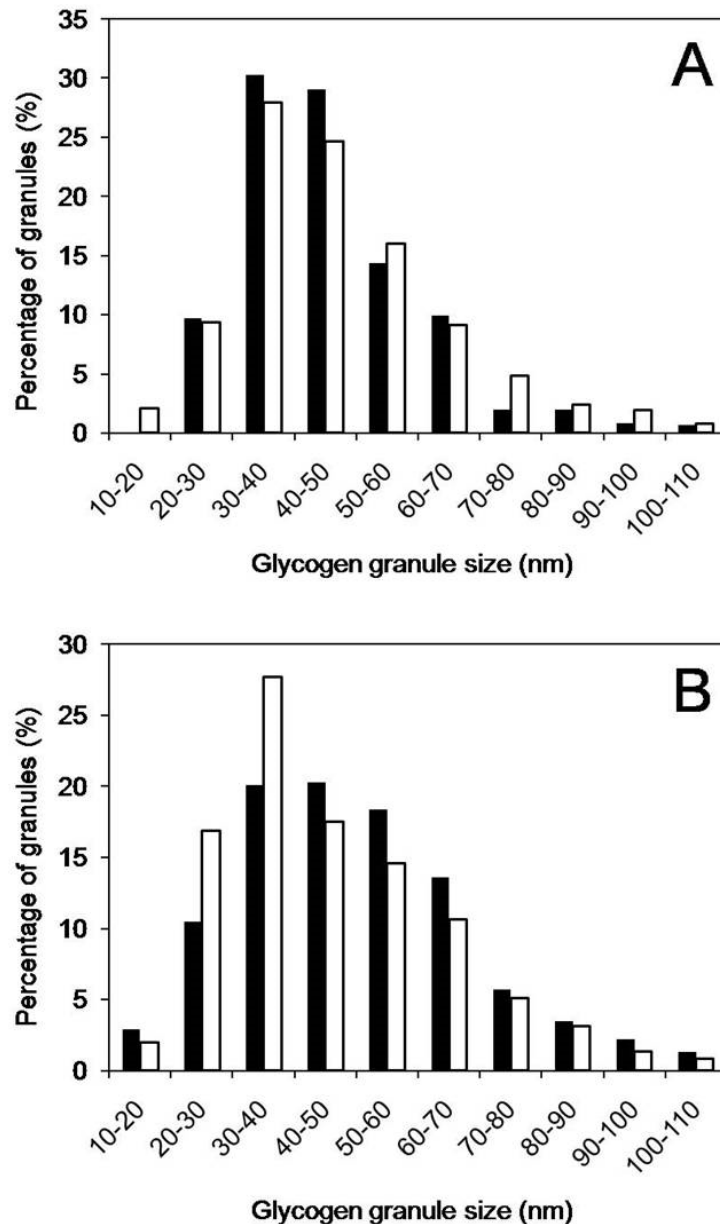


Figure 8: The size of glycogen granules isolated from skin fibroblasts. The diameter of glycogen granules purified from Pompe and unaffected fibroblasts was determined and presented as the percentage of glycogen granules within each size range (10 nm divisions). The diameter of glycogen granules purified from non-glycogen-depleted Pompe (filled bars) and non-depleted unaffected (open bars) fibroblasts are shown in panel (A). The diameter of glycogen granules purified from depleted Pompe (filled bars) and depleted unaffected (open bars) cells are shown in panel (B). The diameter of >800 glycogen granules was evaluated for each group.

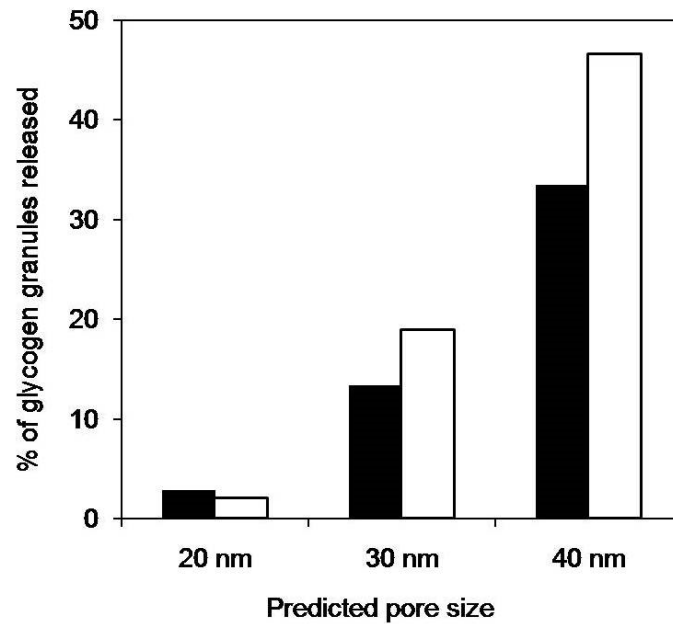


Figure 9: The predicted theoretical maximum number of glycogen granules to be exocytosed from calcimycin-treated skin fibroblasts. The percentage of glycogen granules small enough to pass through calcimycin-induced exocytic pores in Pompe (filled bars) and unaffected (open bars) cells. The pore sizes of 20, 30 and 40 nm were based on the exocytic release of dextran beads from skin fibroblasts (see Fig. 6).

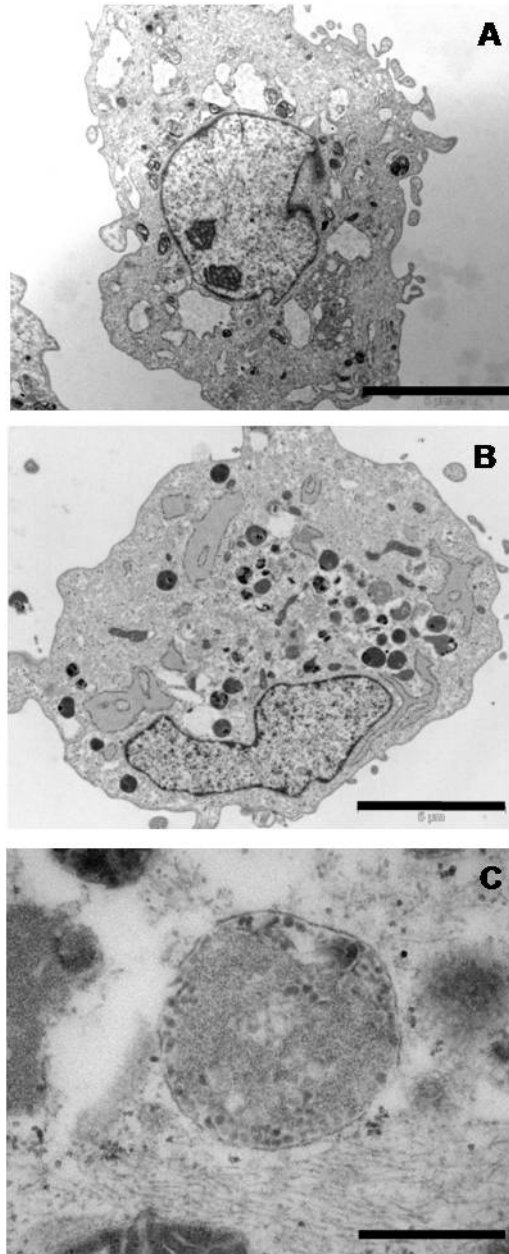


Figure 10: Glycogen accumulation in a Pompe skin fibroblast cell. Electron micrographs of a human unaffected control (A) and Pompe (B) skin fibroblast. Glycogen-filled vesicle inside a Pompe skin fibroblast cell (C). Glycogen can be visualised as electron-dense vacuoles in panels B and C. Size bar is equivalent to 5 μm for A and B and 300 nm for panel C.