Diltiazem, a L-type Ca²⁺ channel blocker, also acts as a pharmacological chaperone in Gaucher patient cells

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Abstract

Recently, inhibition of L-type Ca²⁺ channels, using either Diltiazem or Verapamil, has been reported to partially restore mutant glucocerebrosidase activity in cells from patients with Gaucher disease homozygous for the N370S or L444P alleles, as well as cells from patients with two other lysosomal storage diseases. It was hypothesized that these drugs act on the endoplasmic reticulum, increasing its folding efficiency, inhibited due to altered calcium homeostasis. Several other laboratories have reported that cells carrying either the N370S or the F213I alleles are amenable to enzyme enhancement therapy with pharmacological chaperones, whereas cells homozygous for L444P respond poorly. We found that Verapamil treatment does not enhance mutant enzyme activity in any of the cell lines tested, while Diltiazem moderately increases activity in normal cells, and in N370S/N370S and F213I/L444P, but not in L444P/L444P Gaucher cells, or in either of two adult Tay-Sachs disease cell lines. Since the mode of action of pharmacological chaperones and Diltiazem are believed to be different, we examined the possibility that they could act in concert. Diltiazem co-administered with known chaperones failed to increase enzyme activities above that reached by chaperone-treatment alone in any of the patient cell lines. Thus, we reexamined the possibility that Diltiazem acts as a pharmacological chaperone. We found that, at the acidic pH of lysosomes, Diltiazem was not an inhibitor, nor did its presence increase the heat stability of glucocerebrosidase. However, at neutral pH, found in the endoplasmic reticulum, Diltiazem exhibited both of these properties. Thus Diltiazem exhibits the biochemical characteristics of a glucocerebrosidase pharmacological chaperone.

Keywords

Lysosomal storage disease; Tay-Sachs disease; Protein folding; Endoplasmic reticulum quality control; Enzyme enhancement therapy

The endoplasmic reticulum (ER) is a continuous membrane system consisting of several domains that perform different functions, including maintaining cellular homeostasis by integrating a variety of internal and external signals [1]. Specialized functions of the ER

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include the storage and regulation of intracellular Ca^{2+} , $[\text{Ca}^{2+}]_i$ [2], as well as the biosynthesis of lipids for membranes [3] and proteins destined for the lysosome, plasma membrane [4] or secretion [5]. In this latter role the ER can be looked on as a protein folding factory that imposes strict quality control (ERQC) on its products, ensuring that only properly folded and assembled, i.e., functional, proteins are delivered to their cellular destinations [6]. Non-functional proteins are not engaged by the intracellular transport machinery of the ERQC, but are instead transferred to either the ubiquitin-proteasome system or the macroautophagy-lysosomal system for degradation [7]. These systems are part of the ER associated degradation component of the ERQC. Additionally the ERQC retains an oxidizing environment to facilitate disulfide bond formation, and a system of resident protein chaperones that assist in protein folding and in the prevention of protein-aggregation. Recently a mathematical model (FoldEx) of the ERQC, based on the concept of three competing component systems, has been published [8].

A number of biochemical and physiological stimuli, such as perturbation in Ca^{2+} homeostasis or redox status, elevated secretory protein synthesis, expression of misfolded proteins, sugar/glucose deprivation, and overloading of cholesterol can disrupt ER homeostasis and impose stress on the ER, leading to the accumulation of misfolded proteins in its lumen. ER-stress is believed to be involved in the pathogenesis of numerous human genetic diseases (see Table 1 in [9]), e.g., diabetes mellitus; and several neuronal diseases, e.g., Alzheimer and Parkinson. The occurrence of the unfolded protein response and/or ER-stress in several lysosomal storage diseases (LSDs) has also been reported; e.g., Gaucher disease (GD) [10,11], GM1 [12,13] and GM2 (Tay-Sachs and Sandhoff) [14] gangliosidosis, and Batten disease (CLN1) [15,16].

Earlier reports associating ER dysfunction with the pathophysiology of LSDs focused on the ability of the stored substrate to increase $[\text{Ca}^{2+}]_i$, resulting in increased calcium signaling, apoptosis and neurodegeneration. In the case of Tay-Sachs/Sandhoff disease, GM2 ganglioside storage was reported to accomplish this by inhibiting the uptake of cytosolic Ca^{2+} by the SERCA pump in the ER [14]. In the case of glucocerebroside storage in GD, $[\text{Ca}^{2+}]_i$ was reportedly increased due to an enhancement in the ryanodine-receptor release of Ca^{2+} from ER-stores [17]. Wei et al. [18] have recently shown that cell death by apoptosis in both neurodegenerative and non-neurodegenerative LSDs is mediated by ER- and oxidative-stresses. Reactive oxygen species have also been reported to enhance intracellular calcium signaling through both inhibiting the ability of the SERCA pump to remove Ca^{2+} from the cytosol, and stimulating the ability of ryanodine-receptors to release Ca^{2+} from ER-stores (reviewed in [19]). Interestingly, although fibroblasts synthesize and thus can store little GM1 or GM2 ganglioside [20], these stress pathways were still reported to be active in cells from GM1 and GM2 gangliosidosis patients, as well as in fibroblasts from GD patients [18]. Thus these data suggest that there are additional factors associated with LSDs, e.g., the presence of mutant misfolded lysosomal protein in the ER [10,11], that can also induce these cellular stress pathways.

GD is the most common LSD [21]. The biochemical hallmark of GD is the storage of glucocerebroside in the tissues of the reticulo-endothelial system and the brain arising from the deficiency of membrane-associated lysosomal glucocerebrosidase (Gcc). Although GD

represents a broad and continuous spectrum of clinical involvement, three main clinical phenotypes are generally recognized: Type I, chronic non-neuronopathic; II, acute neuronopathic; and III, subacute neuronopathic [22]. Thus, Type II patients present with the most severe phenotype, followed by III, and then Type I. Type I GD (incidence ~1/50,000) accounts for the bulk of the patients. Approximately 90% of all GD patients carry one of just three alleles, i.e., N370S (Type I), F213I (Type II/III), L444P (Types II/III), with N370S alone accounting for ~75% of mutant alleles (OMIM 606463). The highest carrier frequency for GD occurs amongst Ashkenazi Jewish adults (~1/11) [23,24].

Enzyme enhancement therapy (EET) [25] and more recently, the restoration of calcium homeostasis [26], are two therapeutic approaches recently put forward for GD [27] and other LSDs [28]. They both attempt to use small molecules to enhance the folding efficiency of the patient's own mutant enzyme in the ER. Thus, these approaches also have the potential to reduce ER-stress associated with misfolded protein storage in the ER, as well as substrate storage in the lysosome. However, only genotypes that allow the production of some residual enzyme activity, e.g., missense mutations, are amenable to these therapies. Both approaches have been reported to target the ERQC in different ways, and if their proposed mechanisms of action are correct, they could act additively or even synergistically. The aim of EET is to increase the stability of the native conformation of the mutant enzyme in the ER, allowing more of it to be engaged by the ER transport machinery for transport to the lysosome [27,29]. To date successful chaperones have also been competitive inhibitors of their target enzymes, i.e., pharmacological chaperones (PCs). It is believed that once the PC-enzyme complex reaches the lysosome, the large amounts of stored substrate(s) will displace the PC and continue to stabilize the enzyme [28]. EET has shown promising preclinical results in at least four enzyme deficiencies [28,30] and several Phase I and Phase II clinical trials are underway, e.g., <http://www.amicustherapeutics.com/clinicaltrials/overview.asp>.

Mu et al. [26] recently reported that inhibition of L-type Ca^{2+} channels in the plasma membrane, using either Diltiazem or Verapamil (both FDA-approved hypertension drugs), partially restored mutant Gcc activity in Gaucher patient cells homozygous for the N370S or L444P alleles, as well as two other LSDs (α -mannosidosis and mucopolysaccharidosis type IIIA), presumably through increasing the folding efficiency segment of the ERQC [8], which may be impaired due to altered calcium homeostasis. This is particularly significant as the N370S Type I allele [27,31–33], and the F213I Type II/III allele [31,34] (not tested by Mu et al.), are amenable to EET; whereas the L444P Type II/III allele has not responded significantly to PCs [27,32–34].

In this report we examined the possibility that specific PCs and Diltiazem could act in concert to further enhance mutant enzyme activity in cells from patients with either of two LSDs, GD and adult Tay-Sachs disease (ATSD). We report that Verapamil alone produces little if any enhancement of activity in any of the cell lines tested. Diltiazem alone moderately increases enzyme activity and its transport to lysosomes in N370S/N370S and F213I/L444P GD cells. In contrast, Diltiazem has little effect on residual Gcc in L444P/L444P GD cells or on β -hexosaminidase A (Hex A) in any of the ATSD cell line tested. Diltiazem treatment also did not result in any further enhancement of activities in the GD or ATSD lines when co-administered with known PCs; i.e., isofagomine (IFG) [33], GD;

pyrimethamine (PYR) [35], ATSD. The close similarity of Diltiazem, a benzothiazepine, to a Gcc inhibitor we recently identified with a benzodiazepine backbone ([36], see Supplemental Table 1, compound BTB 03346) suggested that it may also act as a PC. Re-examination of Diltiazem as a potential PC for GD revealed that, while it does not express the biochemical characteristics of a PC at pH 4.5, these characteristics become evident at the neutral pH found in the ER.

Materials and methods

Chemical reagents

Diltiazem and Verapamil hydrochloride were purchased from Tocris Bioscience (USA), Isofagomine hydrochloride from Toronto Research Chemicals Inc. (Canada) and pyrimethamine from Sigma-Aldrich Ltd. (Canada). These compounds were dissolved either in DMSO or ethanol according to their respective solubility. The following fluorogenic substrates were obtained from Sigma-Aldrich Ltd. (Canada) 4-methylumbelliferyl- β -D-glucoside (4MUGlu), 4-methylumbelliferyl- β -D-galactopyranoside (4MUGal), 4-methylumbelliferyl-N-acetyl- β -glucosaminide (4MUG) and used to assay the lysosomal enzymes glucocerebrosidase (Gcc), β -galactosidase (β -Gal), total β -hexosaminidase (Hex A plus B), respectively. Hex A was measured using 4-methylumbelliferyl-sulfo-2-acetamido-2-deoxy- β -D-glucopyranoside (4MUGS) from Toronto Research Chemicals Inc. (Canada). Cerezyme, modified human recombinant Gcc, was purchased from Genzyme Corporation (USA). Taurodeoxycholic acid (TC) sodium salt and dimethylsulfoxide (DMSO) were from Calbiochem (USA) and EMD Chemicals Inc. (Germany), respectively. Other chemicals used were analytical grade reagents from general laboratory suppliers.

Cell lines, tissue culture and small molecule treatments

Primary skin fibroblasts established from a Type I Gaucher Disease patient homozygous for the N370S mutation was provided by the HSC tissue culture services. The GD type II fibroblast cell line homozygous for the L444P (GM07968) mutation was purchased from Coriell Institute Biorepository (USA). The GD type III cell line with the genotype F213I/L444P was a gift from F. Choy (Victoria, Canada). Adult Tay-Sachs disease (ATSD) fibroblasts displaying either the α G269S/G269S or α R499H/null mutations were from our own collection [35]. Cultures of primary wild-type fibroblast cell lines (WT, control) were provided by the HSC tissue culture services.

All cells were grown in α -minimal essential medium (α -MEM) from Wisent Inc. (Canada) in the presence of 5% antibiotics (penicillin and streptomycin, Gibco BRL Canada), supplemented with 10% Fetal Bovine Serum (growth medium) (FBS, Wisent Inc., Canada) and incubated at 37 °C in a humidified atmosphere with 5% CO₂. For treatment or co-treatment of normal, or mutant GD or ATSD fibroblast lines with candidate drugs the cells were processed as follows. Cell lines grown to 60–80% confluency in 24-well plates (Falcon BD, Canada), were rinsed with phosphate buffer saline (PBS, Wisent Inc. Canada) and growth medium either containing the drug(s) to be tested at defined concentration or solvent only (mock treatment) was added. The cells were returned to the incubator for the specific period of time dictated by the experiment, then rinsed with cold PBS, processed for lysate

preparation and assayed immediately. The range of concentrations used, the drugs tested and the length of incubation in presence of the small molecules are indicated in the text or in the legends to the figures. For the determination of the specific activity of Gcc in the GD cell lines, total cell lysates were prepared from cell pellets (10 cm Petri dishes) by five freezing–thawing cycles in PBS containing 0.1% of TC (w/v) followed by centrifugation.

Enzyme assays and protein determination

Enzyme activity determinations after drug treatment (Gcc, β -Gal, total Hex and Hex A) were performed using cell lysates. Cells grown/treated in 24-well plates were rinsed three times with cold PBS, then 150 μ L of lysis buffer was added to each well and the plate was incubated for 1 h at 4 °C. Cell lysis buffers were Mc Ilvaine citrate (0.1 M)—phosphate buffer (0.2 M) (CP) at the optimum pH corresponding to the enzyme of interest (4.1 for Hex or Hex A, 4.3 for β -Gal, 4.5 for Gcc) containing TC 0.4% (w/v) and Triton X-100 (TX) 0.4% (v/v). Gcc, Hex and β -Gal enzyme activities were determined in 96-well plates (in triplicate) by hydrolysis of their respective fluorescent substrate. Stock solutions of the fluorogenic substrates were dissolved in CP buffer at 3.2 mM for 4MUG and 4MUGS, at 0.56 mM for 4MUGal or in water at 20 mM for 4MUGlu. Enzyme activities were measured using an aliquot (2–10 μ L) of cell lysate complemented with CP and BSA (0.25% w/v) to a final volume of 25 μ L then an equal volume of fluorescent substrate was added and the reaction incubated at 37 °C. The reactions were stopped by addition of 200 μ L of 2-amino-2-methyl-1-propanol (MAP; 0.1 M, pH 10.5). Fluorescence of the 4-methylumbelliferone liberated (MU) was measured with a Gemini EM Microplate Spectrofluorimeter (Molecular Devices Inc., USA) with the excitation and emission wavelengths set at 365 and 450 nm, respectively, and analyzed using the SoftMax Pro Software (USA) coupled to the spectrofluorimeter. The volume of cell lysate and the time of incubation was adjusted to obtain meaningful fluorescence readings relative to the level of enzyme present (normal activity or mutant enzyme with less than 1% of WT). Specific activities of GD cell lines were calculated from the fluorescence of a MU standard solution and expressed as nmol/h/mg of total protein in the lysate (U/mg). Protein determinations were performed either with the Bio-Rad Protein assay (Biorad, USA) or the BCA protein assay (Pierce, USA) when TX was present with bovine serum albumin as standard.

Effect of pH on dose response curves of Cerezyme to Diltiazem

A 2-Unit Cerezyme stock solution in PBS/BSA/TC was prepared and diluted 1/500 (4 mU/mL) in CP buffer with TC and TX, pH 4.5–6.5, (5 dilutions). A 3-fold serial dilution in PBS was performed from the 10 mM Diltiazem stock. Assays were performed in 96-well plates and in triplicate. For each pH tested, 10 μ L of each Diltiazem dilution, 10 μ L of the Cerezyme dilution and 5 μ L of CP buffer (TC and TX pH 4.5–6.5) were mixed, then 25 μ L of 4MUGlu was added to start the reaction. After 1 h at 37 °C the reaction was stopped and the fluorescence measured as above. The enzyme activity measured for each time point within the same pH range of Diltiazem concentration was calculated relative to the enzyme activity measured in the absence of the drug. Data, expressed as a mean of triplicate assays plus or minus one standard deviation, were used to generate dose response curves. Non-linear regression of the logarithmic value of the fraction of the remaining enzyme calculated

at different Diltiazem concentrations for each pH relative to the enzyme activity without Diltiazem were generated using Prism (Graph Pad Software, Inc.) to obtain IC50 values.

Heat inactivation assays

Measuring the ability of increasing concentrations of Diltiazem to attenuate Cerezyme heat denaturation at 50 °C assessed the effect of Diltiazem on the stability of Gcc. All the steps were carried out on ice until the final enzyme assays. Cerezyme working mixes were prepared containing 8 mU/mL of Gcc with PBS/BSA/TC (pH 7 or 4.5) and 0–500 µM of Diltiazem. Mixes were distributed in pre-labeled sets of PCR tubes on ice (five sets of three tubes per drug concentration). For each Diltiazem concentration, four sets of PCR tubes containing the enzyme/Diltiazem mix (20 µL) were simultaneously transferred to a PCR machine pre-heated at 50 °C and the last set of tubes kept on ice as zero time points. Then one set of PCR tubes was removed after exactly 5, 10, 15 and 30 min from the PCR machine and immediately cooled on ice. Determination of Gcc activity was performed using 10 µL from each PCR tube as described above, but assayed at pH 5.5 (the pH optimum for Cerezyme, data not shown). The enzyme activity remaining for each time point after heat inactivation was calculated relative to the enzyme activity present at time zero. Data were expressed as mean of the triplicate assays plus or minus one standard deviation. Linear regression curves of the logarithmic value of the fraction of the remaining enzyme calculated at different time points relative to the zero time point were generated using Prism to obtain the time at which 50% of enzyme activity had been denatured ($T_{1/2}$).

Indirect immunofluorescence staining and confocal microscopy imaging

Indirect immunofluorescence and confocal microscopy imaging were performed as previously described [37]. Primary/secondary antibodies and nuclear staining were as previously reported [36]. The primary Gcc antibody used was a rabbit polyclonal generated in our laboratory, and as judged by Western blots, cross-reacts with all the mutant forms of Gcc present in the cell lines analyzed in this report (data not shown).

Results and discussion

In order to test the hypothesis that PCs and inhibitors of L-type Ca^{2+} channels may have additive or even synergistic effects, we selected three GD cell lines representing the spectrum of clinical phenotypes and re-evaluated the effects of Diltiazem or Verapamil treatment on their residual Gcc levels. The Type I GD line (N370S/N370S) we used had a specific Gcc activity level of 50 nmol MUGlu/h/mg total protein (U/mg) (22% of normal, based on the 232 U/mg of Gcc activity in our control). The Type III line (F213I/L444P) had a specific activity of 12 U/mg of Gcc (5%), and the Type II line (L444P/L444P) had 2 U/mg (~1%).

It is difficult to precisely compare specific activity values obtained by different laboratories, because these are highly dependent on the assay condition used [32]. Additionally, even when measured by the same laboratory, the levels of residual activity in GD cell lines from different patients, even with the same genotype vary substantially. For example in one study Gcc activity in normal fibroblasts ($n = 4$) varied from 165–526 U/mg. The residual Gcc

activity in L444P homozygous fibroblasts ($n = 3$) ranged from 10–19 U/mg (2–11% of normal) and in N370S/N370S cells ($n = 3$) from 9–25 U/mg (2–15% of normal) [38]. Another study [39] reported activity ranges of from <1% to 13% in 32 cell lines from L444P/L444P patients. Despite this variability in Gcc measurements several laboratories have reported levels of Gcc enhancement in the order of 2- to 4-fold after treatment with 10–100 μ M of isofagomine (IFG) in N370S/N370S GD cells and little significant enhancement over the same range in L444P/L444P cells [32,33]. We recently reported an ~5-fold enhancement of Gcc activity after treating F213I/L444P GD cells with 25 μ M IFG [31].

Equal numbers of normal or GD cells were treated for 7 days (two doses) with 5–100 μ M of Diltiazem. In normal cells, while Gcc was enhanced 1.7 ± 0.2 -fold at 5–10 μ M, the levels of other lysosomal enzymes, i.e., Hex and β -Gal, were unaffected below 20 μ M and decreased thereafter due to toxicity (data not shown). Treatment of N370S/N370S GD cells resulted in a 1.6 ± 0.1 -fold level of Gcc enhancement between 10 and 50 μ M (Fig. 1A) with toxicity becoming apparent at >50 μ M (based on visual observations, lower total protein and decreased β -Gal and/or Hex (data not shown), as well as Gcc enzyme activity). Mu et al. reported that Diltiazem treatment produced enhancements of ~2.5-fold in both normal and N370S/N370S cells at 10 μ M. Similar treatment of N370S/N370S GD cells with Verapamil, which was toxic above 20 μ M, produced little if any enhancement (maximum of 1.2 ± 0.2 -fold at 10 μ M) (Fig. 1A). Mu et al. reported 2-fold enhancement at 3 μ M. It is also difficult to rationalize the observed enhancement of Gcc activity with Diltiazem in normal cells, as calcium homeostasis in these cells should be normal.

We next used either Verapamil or Diltiazem to treat L444P/L444P GD cells. There was no detectable enhancement with the former, and the latter drug produced an insignificant 1.2 ± 0.2 -fold increase at 5 and 10 μ M (Fig. 1B), as compared to the 1.5- (Verapamil) and 2.3-fold (Diltiazem) increases reported by Mu et al. Additionally we treated F213I/L444P GD cells with both drugs. We detected no enhancement with Verapamil and a 1.4 ± 0.1 -fold increase with Diltiazem at 10–50 μ M (Fig. 1C). While the F213I/L444P cells showed no signs of toxicity up to the maximum 50 μ M used to treat then, this level of Diltiazem had obvious toxic effects on homozygous N370S and L444P GD cells. These data indicate that Verapamil is ineffective at enhancing intracellular levels of Gcc contained any of the three common missense mutations associated with GD Type I, II or III. On the other hand, Diltiazem treatment produces a small increase in Gcc levels carrying the N370S or F213I mutations, but is ineffective at enhancing L444P Gcc activity in GD patient cells.

The ability of Verapamil or Diltiazem to enhance two missense mutations associated with ATSD was also evaluated. We have shown that several small molecules [40] including pyrimethamine [35] acting as PCs can increase levels in α G269S by ~3-fold, but are relatively ineffective (maximum of a 1.2-fold increase) in enhancing α R499H missense mutations in the α -subunit of Hex A after 5 days of treatment [35]. Whereas the ATSD α G269S mutation like N370S in GD, produces a soluble but unstable enzyme [41,42]; the α R499H mutation tends to produce aggregate-prone protein [43]. Little or no enhancement was detected in α G269S/G269S (1.2 ± 0.1 -fold) or α R449H/null (1.2 ± 0.2) ATSD cells after treatment with 5–100 μ M Diltiazem. Interestingly, while treatment with 30 μ M

Diltiazem produced obvious toxic effects in α R499H cells, α G269S cells were resistant to the drug up to levels of 100 μ M (data not shown).

Finally we evaluated the effects of co-treatment of both GD and ATSD cells with previously described PCs and Diltiazem. Cells were again treated for 7 days (two doses) with 5–30 μ M of Diltiazem and either 10 μ M IFG (GD) or 8 μ M (2 μ g/mL) of pyrimethamine (ATSD). Both of the PCs were used at suboptimal concentrations to preclude the possibility of reaching some upper level of enhancement in mutant cells. We detected no enhancement of mutant enzyme activity above that obtained by treatment of the cells with the appropriate PC alone, when cells were co-treated with a PC and various concentrations of Diltiazem (Fig. 2A–C) (data not shown for ATSD). Interestingly, Diltiazem co-treatment appeared to inhibit the enhancement of F231I/L444P GD cells by IFG (Fig. 2C).

The above data were confirmed by indirect immunofluorescence and confocal microscopy. Normal cells and those containing the three GD genotypes were co-stained with antibodies against Gcc and a marker for lysosomes, LAMP-1. A representative cell is shown at high magnification in each panel of Figs. 3 and 4 to allow for the degree of co-localization of the two stains to be clearly evaluated. To provide a qualitative sense of protein levels, the confocal microscope settings were maintained for treated and untreated normal, N370S/N370S and F213I/L444P cells when recording Gcc staining. The Gcc signal had to be increased in L444P/L444P cells in order to produce a recordable signal. However, all settings were maintained between treated and untreated L444P/L444P cells. The Gcc signals were increased by IFG and to a lesser extent by Diltiazem treatment in wild-type cells. Verapamil produced little change in intensity. Importantly, these increases in the Gcc signal co-localize (indicated by yellow color) with LAMP-1 (Fig. 3). In untreated N370S cells (DMSO only), Gcc staining was weaker, but a significant amount of the residual protein did co-localize with LAMP-1 (Fig. 3). IFG, and to a lesser extent Diltiazem, again increased total Gcc staining which co-localized with LAMP-1 (Fig. 3). Verapamil on the other hand, had little effect on the level of Gcc staining or on its co-localization with LAMP-1 (Fig. 3). F213I/L444P GD cells produced results similar to N370S cells, but with less initial co-localization of residual Gcc staining with LAMP-1 in untreated cells. Interestingly, unlike the increased Gcc staining seen after either the Diltiazem or IFG treatment alone, in co-treated cells a significant amount of the increased staining did not co-localize with LAMP-1 (Fig. 4). This observation coupled with the apparent inhibition of IFG-enhancement of activity in co-treated cells (Fig. 2C) suggests that much of the increased Gcc staining corresponds to inactive, misfolded protein retained in the ER. L444P/L444P cells produced the lowest intensity of Gcc staining which did not significantly increase by either Diltiazem or IFG treatment. As well, neither treatment produced a visible increase in Gcc co-localization with LAMP-1 (Fig. 4). These data confirm that, in our hands, treatment of these cells with Diltiazem, like IFG, is ineffective at enhancing the levels of lysosomal L444P Gcc.

The above data suggest that Diltiazem may be acting as a PC, despite the reported lack of inhibitory effect of the drug on Gcc activity (a hallmark of PCs) [26]. Thus, we re-evaluated Diltiazem as an inhibitor of Gcc. At pH 4.5 Gcc was not inhibited by even 2000 μ M Diltiazem, but actually appeared to be activated (~1.5-fold) by it at high concentrations (Fig. 5). However, Diltiazem contains a primary amine and two compounds we recently identified

by high throughput screening of the Maybridge library as PCs for Gcc, 5-[(4-methylphenyl)thio]quina-zoline 2,4-diamine (IC₅₀ ~8 μ M at pH 5.5) and 5-(3,5-dichlorophenoxy)-*N*-(4-pyridinyl)-2-furamide (IC₅₀ ~5 μ M at pH 5.5) [36], also contain either a primary or a secondary amine as part of their structure, and inhibit Gcc best at neutral pH [36]. Interestingly, IFG contains a secondary amine and similarly has been reported to inhibit Gcc best at neutral pH [33]. Thus we further tested the ability of Diltiazem to inhibit Gcc (Cerezyme) at increasing pH. At pH 5.0 inhibition became obvious at >250 μ M and was sufficiently high at pH 6.5 to calculate an IC₅₀ ~160 μ M (Fig. 5). We then applied a more general test for potential PC-activity, the ability to attenuate the heat inactivation of the target enzyme [29], at pH 4.5 as compared to pH 7.0. Interestingly at 50 °C, Gcc has a half-life ($T_{1/2}$) of 3.3 min at pH 4.5, but at pH 7.0 it drops to only 1.7 min. Thus Gcc appears to be more stable at the acidic pH of the lysosome than at the neutral pH of the ER. The addition of 100 μ M of Diltiazem had little effect on the $T_{1/2}$ of Gcc at pH 4.5, increasing it by ~9%. However, at pH 7.0, the $T_{1/2}$ was increased by 53% and continued to rise with increasing concentration of Diltiazem. At the maximum concentration examined, 500 μ M, $T_{1/2}$ was increased 300% to 6.8 min. At this concentration the $T_{1/2}$ at pH 4.5 was 3.8 min., an increase of only 15% (Fig. 6). The fact that Diltiazem clearly acts as a Gcc inhibitor and attenuates its thermal denaturation at the neutral pH found in the ER strongly suggests that Diltiazem is also functioning as a PC in GD cells.

In summary we have shown that Diltiazem is the only L-type Ca²⁺ channel blocker tested that enhances some mutant forms of Gcc, but not Hex A activity in patient cells. Like IFG, a known PC for Gcc, Diltiazem was effective in enhancing N370S or F213I Gcc, but not L444P Gcc activity in the lysosomes of GD cells. Co-treatment of IFG and Diltiazem did not result in any increase in Gcc activity over that of cells treated with IFG alone. Unlike IFG, Diltiazem did not inhibit Gcc or protect it against heat denaturation at pH 4.5. However, like IFG, Diltiazem did exhibit both of these properties at pH 6.5–7. We conclude that the Gcc enhancement mechanism of Diltiazem is the same as that of IFG or either of the other two non-carbohydrate based compounds we have identified by screening of a small molecule library [31,36], i.e., Diltiazem is a PC for Gcc in GD cells. Furthermore Diltiazem represents a PC with a non-carbohydrate framework that has already been evaluated in humans.

Acknowledgments

This study was funded by salary grants from The New Hope Research Foundation and the David M.C. Ju Foundation to B.R. and by a Canadian Institutes of Health Research Team Grant (CTP-82944) to D.M. The authors thank Dr Michael Tropak for his helpful suggestions and to Dr. John Callahan for his assistance in preparing this article. The LAMP-1 antibody used was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

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Fig. 1.

Effect of two L-type calcium channel blockers, Diltiazem and Verapamil, on the Gcc activity of the three main GD phenotypes. Primary human fibroblasts were grown in the presence of 0 to 50 μ M of either Diltiazem (solid black columns) or Verapamil (lightly dotted columns) for 7 days with a change of media after 3 days (total two doses). (A) Fibroblasts from a type I GD patient (N370S/N370S); (B) Fibroblasts from a type II GD patient (L444P/L444P); (C) Fibroblasts from a type III GD patient (F213I/L444P). Data are expressed as fold increase of the Gcc activity in presence of the drug relative to the enzyme activity in mock-treated cells, i.e., a y-axis value of 1 indicates no effect of the drug on Gcc. Indicated values represent the mean $\pm$ 1 standard deviation of triplicate assays.

Fig. 2.

Co treatment for 7 days (total two doses) of GD fibroblasts with Diltiazem and IFG. Primary human fibroblasts were grown in the presence of 10 μM of IFG alone (zero column) or in combination with 5–30 μM of Diltiazem. (A) Fibroblasts from a type I GD patient (N370S/N370S); (B) Fibroblasts from a type II GD patient (L444P/L444P); (C) Fibroblasts from a type III GD patient (F213I/L444P). Data are expressed as fold increase of the Gcc activity in presence of IFG only or both drugs relative to the enzyme activity in mock-treated cells. Indicated values represent the mean $\pm$ 1 standard deviation of triplicate assays.

Fig. 3.

Effect of Diltiazem, IFG or Verapamil treatment for 7 days (two doses) on lysosomal incorporation of Gcc in control and type I GD. Wild-type and N370S/N370S GD patients fibroblast were treated with DMSO, Diltiazem (20 μ M), IGF (10 μ M) or Verapamil (10 μ M). The primary IgGs against Gcc (rabbit polyclonal) or the lysosomal marker LAMP1 (mouse monoclonal) are visualized in green or red, respectively. In the merged images, yellow denotes co-localization of the two proteins in the lysosomes. Nuclei appear in blue.

Fig. 4.

Effect of Verapamil, Diltiazem and/or IFG treatment for 7 days (two doses) on Gcc lysosomal localization in type II and III GD. Type III GD fibroblasts (F213I/L444P) were treated with DMSO, Diltiazem (50 μ M), IFG (10 μ M) or Verapamil (5 μ M), or co-treated with Diltiazem (50 μ M) and IFG (10 μ M). Type II GD fibroblasts (L444P/L444P) were treated with DMSO, Diltiazem (10 μ M) or IFG (10 μ M). The primary IgGs against Gcc or the lysosomal marker LAMP1 are visualized in green or red, respectively. In the merged images, yellow denotes co-localization of the two proteins in the lysosomes. Nuclei appear in blue.

Fig. 5.

Variability with pH of the inhibitory effects of Diltiazem on Gcc enzyme activity *in vitro*. Constant amounts of Cerezyme were incubated with increasing concentration of Diltiazem (0–2000 μM) at a range of pHs; pH 4.5 (solid squares and line), pH 5.0 (triangles and dashed line), pH 5.5 (solid triangles and line), pH 6.0 (solid diamonds and dotted line), and pH 6.5 (solid circles and line). Each assay was done in triplicate. Data are expressed as the mean $\pm$ 1 standard deviation of the Gcc activity relative to untreated controls, versus the log of the Diltiazem concentrations used at each defined pH.

Fig. 6.

Attenuation of heat inactivation of Cerezyme by Diltiazem at pH 4.5 and 7. Constant amounts of Cerezyme in the presence of four different concentrations of Diltiazem at pH 4.5 (A) or at pH 7 (B) were heated at 50 °C for up to 30 min. Diltiazem concentrations were 0 μ M (open columns), 100 μ M (diagonally striped columns), 250 μ M (cross hatched columns) and 500 μ M (solid columns). Each assay was done in triplicate. Data are expressed as the mean $\pm$ 1 standard deviation of the Gcc activity relative to unheated controls containing the same concentrations of Diltiazem versus time spent at 50 °C. (C); the $T_{1/2}$ values obtained from the equation best-fit lines generated from plotting the log of the remaining Gcc activity versus heating time at pH 4.5 (checked columns) or pH 7 (solid columns). Standard deviations were calculated using those of the slopes and intercepts of the best-fit lines.