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Lacidipine Remodels Protein Folding and Ca^{2+} Homeostasis in Gaucher's Disease Fibroblasts: A Mechanism to Rescue Mutant Glucocerebrosidase

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SUMMARY

The hallmark of Gaucher's disease cellular pathogenesis is the lysosomal accumulation of glucosylceramide, which is caused by misfolding of mutated glucocerebrosidase (GC) and loss of lysosomal GC activity, and leads to depletion of $[Ca^{2+}]_{ER}$. We demonstrate that modulation of Ca^{2+} homeostasis and enhancement of the cellular folding capacity synergize to rescue the folding of mutated GC variants. Lacidipine, an L-type Ca^{2+} channel blocker that also inhibits $[Ca^{2+}]_{ER}$ efflux, enhances folding, trafficking, and activity of degradation-prone GC variants. Lacidipine remodels mutated GC proteostasis by simultaneously activating a series of distinct molecular mechanisms, namely modulation of Ca^{2+} homeostasis, upregulation of the ER chaperone BiP, and moderate induction of the unfolded protein response. However, unlike previously reported proteostasis regulators, lacidipine treatment is not cytotoxic but prevents apoptosis induction typically associated with sustained activation of the unfolded protein response.

INTRODUCTION

Gaucher's disease (GD) is characterized by deficient lysosomal glucocerebrosidase (GC) activity and accumulation of GC substrate, glucosylceramide (Schueler et al., 2004). Mutations in GC-encoding gene (GBA; Hruska et al. [2008]) result in inactive GC variants, which are typically retrotranslocated from the endoplasmic reticulum (ER) to the cytoplasm for ER-associated degradation (ERAD). A number of characterized missense mutations destabilize GC native structure without directly impairing its catalytic activity (Schmitz et al., 2005). As a result, these unstable GC variants retain biologic activity if forced to fold into their native 3D structure (Sawkar et al., 2002, 2006; Yu et al., 2007). Rescuing the function of mutated GC variants is an appealing alternative to the currently available therapeutic options (mainly enzyme replacement therapy [Cerezyme®]), which are inadequate for the treatment of neuronopathic forms of GD (Sidransky et al., 2007). Hence, considerable effort has been recently devoted to the design of strategies to rescue cellular folding, trafficking, and activity of mutated GC variants associated with neuronopathic GD (Mu et al., 2008a, 2008b; Ong et al., 2010; Wang et al., 2011), the most common being the severely destabilized L444P GC variant, which is associated with complete loss of activity and neuronopathic symptoms in homozygous patients (Grabowski, 1997).

Ca^{2+} ions, and particularly the gradient of $[Ca^{2+}]$ between the ER (1 mM) and the cytoplasm (100 nM), play a signaling role in a number of fundamental cellular activities, including protein folding in the ER (Berridge et al., 1998; Bygrave and Benedetti, 1996). Ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate (IP3) receptors on the ER membrane regulate $[Ca^{2+}]_{ER}$ efflux, whereas Ca^{2+}-ATPases (SERCA pumps) transfer Ca^{2+} from the cytoplasm into the ER (Baumann and Walz, 2001) (Figure 1A). In GD neurons, glucosylceramide accumulation causes excessive $[Ca^{2+}]_{ER}$ efflux via RyRs (Korkotian et al., 1999; Lloyd-Evans et al., 2003; Pelled et al., 2005). The correlation between the extent of GC variants' residual activities in patient-derived fibroblasts and the clinical severity of the disease, including the occurrence of neuronopathic symptoms, has been established (Beutler et al., 1984; Meivar-Levy et al., 1994; Michelakakis et al., 1995). Hence, patient-derived fibroblasts have been repeatedly used to investigate how Ca^{2+} homeostasis modulation affects the folding of mutated GC variants (Mu et al., 2008a; Ong et al., 2010; Wang et al., 2011).

Ca^{2+} homeostasis influences the biogenesis of secretory proteins and the activity of a number of ER chaperones (Michalak et al., 2002). We previously suggested that impairment of Ca^{2+} homeostasis in GD fibroblasts, by compromising ER folding, hampers rescue of highly unstable, degradation-prone L444P GC folding (Wang et al., 2011). The application of RyR blockers was shown to counteract the effect of glucosylceramide accumulation on $[Ca^{2+}]_{ER}$ efflux, reestablish Ca^{2+} homeostasis, and create an environment more conducive to native folding of L444P GC. However, it did not lead to a substantial rescue of L444P GC folding (Wang et al., 2011).

L-type Ca^{2+} channel (LTCC) (Figure 1A) blockers bind to high voltage-activated channels on the plasma membrane and lower cytosolic-free $[Ca^{2+}]$ (Hockerman et al., 1997; Triggle, 2006). Phenylalkylamines, benzothiazepines, and 1,4-dihydropyridines are the three main classes of LTCC blockers and include molecules that bind to three distinct LTCC receptor sites (Hockerman

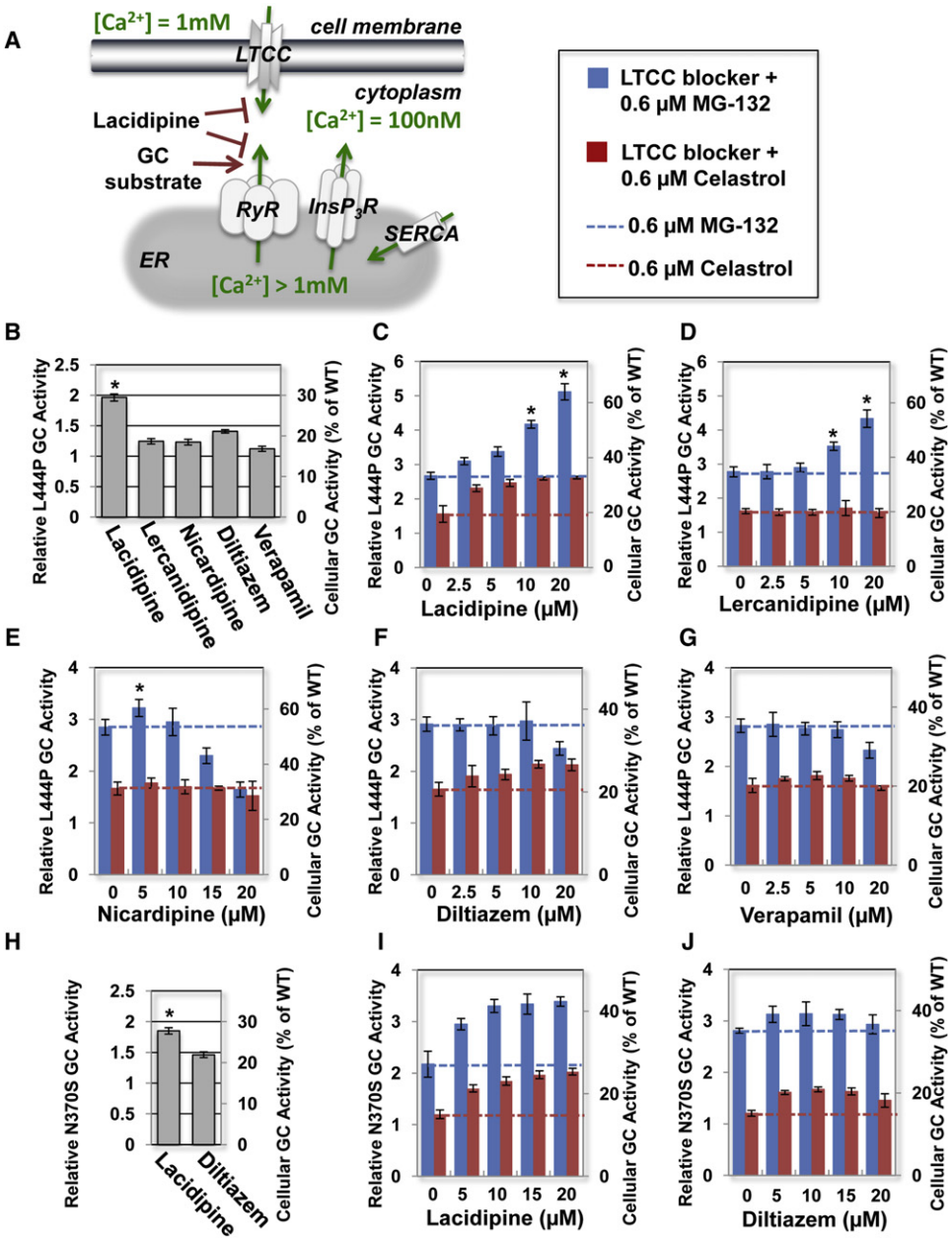

Figure 1. Treatment of GD Patient-Derived Fibroblasts with LTCC Blockers Enhances Mutated GC Activity

(A) $[Ca^{2+}]_{ER}$ is regulated by RyRs, IP_3 receptors, and SERCA pumps. L-type voltage-gated Ca^{2+} channels (LTCCs) mediate Ca^{2+} flow through the plasma membrane. Lacidipine inhibits extracellular Ca^{2+} influx through LTCCs and ER Ca^{2+} efflux through RyRs.

(B–G) Relative L444P GC activities in cells treated with (B) lacidipine (20 μM), lercanidipine (20 μM), and nicardipine (20 μM) for 72 hr, and diltiazem (10 μM) and verapamil (5 μM) for 120 hr. Relative GC activities were evaluated by normalizing GC activities measured in treated cells to the activity of untreated cells (left y axis) ($p < 0.01$ if not specified; *$p < 0.001$). The corresponding fraction of WT GC activity is reported (right y axis). Experiments were repeated three times, and data points are reported as mean ± SD. Relative L444P GC activities were also evaluated in L444P GC cells treated with a proteostasis regulator (MG-132, 0.6 μM; celastrol, 0.6 μM) and an LTCC blocker: (C) lacidipine, (D) lercanidipine, and (E) nicardipine for 72 hr; and (F) diltiazem and (G) verapamil for 120 hr. GC activities were obtained as described in (B) and are reported as mean ± SD.

(H–J) Relative N370S GC activities in cells treated with (H) lacidipine (20 μM) and diltiazem (10 μM) for 72 hr. Relative N370S GC activities in cells treated a proteostasis regulator (MG-132, 0.6 μM; celastrol, 0.6 μM) and an LTCC blocker: (I) lacidipine, and (J) diltiazem.

See Figures S1 and S2.

et al., 1997). Verapamil and diltiazem, prototypes of phenylalkylamines and benzothiazepines, respectively, are FDA-approved drugs for the treatment of hypertension and cardiac arrhythmias (Hockerman et al., 1997), and were previously reported to partially rescue the folding of GC variants, but their mechanism remains elusive (Mu et al., 2008a; Sun et al., 2009). We asked

whether this reported rescue of mutated GC folding is a general property of LTCC blockers and whether their ability to modulate intracellular [Ca^{2+}] correlates with the resulting increase in mutated GC activity. We investigated LTCC blockers with 1,4-dihydropyridine structure including the prototype nifedipine and a series of second- and third-generation derivatives (Epstein, 1999; Pepine, 1989). We found that cell treatment with lacidipine, a third-generation 1,4-dihydropyridine that antagonizes Ca^{2+} mobilization through LTCCs and RyRs (Gunther et al., 2008; Wishart et al., 2008) (Figure 1A), results in enhanced folding, trafficking, and activity of mutated GC variants. Particularly, we show here that lacidipine functions as a proteostasis regulator in patient-derived GD fibroblasts and rescues L444P GC folding with considerably higher efficiency than any other Ca^{2+} channel blocker reported in this study and previously (Mu et al., 2008a; Ong et al., 2010; Wang et al., 2011). By investigating lacidipine-induced modulation of intracellular [Ca^{2+}] and of cellular folding pathways, we demonstrated that lacidipine functions by lowering cytoplasmic [Ca^{2+}], remodeling the expression of ER chaperone and the unfolded protein response (UPR), and reducing cellular toxicity and apoptosis induction, thus activating mechanistically different cellular events previously reported in association with distinct small molecule proteostasis regulators (Wang et al., 2011).

RESULTS

Treatment with Small Molecule LTCC and RyR Blockers Enhances Folding, Trafficking, and Activity of Mutated GC in Patient-Derived Fibroblasts

We investigated a series of LTCC blockers with 1,4-dihydropyridine structure, particularly lacidipine, lercanidipine, nicardipine, nifedipine, and nitrendipine (Triggle, 2003). Patient-derived fibroblasts harboring L444P GC were treated with a range of Ca^{2+} blocker concentrations for 5 days, and GC activities were evaluated every 24 hr with the intact cell GC enzymatic activity assay (Mu et al., 2008b) (see Figure S1 available online). Verapamil and diltiazem, prototypes of the other two classes of LTCC blockers (phenylalkylamines and benzothiazepines, respectively), were included for comparison because they were previously reported to partially rescue mutated GC folding (Mu et al., 2008a). Culturing conditions resulting in maximal rescue of L444P GC activity are reported in Figure 1B. L444P GC activity was observed to increase up to 2.0-fold in cells treated with lacidipine (20 μM, final medium concentration; $p < 0.001$) for 72 hr compared to untreated cells, which corresponds to about 25% of the WT cellular activity, and is expected to ameliorate GD symptoms (Schueler et al., 2004). A milder increase in L444P GC activity (1.2-fold; $p < 0.01$) was detected in the same cells treated with lercanidipine and nicardipine (20 μM) for 72 hr, compared to untreated cells (Figure 1B). Nifedipine and nitrendipine treatment failed to rescue the activity of L444P GC (data not shown). Lacidipine was observed to enhance the activity of L444P GC to a considerably higher degree than diltiazem and verapamil tested under the same conditions (Figure S1). Maximal L444P GC activity increase was observed upon diltiazem (10 μM, 1.4-fold) and verapamil (5 μM, 1.1-fold) treatment for 120 hr (Figure 1B).

Similar to diltiazem and verapamil, lacidipine blocks LTCCs on the plasma membrane as well as RyRs on the ER membrane (Gunther et al., 2008). Nicardipine and lercanidipine are known to block LTCCs and were reported to interfere with the release of Ca^{2+} from the ER (Wishart et al., 2008), whereas nifedipine and nitrendipine are thought to only interact with LTCCs (Gunther et al., 2008). These reported binding interactions, together with results from the GC activity assays reported above (Figure 1B), suggest a correlation between the mechanism of Ca^{2+} mobilization and the extent of L444P GC folding rescue. Specifically, a higher increase in L444P GC activity results from treatment with Ca^{2+} blockers that antagonize both LTCCs and RyRs.

We asked whether treatment with proteostasis regulators, such as MG-132 and celastrol, applied in combination with LTCC blockers enhances the rescue of L444P GC folding, as previously demonstrated for RyR blockers (Wang et al., 2011). Proteostasis regulation was achieved via cell treatment with either MG-132 (0.6 μM) or celastrol (0.6 μM), which are known to rescue L444P GC folding through a mechanism distinct from Ca^{2+} homeostasis modulation (Mu et al., 2008b). Patient-derived fibroblasts were cultured in medium supplemented with an LTCC blocker and a proteostasis regulator for up to 5 days, and GC activity was measured every 24 hr (Figures 1C–1G; Figure S2). Coadministration of lacidipine (20 μM) and MG-132 for 72 hr resulted in a dramatic increase in L444P GC activity compared to untreated cells (5.1-fold; $p < 0.001$) (Figure 1C), which corresponds to 64% of wild-type GC activity and is significantly higher than what was observed treating the cells only with lacidipine (2.0-fold, Figure 1B) or MG-132 (2.7-fold, Figure 1C). Addition of lacidipine was observed to also enhance celastrol-mediated increase in L444P GC activity (2.6-fold, Figure 1C). Interestingly, lercanidipine (20 μM) and nicardipine (5 μM) enhanced MG-132-mediated L444P GC activity rescue (4.3- and 3.2-fold [$p < 0.001$], respectively) but failed to improve celastrol activity (Figures 1D and 1E). Diltiazem and verapamil were observed to synergize with proteostasis regulators with lower efficiency than 1,4-dihydropyridines. Particularly, treatment with diltiazem (10 μM) and verapamil (5 μM) moderately enhanced celastrol-mediated L444P GC folding rescue (2.1- and 1.9-fold, respectively), and failed to alter MG-132-mediated rescue (Figures 1F and 1G).

N370S GC is the most common GC variant exhibiting low residual activity (Meivar-Levy et al., 1994). Cellular folding rescue and enhancement of N370S GC activity were previously reported (Mu et al., 2008a, 2008b; Offman et al., 2010; Sawkar et al., 2002; Wang et al., 2011; Yu et al., 2007). As opposed to L444P GC, N370S GC folding was previously shown to be amenable to rescue with GC-specific chemical chaperones, suggesting that the location and nature of these two mutations have different destabilizing effects on the enzyme's native folding and cellular trafficking (Sawkar et al., 2002, 2005). In addition, patients with GD carrying the N370S GC variant never present neuronopathic GD symptoms typically associated with L444P GC (Michelakakis et al., 1995). To verify whether lacidipine-mediated rescue of mutated GC folding is restricted to the L444P GC variant, GD patient-derived fibroblasts carrying N370S GC were cultured in the presence of lacidipine and proteostasis regulators, and GC activities were measured. Diltiazem

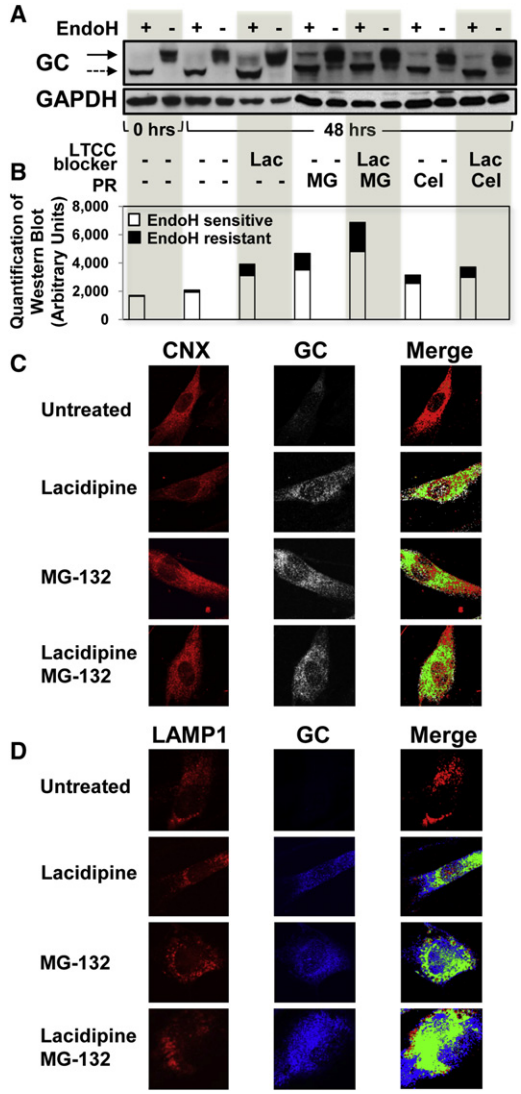

Figure 2. Treatment of GD Patient-Derived Fibroblasts with Lacidipine Promotes L444P GC Folding, Glycosylation, and Trafficking

(A) Western blot analyses of EndoH-treated and untreated total protein content of L444P GC fibroblasts cultured with lacidipine (20 μM), MG-132 (0.6 μM), and celastrol (0.6 μM) for 48 hr and detected using GC-specific antibody. The solid and dashed arrows indicate, respectively, EndoH-resistant and EndoH-sensitive bands. PR, proteostasis regulator; Lac, lacidipine; Dil, diltiazem; MG, MG-132; Cel, celastrol.

(B) Quantification of GC bands detected by western blot. Lower MW, EndoH-sensitive bands corresponding to ER-retained GC were quantified and are reported in the white portion of the bars, and quantification of higher MW, EndoH-resistant bands corresponding to lysosomal GC are reported in the black top portions. Band analyses and quantifications were conducted using NIH ImageJ analysis software.

(C and D) Immunofluorescence microscopy of (C) GC and CNX (an ER marker), and (D) GC and LAMP1 (a lysosomal marker) in L444P GC fibroblasts. Cells were treated with lacidipine (20 μM) and MG-132 (0.6 μM) for 48 hr. Colocalization of CNX (red, column 1) and GC (gray, column 2) is shown in green (column 3). Colocalization of LAMP1 (red, column 1) and GC (blue, column 2) is also shown in green (column 3).

was previously shown to cause increase in N370S GC folding (Mu et al., 2008a), hence it is reported here for comparison. Similar to what was reported above for L444P GC fibroblasts, lacidipine (20 μM) treatment for 72 hr resulted in an increase in N370S GC activity (1.8-fold; $p < 0.001$) (Figure 1H), which was enhanced by the addition of MG-132 and celastrol (3.4- and 2.0-fold, respectively, Figure 1I). Diltiazem (10 μM, 1.4-fold; Figure 1H)-mediated increase in N370S GC activity was enhanced by the addition of MG-132 and celastrol (3.1- and 1.7-fold, respectively, Figure 1J). These results suggest that lacidipine rescues the folding and activity of different mutated GC variants and, thus, functions as a proteostasis regulator in GD patient-derived fibroblasts.

In order to confirm that the increase in activity detected in cells treated with lacidipine results from rescue of mutated GC folding and trafficking to the lysosome, we tested L444P GC glycosylation state and cellular localization.

GC glycosylation state was investigated by endoglycosidase H (EndoH) treatment. EndoH hydrolyzes high mannose, immature N-linked glycoproteins. EndoH treatment followed by GC detection by western blot typically reveals a low MW band corresponding to partially glycosylated, ER-retained GC (EndoH-sensitive) and a high MW band corresponding to fully glycosylated, lysosomal GC (EndoH-resistant) (Maley et al., 1989). The total protein content of cells cultured in media supplemented with lacidipine (20 μM), MG-132 (0.6 μM), celastrol (0.6 μM), or a combination thereof for 48 hr was subjected to EndoH treatment, and GC was detected by western blot. A representative western blot (Figure 2A) and quantification of EndoH-resistant and EndoH-sensitive GC bands (Figure 2B) were reported. In untreated cells nearly all L444P GC was detected as EndoH sensitive, as expected (Mu et al., 2008b). However, a band corresponding to EndoH-resistant L444P GC was detected in cells treated with lacidipine, and its intensity was comparable to that detected in cells cultured with MG-132 or celastrol (the results obtained from the experiments conducted with MG-132 and celastrol have been previously shown by Mu et al. [2008b], and are reported here for comparison). Interestingly, lacidipine treatment resulted in an ~1.5-fold increase of total L444P GC and a decrease of EndoH-sensitive fraction to 80% of total GC. Cotreatment with lacidipine and MG-132 was observed to cause a 2.5- and 1.7-fold increase in the EndoH-resistant pool of L444P GC, compared to cells treated only with lacidipine and MG-132, respectively (Figures 2A and 2B). This increase in mature, fully glycosylated GC correlates with results obtained from GC enzymatic assays.

L444P GC cellular localization was evaluated using immunofluorescence microscopy of L444P GC patient-derived fibroblasts treated with lacidipine (20 μM) and MG-132 (0.6 μM) for 48 hr and using antibodies specific for GC, for an ER marker (Calnexin, CNX), and for a lysosomal marker (LAMP-1). Colocalization of GC and CNX (Figure 2C) and GC and LAMP-1 (Figure 2D) is reported in green. L444P GC was barely detectable in untreated cells due to extensive ERAD (Figures 2C and 2D), as previously reported (Michelakakis et al., 1995). Analysis of merged images revealed the presence of a large pool of enzyme in the ER (Figure 2C) and in the lysosome (Figure 2D) in lacidipine-treated cells, suggesting that lacidipine treatment increases the pool of folded L444P GC that escapes ERAD

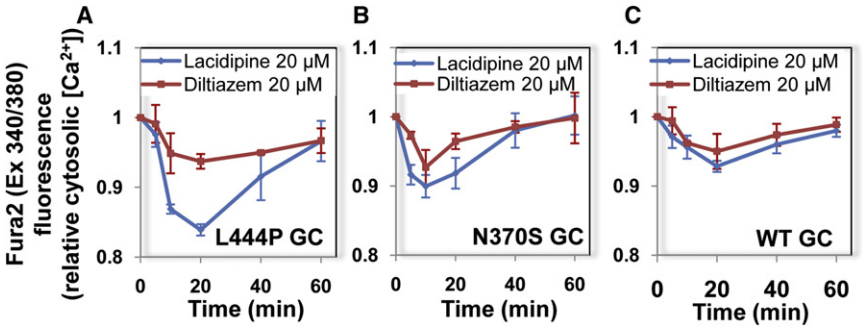

Figure 3. LTCC Blockers Reduce Cytosolic [Ca^{2+}] Levels in Patient-Derived Fibroblasts

(A) L444P GD, (B) N370S GD, and (C) WT fibroblasts were cultured with lacidipine (20 μM) and diltiazem (20 μM) for 5, 10, 20, 40, and 60 min, respectively. Cytosolic [Ca^{2+}] was evaluated by measuring excitation 340/380 ratio of Fura-2 acetoxymethyl ester and normalized to that at time zero. The data are reported as mean ± SD.

and traffics to the lysosomes. Moreover, cotreatment with lacidipine and MG-132 further increased the pool of ER and lysosomal GC (Figures 2C and 2D), demonstrating that these two molecules synergize to rescue L444P GC folding and trafficking, and confirming the results obtained from enzymatic assays (Figures 1B and 1C).

We previously showed that RyR inhibition creates an environment more amenable to L444P GC proteostasis than that of the ER of untreated GD fibroblasts (Wang et al., 2011). The results reported here suggest that combining inhibition of RyRs and LTCCs enables direct rescue of L444P GC proteostasis. However, lacidipine treatment seems to rescue L444P GC folding and activity more efficiently than the other LTCC blockers tested. This suggests that lacidipine is a more potent modulator of intracellular [Ca^{2+}] than other Ca^{2+} blockers used here and previously (Mu et al., 2008a; Ong et al., 2010; Wang et al., 2011) or that cell treatment with lacidipine rescues mutant GC folding by activating other cellular mechanisms that influence the mutated GC folding free-energy diagram. The following studies were conducted to investigate these hypotheses. Diltiazem was used as comparison in these studies because, although it also inhibits LTCCs and RyRs and was reported to enhance the folding of mutated GC variants (Mu et al., 2008a), it is shown here to rescue L444P GC folding to a significantly lower extent than lacidipine. In addition the mechanism involved in diltiazem-mediated GC variant folding rescue still remains elusive.

Lacidipine Depletes Cytosolic-Free [Ca^{2+}] in GD Patient-Derived Fibroblasts

Glucosylceramide buildup causes $[Ca^{2+}]_{ER}$ efflux and elevation of cytosolic [Ca^{2+}] in GD cells (Korkotian et al., 1999). Lacidipine and diltiazem, by binding to LTCCs and RyRs, are expected to lower cytosolic [Ca^{2+}] and increase $[Ca^{2+}]_{ER}$, respectively. We asked whether the larger increase in mutated GC variants activity caused by cell treatment with lacidipine compared to diltiazem correlates with their different effect on intracellular Ca^{2+} mobilization. Cytosolic-free [Ca^{2+}] was evaluated by monitoring changes in Fura-2 fluorescence (Ong et al., 2010) in L444P, N370S, and wild-type GC fibroblasts treated with lacidipine or diltiazem (Figure 3). Lacidipine treatment was observed to deplete cytosolic [Ca^{2+}] with higher efficiency than diltiazem treatment in all cell types. In addition, depletion of cytosolic [Ca^{2+}] is markedly more enhanced in L444P GC than in N370S GC cells, suggesting a correlation between LTCC blocker-mediated Ca^{2+} homeostasis modulation and rescue of mutated GC variants' folding.

Lacidipine Treatment Upregulates BiP Expression in L444P GC Fibroblasts

We previously reported that the ER luminal chaperone BiP plays a key role in L444P GC folding. Upregulation of BiP expression, in combination with moderate UPR induction through MG-132 treatment, was shown to dramatically enhance the folding of L444P GC (Wang et al., 2011). We asked whether cell treatment with lacidipine influences the expression of ER chaperones and conducted quantitative RT-PCR analyses to measure the expression of the representative chaperones BiP, CNX, and Calreticulin (CRT) in L444P GC fibroblasts treated with lacidipine (20 μM), diltiazem (10 μM), MG-132 (0.6 μM), celastrol (0.6 μM), or a combination thereof (Figures 4A–4C). BiP expression (Figure 4A) was dramatically upregulated by lacidipine treatment (5.6-fold; $p < 0.01$), and lacidipine and MG-132 cotreatment (13.1-fold; $p < 0.01$). Diltiazem treatment resulted in a milder increase in BiP expression (1.8-fold), even when used in combination with MG-132 (3.1-fold). Although celastrol treatment was observed to cause a modest increase in BiP expression (1.9-fold), supplementing celastrol-containing medium with a LTCC blocker did not influence BiP transcription.

ER chaperone expression in cells treated with lacidipine (20 μM), diltiazem (10 μM), and MG-132 (0.6 μM) was confirmed by western blot using chaperone-specific antibodies (Figure 4D). BiP protein accumulation was enhanced by treatment with lacidipine alone or in combination with MG-132 compared to untreated cells but only slightly enhanced by diltiazem and MG-132 treatment. CNX and CRT protein levels did not seem to be drastically altered. These results are consistent with RT-PCR analyses and confirm the key role of BiP expression in promoting native folding of L444P GC (Wang et al., 2011).

As opposed to what was previously reported for cells treated with RyR blockers, which despite dramatically enhancing MG-132-mediated L444P GC folding rescue do not directly modulate the expression of ER chaperones (Wang et al., 2011), these results indicate that lacidipine's mechanism of action is based on extensive remodeling of ER chaperone pathways. However, we found that although cell treatment with lacidipine significantly enhances BiP expression, it does not alter its cellular localization. Particularly, immunofluorescence studies conducted to test BiP localization in the ER and in the Golgi revealed that BiP is still primarily localized in the ER (Figure S3).

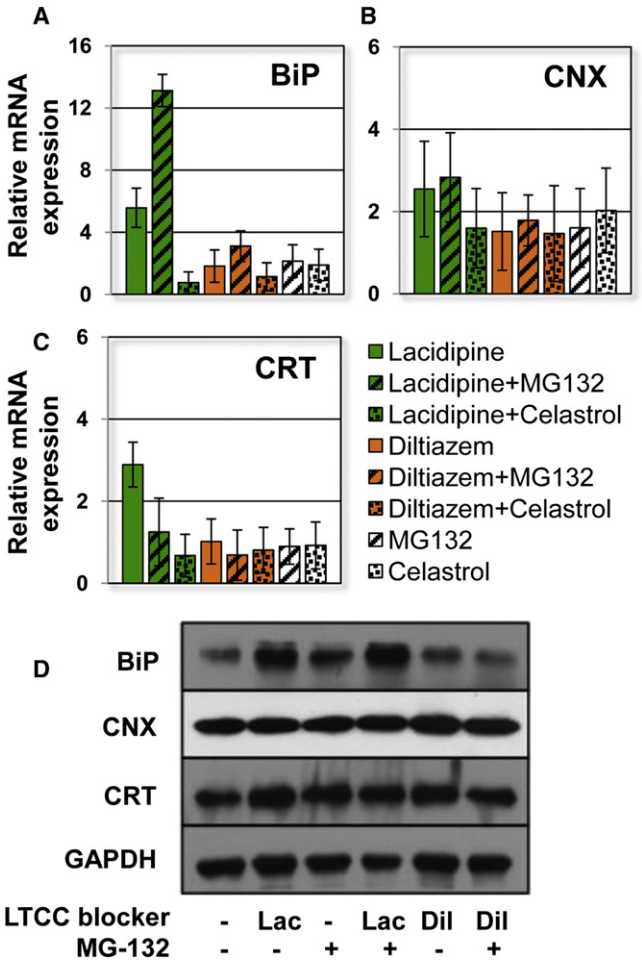

Figure 4. Treatment of Patient-Derived Fibroblasts with Lacidipine Upregulates BiP Expression

(A–C) Relative mRNA expression levels of (A) BiP ($p < 0.01$), (B) CNX ($p < 0.05$), and (C) CRT ($p < 0.05$) in L444P GC fibroblasts treated with lacidipine (20 μM), diltiazem (10 μM), MG-132 (0.6 μM), and celastrol (0.6 μM) for 24 hr were obtained by quantitative RT-PCR, corrected by the expression of the housekeeping gene GAPDH, and normalized to those of untreated cells. The data are reported as mean ± SD.

(D) Western blot analyses of BiP, CNX, CRT, and GAPDH (used as loading control) accumulation in cells treated with lacidipine (20 μM) and MG-132 (0.6 μM) for 48 hr. Lac, lacidipine; Dil, diltiazem.

See Figure S3.

Lacidipine Treatment Causes Modest Activation of All Three Arms of the UPR but Does Not Induce Cytotoxicity in L444P GC Patient-Derived Fibroblasts

The UPR is a tripartite signal-transduction cascade activated in response to the accumulation of misfolded proteins in the ER. UPR induction is mediated by the activation of three integral ER membrane proteins, namely inositol requiring kinase 1 (IRE1), activating transcription factor 6 (ATF6), and double-stranded RNA-activated ER kinase (PERK) (Schroder and Kaufman, 2005), which lead to the upregulation of UPR-related genes, including chaperones and ERAD proteins. The expression of ATF6, PERK, and IRE1 was investigated to evaluate UPR induction in GD patient-derived fibroblasts treated with lacidipine and diltiazem. Lacidipine was found to activate two of the three arms of the UPR and with higher efficiency than diltiazem. The increase in expression of UPR-associated genes in cells treated with lacidipine was considerably enhanced by the addition of MG-132. These results correlate with measurements of L444P GC activity reported above, in which maximal increase was obtained upon cotreatment with lacidipine and MG-132 (Figure 1).

Activation of IRE1 causes X-box binding protein-1 (Xbp-1) mRNA cleavage (Ron and Walter, 2007). The product of Xbp-1 spliced mRNA acts as an activator of UPR target genes, whereas the product of the unspliced Xbp-1 precursor acts as a repressor (Ron and Walter, 2007). RT-PCR experiments followed by gel electrophoresis were conducted to evaluate the accumulation of the spliced and unspliced forms of Xbp-1 in L444P GC fibroblasts treated with LTCC blockers (lacidipine [20 μM] or diltiazem [10 μM]) and a proteostasis regulator (MG-132 [0.6 μM] or celastrol [0.6 μM]) for 24 hr (Figure 5A). Treatment with MG-132 enhanced Xbp-1 splicing in L444P GC fibroblasts, as previously reported (Mu et al., 2008b). Spliced Xbp-1 was barely detectable in cells treated with lacidipine. However, a 4.8-fold increase in spliced Xbp-1 accumulation was observed upon cotreatment with lacidipine and MG-132 compared to treatment with MG-132 only, recapitulating the synergistic effect of lacidipine and MG-132 observed in enzymatic assays (Figures 5A and 5B). A 4.3-fold increase in spliced Xbp-1 was detected in L444P GC fibroblasts treated with diltiazem and MG-132 compared to that measured in fibroblasts treated with MG-132 only. Although cell treatment with celastrol causes increase in Xbp-1 splicing and in L444P GC folding rescue (Mu et al., 2008b), addition of celastrol to the media of lacidipine- or diltiazem-treated cells did not increase Xbp-1 splicing. Taken together, these results suggest a synergistic effect of LTCC blockers lacidipine and diltiazem and the proteostasis regulator MG-132 on the activation of the IRE1 arm of the UPR in L444P GC patient-derived fibroblasts. Among the culturing conditions investigated, the highest degree of Xbp-1 splicing was observed in cells displaying the maximum increase of L444P GC activity (lacidipine and MG-132 treatment, Figure 1), suggesting a key role of IRE1 activation in rescuing L444P GC folding.

The second arm of the UPR is mediated by ATF6 activation (Ron and Walter, 2007). Quantitative RT-PCR was used to evaluate ATF6 expression in cells treated as described above. Lacidipine treatment resulted in ATF6 upregulation (2.1-fold), which was further enhanced by the addition of MG-132 (3.5-fold), suggesting that the ATF6 arm of the UPR is also activated by treatment with lacidipine, particularly when used in combination with MG-132 (Figure 5C). Diltiazem treatment barely affected ATF6 expression, and the addition of neither MG-132 nor celastrol to diltiazem-supplemented media caused significant changes.

The third branch of the UPR is induced by PERK oligomerization and phosphorylation of the eukaryotic translation initiation factor-2 (eIF2α). eIF2α induces the expression of the transcription factor ATF4 and a subset of ATF4 target genes, including CHOP (Ron and Walter, 2007). Lacidipine treatment caused CHOP upregulation (3.7-fold), which was considerably enhanced by the addition of MG-132 (5.0-fold), indicating that the PERK arm of the UPR is activated in response to lacidipine treatment (Figure 5D). Treatment with diltiazem alone or in

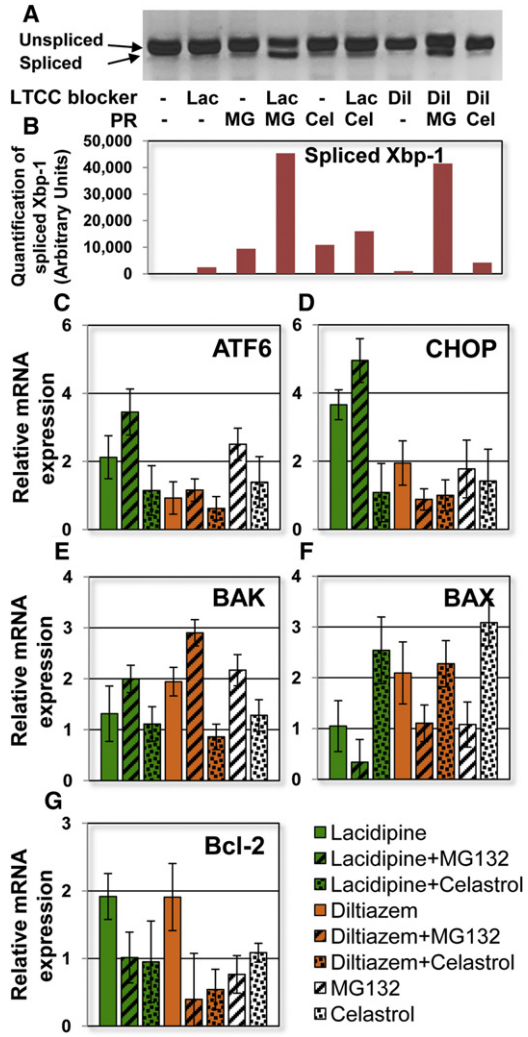

Figure 5. Treatment of Patient-Derived L444P GC Fibroblasts with Lacidipine Results in Upregulation of the UPR without Induction of Apoptosis

Cells were treated with lacidipine (20 μM), diltiazem (10 μM), MG-132 (0.6 μM), and celastrol (0.6 μM) for 24 hr.

(A) Xbp-1 expression and splicing were determined by RT-PCR followed by gel electrophoresis.

(B) Quantification of spliced Xbp-1 band intensities was conducted using the NIH ImageJ analysis software. PR, proteostasis regulator; Lac, lacidipine; Dil, diltiazem; MG, MG-132; Cel, celastrol.

(C–G) Relative mRNA expression levels of (C) ATF6, (D) CHOP, (E) BAK, (F) BAX, and (G) Bcl-2 were obtained by quantitative RT-PCR and calculated as described in Figure 4. The data are reported as mean ± SD ($p < 0.01$).

combination with a proteostasis regulator did not cause significant changes in CHOP expression (Figure 5D).

Prolonged induction of the UPR and inability of the ER folding capacity to cope with the load of misfolded proteins lead to apoptosis. A number of genes are involved in the regulation of apoptosis induction, including the proapoptotic genes encoding for Bcl-2 homologous antagonist (BAK) and Bcl-2-associated X protein (BAX) (Scorrano et al., 2003), and the antiapoptotic gene encoding the apoptosis regulator Bcl-2 (Rodriguez et al., 2011). The expression of BAK, BAX, and Bcl-2 was investigated in cells treated with LTCC blockers and proteostasis regulators as described before. MG-132 and celastrol treatments caused upregulation of the proapoptotic proteins BAK and BAX. Specifically, MG-132 induced upregulation of BAK (2.1-fold, Figure 5E), and celastrol induced upregulation of BAX (3.1-fold, Figure 5F). Lacidipine did not significantly alter either BAX or BAK expression, whereas diltiazem caused upregulation of both BAK (1.9-fold) and BAX (2.1-fold). Cotreatment with lacidipine and MG-132 was compared to treatment with MG-132. When cells were cotreated with lacidipine and MG-132, BAK expression was barely altered, but BAX expression was observed to decrease, compared to cells treated with MG-132 only. This is interesting because lacidipine and MG-132 were shown to have a synergic effect on the rescue of L444P GC activity (Figure 1C) and on UPR activation (Figures 5A–5D). Because enhanced UPR activation typically leads to enhanced apoptosis induction, both BAX and BAK expression would be expected to increase in cells treated under these conditions. Particularly, BAX expression was lowered 2.9-fold in cells treated with lacidipine and MG-132, suggesting that the mechanism of lacidipine-mediated L444P GC folding rescue involves inhibition of apoptosis via BAX downregulation. Interestingly, when diltiazem was used in combination with a proteostasis regulator, MG-132-mediated BAK upregulation was enhanced (2.9-fold), but celastrol-mediated BAX upregulation was lowered, which may explain why treatment with celastrol, but not with MG-132, results in enhancement of L444P GC activity increase mediated by diltiazem.

The expression of the antiapoptotic Bcl-2 encoding gene was also evaluated (Figure 5G). Bcl-2 contributes to maintaining ER Ca^{2+} homeostasis by reducing $[Ca^{2+}]_{ER}$ efflux (Eckenrode et al., 2010; Rong et al., 2009), and was found to be upregulated in L444P GC fibroblasts cultured with either lacidipine or diltiazem (1.9-fold), underscoring the therapeutic potential of Ca^{2+} homeostasis modulation in L444P GC fibroblasts. MG-132 treatment lowered Bcl-2 expression (0.8-fold), whereas celastrol treatment did not seem to affect it. The addition of a proteostasis regulator to lacidipine-treated cells resulted in lowered Bcl-2 expression, particularly it brought Bcl-2 expression back to the level detected in untreated cells, whereas the addition of a proteostasis regulator to diltiazem-treated cells resulted in substantial downregulation of Bcl-2 (MG-132 and diltiazem: 2.3-fold; celastrol and diltiazem: 2.0-fold). Similar to what was reported above regarding the expression of proapoptotic genes, modulation of Bcl-2 expression correlates with the ability of LTCC blockers to rescue L444P GC folding when used alone or in combination with an UPR-inducing proteostasis modulator.

We next tested whether lacidipine-mediated changes in the expression of pro- and antiapoptotic genes translate in differences in cytotoxicity and cell death, a common marker of cells treated for L444P GC folding rescue through UPR activators, such as MG-132, tunicamycin, and thapsigargin (Wang et al., 2011). L444P GC patient-derived fibroblasts treated with Ca^{2+} blockers and proteostasis regulators as described above were tested using the CytoGLO™ Annexin V-FITC Apoptosis Detection Kit to monitor membrane rearrangement (Annexin V binding) and fragmentation (propidium iodide [PI] binding), which occur during early and late apoptosis, respectively (Table 1; Figure S4).

Table 1. Cell Toxicity Assay

	Annexin V		PI	
Cell Treatment	Population[a]	Binding[b]	Population[a]	Binding[b]
Lacidipine	1.80 ± 0.35	−2.92 ± 1.84	−3.30 ± 0.86	2.46 ± 1.26
MG-132	7.20 ± 0.12	47.36 ± 0.99	7.40 ± 0.38	27.87 ± 0.87
Lacidipine+MG-132	3.55 ± 0.14	40.77 ± 1.07	4.45 ± 0.26	14.53 ± 1.80
Celastrol	1.80 ± 0.86	10.40 ± 0.27	4.70 ± 0.23	18.98 ± 1.18
Lacidipine+celastrol	1.00 ± 0.53	8.34 ± 1.21	4.05 ± 0.95	13.20 ± 0.18

$p < 0.01$. Flow cytometry histograms are reported in Figure S4.
[a] Change (%) in number of cells bound to Annexin V/PI compared with untreated cells.
[b] Change (%) in Annexin V/PI-binding affinity compared with untreated cells.

Treatment of L444P GC fibroblasts with lacidipine (20 μM) did not cause cytotoxicity, and induction of apoptosis was not significantly altered compared to untreated cells. MG-132 (0.6 μM) and celastrol (0.6 μM) resulted in 47.36% and 10.40% increase in Annexin V binding, and 7.40% and 4.70% increase in dead cell population, respectively. The addition of lacidipine to MG-132 or celastrol-treated cells led to a decrease in Annexin V binding to 40.77% and 8.34% and to a decrease in dead cell population to 4.45% and 4.05%, respectively. These results indicate that lacidipine treatment under conditions observed to rescue mutated GC folding and induce UPR not only does not cause cytotoxicity but also partially counteracts the cytotoxic effect of UPR-inducing proteostasis regulators.

Interestingly, lacidipine mechanism of L444P GC folding rescue differs significantly from that of RyR blockers reported previously (Wang et al., 2011). Although cell treatment with RyR blockers does not activate the UPR but rescues GD fibroblasts from UPR-induced toxicity, lacidipine treatment concurrently activates the UPR and ameliorates UPR-induced toxicity in GD fibroblasts.

Lacidipine Treatment Upregulates GC Chromosomal Expression in L444P GC Fibroblasts

As briefly alluded to before (Figures 2A and 2B), the total amount of L444P GC seems to be enhanced by lacidipine treatment. Upregulation of GC gene (GBA) as well as of other genes encoding for lysosomal enzymes involved in lipid metabolism was previously reported in cells treated for the rescue of L444P GC folding through UPR induction. Particularly, we found that a number of genes associated with the development of lysosomal storage disorders, such as Niemann-Pick, Tay-Sachs, and Fabry diseases, were upregulated upon MG-132-induced UPR (Wang et al., 2011). This finding resonates with the general increase in lipid metabolism that normally occurs during UPR (Schroder and Kaufman, 2005) and was suggested as a potentially therapeutic "side-effect" of mutated GC proteostasis regulation via UPR activation (Wang et al., 2011).

Quantitative RT-PCR and western blot analyses were conducted to understand whether the increase in cellular concentration of L444P GC observed in cells treated with lacidipine is due to upregulation of GC expression in addition to L444P GC-enhanced folding and lowered ERAD. L444P GC fibroblasts were treated with lacidipine (20 μM), diltiazem (20 μM), MG-132 (0.6 μM), celastrol (0.6 μM), or a combination thereof (Figure 6A). Lacidipine treatment was observed to enhance GC mRNA expression (3.1-fold) to an extent similar to MG-132 (3.1-fold) or celastrol (3.4-fold). Treatment with diltiazem resulted in a lower increase in GC expression (2.5-fold), most likely reflecting diltiazem's milder effect on UPR induction. Interestingly, addition of MG-132 resulted in increase in both lacidipine and diltiazem-mediated GC upregulation (4.5- and 4.6-fold, respectively), whereas celastrol slightly lowered it (2.9- and 2.2-fold, respectively). These transcriptional changes were confirmed at the translational level by western blot analyses (Figure 6B). L444P GC fibroblasts were cultured with lacidipine (20 μM), MG-132 (0.6 μM), and celastrol (0.6 μM) for 48 hr, and bands detected with a GC-specific antibody were quantified using NIH ImageJ analysis software (Figure 6C). The L444P GC content of lacidipine-treated cells increased about 50% compared to that of untreated cells, similarly to what was observed in MG-132-treated cells. In addition the combination of lacidipine and MG-132 caused a 2.3-fold increase in total L444P GC, which is higher than what was observed in cells treated with either one of these molecules, and is in perfect agreement with the results obtained from quantitative RT-PCR. Celastrol treatment did not significantly affect GC protein accumulation. In summary these data indicate that GC chromosomal expression is enhanced upon treatment with lacidipine, and suggest that GC upregulation contributes to L444P GC folding rescue mediated by UPR induction.

DISCUSSION

Ubiquitously expressed voltage-gated LTCCs support inward current of Ca^{2+} ions. The function of Ca^{2+} ions as an intracellular second messenger has been reported in many cellular processes, ranging from gene expression to cardiac and smooth muscle contraction. Because Ca^{2+} mediates both physiological and pathological events, considerable effort has been devoted to the study of Ca^{2+} channel antagonists, a chemically and pharmacologically heterogeneous group of drugs widely used as therapeutic agents as well as research tools. The prototypical LTCC antagonists are diltiazem (a benzothiazepine), verapamil (a phenylalkylamine), and nifedipine (a 1,4-dihydropyridine) (Triggle, 2006). Diltiazem and verapamil are FDA-approved drugs for the treatment of hypertension and cardiac arrhythmias (Hockerman et al., 1997). They were reported to rescue folding, trafficking, and activity of GC variants in patient-derived fibroblasts (Mu et al., 2008a),but failed to rescue mutated GC activity in mice (Sun et al., 2009). In an effort to discover small molecules that

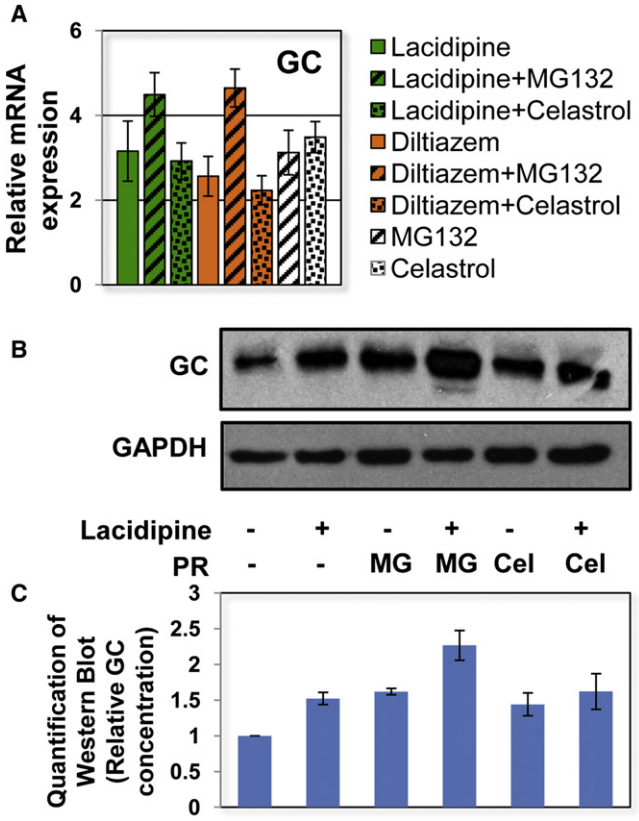

Figure 6. L444P GC Expression Is Upregulated in Fibroblasts Treated with Lacidipine and Diltiazem

(A) Relative GC mRNA expression level was evaluated by quantitative RT-PCR in L444P GC fibroblasts treated with lacidipine (20 μM), diltiazem (10 μM), MG-132 (0.6 μM), and celastrol (0.6 μM) for 24 hr ($p < 0.01$). mRNA expression levels were calculated as described in Figure 4.

(B) Representative western blot analysis of cells treated with lacidipine (20 μM), MG-132 (0.6 μM), and celastrol (0.6 μM) for 48 hr using GC-specific antibody.

(C) Western blots band quantification. GC bands were quantified by NIH ImageJ analysis software. GAPDH expression was used as a loading control. PR, proteostasis regulator; MG, MG-132; Cel, celastrol. The data are reported as mean ± SD.

efficiently rescue the folding of mutated GC variants by enhancing the cellular folding capacity but without inducing cytotoxicity, we tested a series of 1,4-dihydropyridines, a class of LTCC antagonists known to lower intracellular [Ca^{2+}] with higher selectivity than benzothiazepines and phenylalkylamine (Triggle, 2003).

Lacidipine was found to rescue the activity of GC variants carrying the two most common mutations, L444P and N370S, in GD patient-derived fibroblasts. In particular, lacidipine mediates a substantially higher increase in L444P GC activity than what was observed using other LTCC and RyR blockers to date (Mu et al., 2008a; Ong et al., 2010; Wang et al., 2011), and this increase is markedly enhanced by cotreatment with proteostasis modulators MG-132 and celastrol. The nature of LTCC blockers' chemical structure—with lacidipine highly hydrophobic, diltiazem and verapamil charged at physiologic pH (Triggle, 2003)—is likely to influence their cell permeability and explain why treatment of GD fibroblasts with lacidipine resulted in higher depletion of cytosolic [Ca^{2+}] and more effective remodeling of L444P GC proteostasis compared to diltiazem and verapamil.

L444P GC fibroblasts treated with lacidipine (and diltiazem for comparison) were used to conduct mechanistic studies and gain a better understanding of the molecular mechanisms involved in L444P GC proteostasis. We demonstrated that lacidipine-mediated rescue of mutated GC folding (Figures 1 and 2) correlates with its ability to: (1) lower cytoplasmic [Ca^{2+}], thereby counteracting the effect of GC substrate accumulation (Figure 3); (2) enhance the ER's folding capacity via substantial upregulation of BiP expression (Figure 4), confirming that BiP plays a key role in the folding of L444P GC; (3) induce the UPR (Figure 5) and upregulate GC expression (Figure 6); and (4) lower cytotoxicity and limit UPR-mediated apoptosis induction (Figure 5 and Table 1). An analogous mode of action was previously reported to explain the synergic effect of two distinct small molecules, a proteostasis regulator (MG-132) and a RyR blocker (ryanodine), on L444P GC folding rescue. Particularly, MG-132 was reported to induce BiP upregulation and UPR induction, and ryanodine to lower intracellular [Ca^{2+}] and counteract UPR-induced cytotoxicity (Wang et al., 2011), suggesting that combining these different mechanisms of proteostasis regulation is an effective strategy to rescue mutated GC folding. In summary this study sheds light on the cellular pathways involved in mutated GC folding and introduces a novel strategy to rescue mutant GC folding via small molecule treatment that combines remodeling of two general cellular pathways involved in protein homeostasis: protein folding and Ca^{2+} homeostasis.

SIGNIFICANCE

This work describes an efficient strategy to rescue L444P GC folding, the most frequently encountered allele causing neuronopathic forms of Gaucher's disease. Treatment of patient-derived fibroblasts with lacidipine leads to rescue of folding, lysosomal trafficking, and activity of mutated GC variants. By comparing lacidipine to other LTCC blockers previously reported to partially restore the folding of mutated GC, we demonstrate that lacidipine efficiently lowers cytosolic [Ca^{2+}] and, thus, counteracts the deleterious effect of GC substrate accumulation. We profiled the expression of a number of genes involved in cellular folding, ER stress and UPR, and apoptosis induction, and demonstrate that lacidipine remodels GC proteostasis by influencing the expression of ER chaperones and by inducing the UPR. However, unlike previously reported proteostasis regulators, lacidipine limits cytotoxicity and prevents induction of apoptosis typically associated with sustained UPR activation. By uncovering the molecular mechanisms associated with the rescue of L444P GC folding induced by Ca^{2+} homeostasis modulation, this study lays the foundation for the development of strategies to modulate mutated GC folding pathway for therapeutic intervention, which is particularly significant for the treatment of presently incurable neuronopathic forms of Gaucher's disease.

EXPERIMENTAL PROCEDURES

GC Activity Assay

The intact cell GC activity assay was performed as described previously (Mu et al., 2008b) and in the Supplemental Experimental Procedures.

Quantitative RT-PCR

RT-PCR was conducted as described previously (Wang et al., 2011) and in the Supplemental Experimental Procedures, using the primers listed in Table S1.

Western Blot Analyses and Immunofluorescence Assays

Details are provided in the Supplemental Experimental Procedures.

Intracellular [Ca^{2+}] Measurement

Fura-2, AM (AnaSpec) was used to measure cytosolic [Ca^{2+}] according to company's instructions. Briefly, cells were incubated with 5 μM Fura-2, AM and 0.05% (w/v) Pluronic F-127 (Invitrogen) at 37°C for 30 min. Following two washing steps, fluorescence was measured (excitation 340 and 380 nm, emission 510 nm). Fluorescence ratio of excitation 340/380 reflects relative intracellular Ca^{2+} level. Additional details are provided in the Supplemental Experimental Procedures.

Toxicity Assay

Toxicity assays were conducted as described previously (Wang et al., 2011) and in the Supplemental Experimental Procedures.

Statistical Analysis

All data are presented as mean ± SD, and statistical significance was calculated using a two-tailed Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at doi:10.1016/j.chembiol.2011.04.008.

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