Published in final edited form as:

Biochem J. 2012 January 15; 441(2): 685–696. doi:10.1042/BJ20111004.

DEFECTIVE TRAFFICKING OF CONE PHOTORECEPTOR CNG CHANNELS INDUCES THE UNFOLDED PROTEIN RESPONSE AND ER STRESS-ASSOCIATED CELL DEATH

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SYNOPSIS

Mutations that perturb the function of photoreceptor cyclic nucleotide-gated (CNG) channels are associated with several human retinal disorders, but the molecular and cellular mechanisms leading to photoreceptor dysfunction and degeneration remain unclear. Many loss-of-function mutations result in intracellular accumulation of CNG channel subunits. Accumulation of proteins in the endoplasmic reticulum (ER) is known to cause ER stress and trigger the unfolded protein response (UPR), an evolutionarily conserved cellular program that results in either adaptation via increased protein processing capacity or apoptotic cell death. We hypothesize that defective trafficking of cone photoreceptor CNG channels can induce UPR-mediated cell death. To test this idea, CNGA3 subunits bearing the R563H and Q655X mutations were expressed in photoreceptor-derived 661W cells with CNGB3 subunits. Compared to wild type, R563H and Q655X subunits displayed altered degradation rates and/or were retained in the ER. ER retention was associated with increased expression of UPR-related markers of ER stress and with decreased cell viability. Chemical and pharmacological chaperones (TUDCA, 4PBA, and the cGMP analog CPT-cGMP) differentially reduced degradation and/or promoted plasma-membrane localization of defective subunits. Improved subunit maturation was concordant with reduced expression of ER stress markers and improved viability of cells expressing localization-defective channels. These results indicate that ER stress can arise from expression of localization defective CNG channels, and may represent a contributing factor for photoreceptor degeneration.

Keywords

Retinal disease; misfolding; chaperone; UPR; membrane localization; ion channel subunit

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AUTHOR CONTRIBUTIONS

M.D.V. conceived and supervised the project. D.L.D., R.L.B. and M.D.V. designed the experiments. Experiments were carried out by D.L.D. D.L.D., R.L.B. and M.D.V. analyzed the data. D.L.D., R.L.B. and M.D.V. wrote the paper.

INTRODUCTION

Cyclic nucleotide-gated (CNG) channels are critical to the phototransduction cascade that underlies vertebrate vision. These channels open and close in response to light-induced changes in the intracellular cyclic GMP concentration; the resulting changes in membrane potential modulate activity at the photoreceptor synaptic terminal (reviewed in [1]). In mammals, six genes encode two subunit types (A and B), which form heterotetrameric channels. Mutations in photoreceptor CNG channel genes are associated with complete and incomplete achromatopsia (ACHM), progressive cone dystrophy (PCD), and macular degeneration (MD) [2–5]. Forms of ACHM, PCD, and MD linked to mutations in *CNGA3* and *CNGB3* are characterized by intact rod function and limited or absent cone function. ACHM, once considered a stable cone dystrophy, has recently been shown to exhibit progressive cone photoreceptor loss in some patients [2, 3], while PCD and MD are distinguished by cone degeneration [4, 5]. Genetic and clinical heterogeneity of these disorders has complicated phenotype-genotype correlations [4, 6], and the underlying mechanisms causing photoreceptor dysfunction and death in response to CNG channel mutations are not well understood. Functional characterization of CNG channels bearing disease-associated mutations has revealed both gain- and loss-of-function phenotypes [7–12]. Reduced or absent plasma-membrane (PM) localization commonly contributes to the functional deficit of loss-of-function mutations [7, 10–12]. As a prerequisite to assuming their appropriate plasma membrane location, CNG channels must undergo a number of protein maturation steps, including folding, assembly, and trafficking out of the endoplasmic reticulum (ER). Disposal of proteins that cannot successfully mature imposes a metabolic burden on the cell that may contribute to the pathophysiology associated with mutations that give rise to localization-defective channels. For such CNG channel mutations, it remains unexplored whether the loss of channel function is the sole pathogenic culprit, or if the metabolic stress of processing defective proteins also plays a role in disease progression.

Accumulation of proteins in the ER activates the unfolded protein response (UPR), a coordinated signaling program that protects the cell from ER stress (reviewed in [13]). Three resident ER luminal transmembrane proteins serve as proximal sensors of ER stress and initiate eponymous branches of the UPR: protein kinase-like ER kinase (PERK), inositol requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF6). Activated PERK phosphorylates eukaryotic translation initiating factor 2 (eIF2 α), rendering it unable to initiate global mRNA translation and immediately reducing the processing load of the ER. Activation of IRE1 and ATF6 upregulates expression of cytoprotective proteins involved in the processing capacity of the ER, including chaperone proteins (e.g., binding Ig protein, BiP) and the transcription factor X-box protein binding protein 1 (XBP1). Paradoxically, ER stress also induces production of pro-apoptotic signals (e.g., C/EBP homologous protein transcription factor (CHOP)). The speed, magnitude, and maintenance of specific signals determine the balance between adaptive and apoptotic pathways, neither of which is exclusive to any branch of the UPR [14]. If the cell cannot prevent accumulation of misfolded proteins, the prolonged perturbation from ER homeostasis can result in apoptotic cell death (reviewed in [15]).

ER stress has been linked recently to several channelopathies associated with channel mutations that cause trafficking defects, including the I593R mutation in the human *ether-a-go-go* related gene (HERG) associated with long QT syndrome type 2 (LQT2) [16] and the Δ F508 mutation in the cystic fibrosis transmembrane regulator (CFTR), the most common cause of CF [17]. Chemical chaperones, agents that intervene in the UPR to reduce ER stress, have been shown to improve trafficking of CFTR Δ F508 [18]. Furthermore, the function of a number of HERG trafficking-defective channels (e.g., N470D) can be rescued by channel blockers acting as pharmacological chaperones [19]. Such agents bind specific

structural elements within the substrate protein and reduce ER stress by promoting maturation, degradation, and/or trafficking of defective proteins [20–22]. The relative importance of channel activity restoration versus ER stress reduction remains undetermined for these channelopathies.

The involvement of the UPR in the pathophysiology of retinal degenerative disorders also is becoming evident [23–25]. Transgenic expression of autosomal dominant RP-associated P23H rhodopsin, known to accumulate in the ER and form pericentriolar inclusion bodies, induced ER stress in rat retinas [26]. Addition of the pharmacological chaperone and chromophore 11-*cis*-retinal promoted maturation and PM localization of P23H rhodopsin when heterologously expressed in HEK-293 cells [21, 22]. In mouse cone photoreceptor-derived 661W cells, co-expression of putative chaperone proteins reduced ER accumulation of P23H [27]. Recently, visual function was restored in P23H transgenic rats using viral gene delivery of the endogenous chaperone BiP [25]. Despite gathering evidence implicating ER stress in retinal degeneration, this issue remains unexamined in the context of mutant CNG channels that fail to localize to the plasma membrane.

Here, we test the hypothesis that trafficking defective CNG channel subunits cause ER stress and UPR-associated cell death. We observed changes in markers of ER stress and cell viability in photoreceptor-derived cells after heterologous expression of localization defective CNGA3 R563H and Q655X subunits, which have been linked previously to progressive cone dystrophy and complete achromatopsia [6]. Chemical and pharmacological chaperones were found to relieve ER stress and cytotoxicity, and to improve stability, ER exit, and/or PM localization of CNG channel subunits. Our results suggest a connection between expression of localization defective channel subunits and UPR-mediated cytotoxicity.

EXPERIMENTAL

Chemicals

1,4-dithiothreitol (DTT) CAS 27565-41-9, Roche Diagnostics Corporation (Indianapolis, IN); tunicamycin (Tn) CAS 11089-65-9, Calbiochem/EMD Biosciences, Inc. (La Jolla, CA); thapsigargin (Tg) CAS 67526-95-8, Sigma-Aldrich (St. Louis, MO); tauroursodeoxycholate sodium salt (TUDCA) CAS 14605-22-2, Calbiochem; sodium 4-phenylbutyrate (4PBA) CAS 1716-12-7, Calbiochem; CPT-cGMP CAS 51239-26-0, Sigma-Aldrich; cycloheximide CAS 66-81-9, Calbiochem; trypsin CAS 9002-07-7 Sigma-Aldrich; lima bean trypsin inhibitor CAS 9035-81-8, Sigma-Aldrich.

Molecular Biology, Cell Culture, and Transfection

For expression in mammalian cells, the coding sequences for wild type human CNGA3 (accession number Q16281) and CNGB3 (accession number Q9NQW8) subunits [9, 28] were fused with an amino-terminal EGFP or triple-FLAG tag and subcloned into the pcDNA3.1/Zeo(+) plasmid (Invitrogen, Carlsbad, CA) using unique restriction sites. Point mutations in A3 (R563H) and B3 (F525N) were made using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA); the Q655X deletion was made by PCR cassette mutagenesis. The murine cone photoreceptor-derived 661W cell line was a generous gift from Dr. Al-Ubaidi (University of Oklahoma Health Sciences Center). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gemini Bioproducts, Sacramento, CA), penicillin (100 UI/ml) and streptomycin (100 mg/ml; Gibco) at 37 °C in a humidified 5% CO₂ incubator. For pharmacological ER stress induction, cells were seeded in 6-well (10 cm²/well) plates at a density of approximately 2×10^5 cells/well in media supplemented with either 2 mM DTT, 10 µg/ml tunicamycin (Tn), or 0.5 µM thapsigargin

(Tg). At intervals after addition of chaperone agents, cells from duplicate wells were harvested simultaneously for RNA or protein for each treatment. Untreated control cells were harvested at time zero. For CNG channel experiments, cells were co-transfected with pcDNA3.1/Zeo plasmids encoding CNGA3 and CNGB3 subunits at a ratio of 1:1 (to form heteromeric channels) using LipofectAMINE 2000 and OptiMEM (Invitrogen) as per the manufacturer's protocol for cells in suspension. For RNA and protein experiments, cells were plated in 6-well (10 cm²/well) plates at a density of approximately 1.6×10^5 cells/well. For lactate dehydrogenase (LDH) assays, cells were plated in 96-well (0.3 cm²/well) plates at a density of approximately 8×10^3 cells/well. Transfection efficiency was assessed by GFP expression from the reporter plasmid pQBI25-fc2 (Qbiogene Inc.; MP Biomedicals USA, Solon, OH) in a transfection control sample; transfection efficiency was typically ~75%. The pcDNA3.1/Zeo(+) plasmid was used as a control to determine transfection toxicity. The pDsRed2-ER plasmid (Clontech, Mountain View, CA) was used to identify the endoplasmic reticulum in confocal microscopy experiments. For chaperone treatments media was exchanged at three hours post-transfection for DMEM supplemented with 10% FBS with or without chaperone agents (1 mM tauroursodeoxycholate sodium salt (TUDCA), 1 mM sodium 4-phenylbutyrate (PBA), or 1 μ M CPT-cGMP). For CNG channel subunit stability experiments, cycloheximide was added to 75 μ g/ml at 40 hr post-transfection.

RT-qPCR

At 24 hours post-transfection, RNA was extracted and purified using TRIzol (Invitrogen) per manufacturer's protocol. Oligo dT-primed reverse transcription was performed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Platinum Quantitative PCR SuperMix-UDG (Invitrogen) was used to perform qPCR. Briefly, 25 μ L reactions (1 ng of cDNA, 10 μ M primers) were run in triplicate in 96-well plates as follows: 2 min at 50 °C to degrade contaminants, 2 min at 95 °C to deactivate UDG and activate Platinum Taq, followed by 40 cycles of 15 sec at 95 °C melting, 15 sec 58 °C annealing, and 15 sec 72 °C elongation. An iCycler version 2.039 (Bio-Rad Laboratories, Inc., Hercules, CA) thermocycler was used, and threshold cycles (Ct's) determined using iCycler IQ Optical System Software version 3.1 set for Baseline Subtracted Curve Fit. Temperature gradient reactions were used to confirm efficient priming at 58 °C for all primer sets. Specificity of priming was confirmed by melt curve and electrophoretic analysis of products. Primer efficiency was calculated after determining Ct for template concentrations over at least three orders of magnitude. Δ Ct between treated and untreated cells was determined for all genes (except CNG channels), and the Pfaffl Method [29] was used to calculate fold changes using hypoxanthine-guanine phosphoribosyltransferase (HPRT) to standardize amount of RNA [30] in samples. 661W cells do not express detectable levels of CNGA3 mRNA [31] and therefore fold changes cannot be anchored, thus Δ Ct between CNGA3 and HPRT were compared directly between samples. Percentage of spliced XBP1 (XPB1s) was determined using the ratio of fold-changes for spliced:total XBP1.

Primers

mHPRT (+) AGTCCCAGCGTCGTGATTAG;
 mHPRT (–) CCAGCAGGTCAGCAAAGAAC;
 mXPB1s (+) TGAGTCCGCAGCAGGTG;
 mXPB1s (–) GAGGCAACAGTGTCTAGAGTCC;
 mXPB1 (+) AGAACACGCTTGGGAATGG;
 mXPB1 (–) CCACCAGCCTTACTCCACTC;
 mBip (+) TGTTTGTCCCCCTTACACTTGG;

mBip (–) GGCGGTTTTGGTCATTGG;
 mCHOP (+) GTCCCTGCCTTTTCACCTTG;
 mCHOP (–) TCGTTCTCCTGCTCCTTCTC;
 hCNGA3 (+) ATCACCTCGTGTGTTCTTTGG;
 hCNGA3 (–) GGCTGTCTTTGAATCTCTTTG;
 hCNGB3 (+) AGCAACCCCTCCAAGAAAAG;
 hCNGB3 (–) GGGGTTCTTCCTCCACTGC.

Immunoblotting

Cells were pelleted by centrifugation at 300 g for 3 min, gently washed with PBS buffer, and resuspended in 20 μ L ice-cold lysis buffer containing: 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM NaF, 1 mM NaVO₄, 1% NP40, 0.25% Na-deoxycholate; plus 1 protease inhibitor cocktail tablet (complete Mini EDTA-free; Roche Applied Science, Indianapolis, IN) per 50 ml lysis buffer. To each sample, 2 units of Turbo DNase (Ambion Inc.; Applied Biosystems Inc., Foster City, CA) were added. Samples were placed on ice and allowed to lyse for 20 min. Cellular debris was pelleted by centrifugation at 5000 g for 5 min, followed by collection of the supernatants. Immediately, NuPAGE[®] LDS Sample Buffer and Reducing Agent (Invitrogen, Carlsbad, CA) were added, and samples were heated to 70 °C for 10 min prior to gel loading. Proteins were separated by SDS-PAGE using 4–12% Bis-Tris NuPAGE[®] gels in MES-SDS Running Buffer plus Antioxidant (Invitrogen), then transferred to nitrocellulose using NuPage[®] Transfer Buffer (Invitrogen). Blots were incubated 1 hour in 50% Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) in Tris-buffered saline (TBS, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl), then overnight in primary antibody (or 1 hour for anti-actin and anti-CHOP antibodies) in 50% Odyssey Blocking Buffer in TBS plus 0.1% Tween-20 on ice. Blots were washed extensively in TBS + 0.1% Tween-20. Blots were incubated two hours in secondary antibodies in 50% Odyssey Blocking Buffer in TBS with 0.1% Tween-20 and 0.01% SDS on ice, then washed extensively. Proteins were detected using the Odyssey Infrared Imaging System and Software version 2.0 (LI-COR Biosciences) at medium resolution and 5.0 intensity. Protein bands were analyzed and quantitated using LI-COR Software; values were normalized to actin for each lane.

Cell-Surface Biotinylation

At 48 hours post-transfection, cells were gently rinsed three times with chilled (4 °C) Dulbecco's PBS, pH 8.0 to remove media, then treated with 0.01 mg/ml SulfoLink NHS-LC-Biotin (Pierce, Rockford, IL) in chilled DPBS, pH 8.0 for 30 min with gentle rocking at 4 °C. Reactions were terminated by washing cells three times for 5 min with ice-cold 100 mM glycine in PBS, pH 8.0. Cells were gently scraped from plate, transferred to tubes, and harvested for immunoblotting as described above.

Immunohistochemistry and Confocal Microscopy

Transfected cells were grown on poly-L-lysine coated glass coverslips. At 48 hours post-transfection, cells were rinsed with PBS, and then fixed with 4% paraformaldehyde in PBS for six minutes. After three 10 min washes in PBS, cells were incubated with AlexaFluor-conjugated wheat germ agglutinin (WGA) in PBS for 10 minutes, then rinsed twice for 10 minutes with PBS. Cells were then permeabilized for 30 minutes in 0.5% Triton X-100, 1% BSA, and 5% normal goat serum (NGS). Cells were rinsed three times with PBS, then incubated for 1 hour in 0.1% Triton X-100, 1% BSA, 5% NGS, and appropriate dilutions of primary antibodies. Cells were washed three times in PBS, then incubated in 0.1% Triton

X-100, 1% BSA, 5% NGS, and appropriate secondary antibodies at 1:2000. After three 10 min washes in PBS, coverslips were mounted on slides with Prolong Gold AntiFade Reagent (Molecular Probes, Eugene, OR). Images were collected as 0.4–0.6 micron Z-sections using an Olympus FluoView 1000 laser-scanning microscope (Olympus, Tokyo, Japan) in XYZ mode with a 60×/ 1.4 NA oil immersion lens. Line scan intensity and colocalization calculations were performed using FluoView 10-ASW version 1.6 software.

Membrane Isolation and Protease Digestion

Parallel plates of similarly confluent cultures were used to isolate crude membrane fractions. All steps were performed at 4 °C. Briefly, adherent cells were rinsed with PBS and scraped off into a 2 ml solution of 200 mM NaCl, 33 mM NaF, 10 mM EDTA, 50 mM HEPES (pH 7.4 with NaOH) plus a protease inhibitor cocktail (100 μ M phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin A, 1 μ g/ml of leupeptin, 4 μ l/ml of aprotinin). The cells were homogenized and centrifuged at 500 g for 10 min. The membrane fractions were pelleted from the low-speed supernatants by centrifugation at 60,000 r.p.m. in a TLA 100.1 rotor (Beckman) for 1 hour at 4 °C. Membrane pellets were resuspended in 50 μ l TBS. For each protease digest time course, 25 ng of trypsin was added to 70 μ l of a ten-fold membrane dilution. Time point samples of 10 μ l were transferred to microfuge tubes containing 2 μ g of trypsin inhibitor. At end of the time course, samples were analyzed by immunoblotting.

Antibodies

Antibody sources, dilutions, and applications as follows: anti-phospho-PERK, rabbit monoclonal, 1:500 (Cell Signaling Technology, Beverly, MA); anti-GRP78 BiP, rabbit polyclonal, 1:5000 (Abcam, Inc., Cambridge, MA); anti-GADD 153 (CHOP), mouse monoclonal, 1:1000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-actin, mouse monoclonal, 1:5000 (Chemicon/Millipore, Billerica, MA); anti-FLAG M2, mouse monoclonal, 1:50,000 (immunoblot) or 1:5,000 (confocal microscopy) (Sigma-Aldrich, St. Louis, MO); anti-rabbit (IR680), donkey 1:5000 (LI-COR Biosciences, Lincoln, NE); anti-mouse (IR800), donkey 1:10000, (LI-COR Biosciences); AlexaFluor 495 WGA at 5 μ g/ml, (Invitrogen); anti-DsRed, rabbit, 1:500 (Clontech); anti-mouse AlexaFluor 488, goat (Invitrogen, Carlsbad, CA); anti-rabbit AlexaFluor 594, goat (Invitrogen); anti-rabbit AlexaFluor 647, donkey (Invitrogen).

Cell Viability Assay

The lactose dehydrogenase (LDH) cytotoxicity assay kit (Roche Applied Science) was used as per the manufacturer's protocol. Briefly, cultured cells were transfected as described above and plated in 96-well tissue culture plates. Three hours post-transfection, media was exchanged for DMEM supplemented with 1% FBS with or without chaperone agents, with five to ten technical replicates per condition. After 48 hours of incubation, half of the culture media in each well was transferred to the identical well in another 96-well plate. The cells in the original plate were lysed, releasing LDH from intact cells into the remaining half of culture media. LDH activity was measured in both plates as per protocol using a coupled enzymatic reaction that uses LDH-reduced NADH to reduce the tetrazolium salt to formazan, which absorbs light at 490 nm. LDH from dead cells was determined by doubling the amount measured in the media plate. LDH in intact cells was determined by subtracting the LDH measured in the media plate from the LDH measured in the original (lysed cells) plate. Percent cytotoxicity was calculated from the ratio of LDH in media:cells, and normalized to the percent cytotoxicity in cells transfected with empty vector. Chaperone rescue was assessed by comparing percent cell death of chaperone-treated and untreated cells expressing the same CNGA3 subunit.

Statistical Analysis

Data were analyzed using SigmaPlot and SigmaStat (Systat Software Inc, San Jose CA), and expressed as mean $\pm$ SEM. Statistical significance was determined using ANOVA or paired (by biological replicate) Student's t-test; P values < 0.05 were considered significant.

RESULTS

Characterization of Disease-associated R563H and Q655X CNGA3 Mutations

Several mutations in the human CNGA3 channel subunit have been associated with retinal disease. The R563H mutation in CNGA3 lies within the cyclic nucleotide-binding domain (Fig. 1A) and alters cell-surface localization [7], while the Q655X (Q655stop) mutation disrupts a region important for CNG channel assembly [32], but little is known about the mechanistic underpinnings of the disease pathology. To investigate these mechanisms, we transiently expressed FLAG epitope-tagged CNGA3 and FLAG- or EGFP-tagged CNGB3 channel subunits in cone photoreceptor-derived 661W cells. These cells express elements of the phototransduction cascade [31, 33]. Absence of endogenous CNGA3 and minimal expression of CNGB3 channel subunits [31] make 661W cells a particularly useful model for the study of exogenous CNG channels in a cone photoreceptor-derived cellular environment. We determined transient expression levels of FLAG-tagged CNGA3 subunits and found that the mutations did not significantly alter steady-state levels relative to wild-type subunits (Fig. 1B). To quantitate cell-surface localization of CNGA3 subunits, we employed surface biotinylation using membrane-impermeable reagents (Fig. 1B). Relative to wild type, surface localization of R563H and Q655X was significantly reduced ($p < 0.05$ and $p = 0.02$, respectively) (Fig. 1C). Using confocal fluorescence microscopy, we observed significantly reduced PM localization of R563H and Q655X relative to wild-type CNGA3 in 661W cells ($p < 0.006$) (Figs. 1D and E). Next, we co-expressed an ER marker protein (*Discosoma* sp. red fluorescent protein DsRed2 fused at the 3' end to the "KDEL" ER-retention sequence) and evaluated colocalization with CNGA3 subunits using confocal microscopy (Fig. 1F). Compared to wild-type subunits, R563H and Q655X showed significantly greater ER localization ($p < 0.001$) (Fig. 1G). These results demonstrate that CNGA3 R563H and Q655X mutations reduced the trafficking competency and increased ER retention of CNGA3 subunits in 661W cells.

Proteins that fail to fold properly are retained in the ER, where they accumulate or proceed to ER-associated degradation (ERAD) (reviewed in [13]). Thus, we investigated the effects of R563H and Q655X mutations on the stability of CNGA3 and co-expressed CNGB3 subunits. After treatment with cycloheximide to halt protein synthesis, the amount of CNGA3 remaining in 661W cells was quantitated over the course of 24 hours. Core-glycosylated and unglycosylated subunits (Fig. 2A, upper and lower bands, respectively) were observed and their intensities pooled. The degradation rate relative to wild type CNGA3 was increased by the Q655X mutation, but unaffected by the R563H mutation (Figs. 2A and C). Simultaneously, the degradation rate of CNGB3 subunits was increased when coexpressed with R563H or Q655X compared to expression with wild-type CNGA3 (Figs. 2B and D). These results suggest that mutations in CNGA3 can give rise to structural abnormalities subject to the quality-control functions of the ER.

To investigate the effects of these mutations on the folding of CNGA3 subunits, we isolated membranes from cells expressing wild-type, R563H, or Q655X subunits and subjected them to trypsin proteolysis (Fig. 2E). Changes in proteolytic rate are presumed to indicate changes in the accessibility of trypsin-labile sites, which is used as an indicator of changes in protein structure. Recapitulating the stability results, trypsin proteolysis of R563H was indistinguishable from wild type, while Q655X showed a decreased rate of proteolysis (Fig.

2F). Collectively, these data show that structural defects produced by Q655X and R563H increase turnover and/or ER retention of CNG channel subunits.

Characterization of ER Stress in 661W Cells

Since CNGB3 R563H and Q655X subunits were found to be localization defective, we hypothesized that these mutations can cause ER stress and induce the UPR. To confirm the suitability of 661W cells as an ER stress model, we used exogenous agents known to induce ER stress and then characterized the subsequent UPR. Cells were incubated with thapsigargin (Tg), tunicamycin (Tn), or dithiothreitol (DTT) to deplete ER calcium stores, inhibit N-linked glycosylation, or prevent disulfide-bond formation, respectively. Activation of the three branches of the UPR were monitored by quantitating changes in splicing and transcription of XBP1 mRNA, phosphorylation of PERK, and expression of BiP and CHOP. XBP1 splicing increased within the first hour of each treatment (Fig. 3A), indicating a rapid response through the IRE1 branch. Compared to untreated cells, XBP1 transcription increased within 4 hours for each treatment, indicating a similarly responsive ATF6 branch (Fig. 3B). Transcription of BiP and CHOP also increased within 4 hours of treatment (Figs. 3C and D). Protein indicators of the UPR were analyzed by immunoblotting (Fig. 3E). PERK phosphorylation was significantly increased ($p = 0.03$) within 30 minutes, but was reduced ($p = 0.05$) after 6 hours of Tg treatment and was unchanged after 6 hours of Tn treatment compared to levels in untreated cells (Fig. 3F). Levels of BiP increased after 6 hours of Tn treatment ($p = 0.004$) but did not respond to Tg treatment. Expression of CHOP increased after 6 hours of treatment with Tg ($p = 0.02$) or Tn ($p = 0.03$). These data demonstrate an intact tripartite UPR system in 661W cells and support the use of these markers to monitor ER stress associated with expression of CNG channels.

Localization Defective CNG Channels Increase ER Stress

To test the hypothesis that localization-defective channel subunits induce ER stress, we assessed markers in cells transiently expressing wild type CNG channel subunits or mutant subunits exhibiting trafficking defects. We also examined CNGB3 F525N, a mutation previously shown to produce hyperactive but localization-competent channels [8]; no significant differences in protein expression levels between CNGB3 wild-type and F525N subunits were detected (normalized to wild type, 1.00 ± 0.12). Compared to untransfected cells, expression of wild type CNG channels modestly increased splicing of XBP1 and transcription of BiP and CHOP, but these changes were indistinguishable from expression of a GFP-only control ($p = 0.2$, 0.4 , and 0.4 , respectively), suggesting a non-specific effect. Compared to wild-type subunits, both R563H and Q655X induced significant increases in splicing of XBP1 and transcription of BiP, while F525N expression did not (Fig. 4A). CHOP transcription was increased by all three mutations compared to wild-type subunits (Fig. 4A). Transcription levels for XBP1 were not significantly different for any mutant compared to wild type channels ($p = 0.2$, 0.3 , and 0.6 for R563H, Q655X, and F525N, respectively). Finally, we used immunoblotting to examine protein markers of ER stress (Fig. 4B). PERK phosphorylation was increased by expression of R563H and Q655X but not by F525N (Fig. 4C). Similarly, increases in BiP and CHOP protein levels were observed only with expression of R563H or Q655X subunits (Fig. 4C). Elevation of these markers indicates that expression of localization defective CNG channel subunits increased ER stress in 661W cells.

Chaperones Reduce ER Stress Associated with Expression of Localization Defective CNG Channels

A number of studies have shown that chaperone compounds can reduce ER stress associated with expression of proteins with folding and trafficking defects [34]. We therefore tested representative chaperones for the ability to alleviate ER stress induced by expression of

localization defective CNG channel subunits. Chemical chaperones 4-phenylbutyrate (4PBA, 5 mM) and tauroursodeoxycholate (TUDCA, 1 mM), and the CNG channel ligand analog 8-(4-chlorophenylthio)-cGMP (CPT-cGMP, 1 μ M), a putative pharmacological chaperone, were applied to cells expressing CNGA3 wild-type, R563H, or Q655X subunits, and the effects on mRNA markers of ER stress were determined. No significant changes were detected in cells expressing wild type channel subunits (Figs. 5A-C). Splicing of XBP1 mRNA in cells expressing R563H or Q655X was reduced by TUDCA or 4PBA (Fig. 5A). However, CPT-cGMP significantly reduced XBP1 splicing only in cells expressing R563H subunits (Fig. 5A). Transcription of BiP displayed the same pattern of change as XBP1 mRNA splicing (Fig. 5B). Transcription of CHOP in cells expressing R563H was reduced by TUDCA or CPT-cGMP, and by TUDCA or 4PBA in cells expressing Q655X (Fig. 5C). Transcription of XBP1 was not significantly altered by any treatment (data not shown). Next, we measured the effects of chaperone treatment on protein indicators of ER stress (Fig. 6A). No significant changes were detected in cells expressing wild type channel subunits (Figs. 6B and C). Application of CPT-cGMP reduced the level of BiP protein in cells expressing R563H (Fig. 6B). No other changes in BiP protein levels were significant. The level of CHOP protein in cells expressing R563H or Q655X was reduced by 4PBA but not TUDCA treatment, while CPT-cGMP treatment significantly reduced CHOP protein only in cells expressing R563H subunits (Fig. 6C). No significant changes in phosphorylation of PERK were detected (data not shown). Together these data indicate that chaperones can reduce several markers of ER stress in cells expressing localization defective CNG channel subunits and that CPT-cGMP specifically affected cells expressing channels having a mutation in the cyclic nucleotide-binding domain.

Chaperones Improve Maturation and Trafficking of Localization Defective CNG Channels

We next addressed the question of how chaperones reduce ER stress in cells expressing localization defective CNG channel subunits. To examine the working hypothesis that chaperones alleviate ER stress by assisting maturation and trafficking of proteins, we determined the effect of chaperones on the degradation rate and localization of CNGA3 wild-type, R563H, and Q655X subunits. Using cycloheximide treatment to halt protein synthesis (Fig. 7A), we found that the stability of Q655X subunits was significantly increased by TUDCA and 4PBA, but not by CPT-cGMP (Fig. 7B). The stability of wild-type and R563H subunits was not significantly changed by any chaperone treatment (Fig. 7B). Next, we determined the effect of chaperones on cell-surface localization of CNG channels using cell-impermeant biotinylation (Fig. 8A). The surface localization of CNGA3 R563H subunits was restored to wild-type levels by TUDCA and CPT-cGMP (Fig. 8B). Surface localization of Q655X subunits was not significantly improved by chaperone treatment (Fig. 8B). Treatment with 4PBA altered cell morphology; since comparison of surface biotinylation data requires constant surface:volume ratios between samples, the effects of 4PBA on surface localization of CNGA3 subunits could not be determined. However, surface localization of Q655X and R563H were not significantly reduced compared to wild type CNGA3 subunits in cells treated with 4PBA (data not shown). Next, we measured plasma-membrane localization of CNGA3 subunits using confocal microscopy (Fig. 9A). In agreement with cell-surface biotinylation results, PM localization of R563H subunits was increased by TUDCA ($p < 0.001$) and CPT-cGMP ($p < 0.001$) (Fig. 9B). Additionally, PM localization of Q655X subunits was significantly increased with TUDCA ($p < 0.002$) but not CPT-cGMP treatment ($p = 0.6$). Finally, we measured ER retention of CNGA3 subunits by colocalization with an ER marker (DsRed2-ER) using confocal microscopy (Fig. 9C). Concordant with increased PM localization, ER retention of R563H subunits was reduced by TUDCA ($p < 0.001$) and CPT-cGMP ($p < 0.001$) (Fig. 9D). Additionally, ER retention of Q655X subunits was significantly reduced with TUDCA ($p < 0.001$) but not CPT-cGMP treatment ($p = 0.08$). This selectivity of CPT-cGMP rescue for

R563H suggests that the mechanism underlying its chaperone effect is mutation-specific. Collectively, these data show that alleviation of ER stress by chaperones is associated with improvement in the maturation and trafficking of localization defective CNG channel subunits.

Chaperones Reduce Cytotoxicity Associated with Expression of Localization Defective CNG Channels

Our working hypothesis, that ER stress plays a role in cell death arising from localization-defective CNG channels, predicts that alleviation of ER stress would promote viability of cells expressing such channels. To test this prediction in our model, we treated cells expressing CNG channels with chaperones and measured the effect on cell viability. Expression of R563H and Q655X subunits increased cell death relative to cells expressing wild-type channels (Fig. 10). Chemical chaperones TUDCA and 4PBA reduced cytotoxicity arising from R563H or Q655X expression, while the pharmacological chaperone CPT-cGMP rescued the viability of cells expressing R563H but not Q655X subunits (Fig. 10). Viability of cells expressing wild type CNG channels was not significantly affected by chaperone treatment. Together these results show that chaperone agents can improve the viability of cells expressing localization defective CNG channel subunits.

DISCUSSION

In this study, we demonstrate that expression of localization defective CNG channels bearing disease-associated mutations is associated with induction of the UPR and ER stress-related cytotoxicity in a photoreceptor-derived cell line. Expression of CNGA3 R563H or Q655X subunits increased markers of ER stress relative to levels associated with expression of wild-type subunits (Fig. 4). Increased splicing of XBP1 mRNA indicates signaling through the IRE1 branch of the UPR, and increased phosphorylation of PERK indicates activation of the eponymous UPR pathway. Increased expression of BiP and CHOP denotes activation of multiple branches of the UPR, including ATF6. The adaptive function of the UPR prevents cell death until the magnitude of ER stress reaches a tipping point, after which apoptosis is initiated [26]. Compared to the acute ER-stress response (Fig. 3) and rapid cell death induced by chemical agents (data not shown), the milder response to expression of localization defective CNG channel subunits suggests a mostly adaptive rather than apoptotic ER stress response over the short assay period examined here [14, 26]. These results are consistent with the reported disease progression associated with these and related mutations in humans, which can occur over a span of years [2, 3, 6], and support our interpretation that expression of localization defective CNG channel subunits is associated with ER stress.

Our results indicate that induction of the UPR was characteristic of localization-defective but not localization-competent channels. In contrast to trafficking defective CNGA3 subunits, cell death was not increased by expression of wild-type subunits. Similarly, expression of localization competent CNGB3 F525N did not increase markers of ER stress, with the exception of CHOP transcription (Fig. 4). Our previous work has shown that the F525N mutation gives rise to hyperactive CNG channels [8] and promotes calcium-dependent cytotoxicity in 661W cells (Liu, C. and M. Varum, unpublished). Thus, results here are in agreement with experiments demonstrating that elevated intracellular calcium increases transcription [35] and stability of CHOP mRNA [36] without inducing a coordinated ER stress response. The specificity of the ER stress response to localization-defective subunits indicates that it is not a general feature of CNG channel expression.

We treated channel-expressing cells with chaperone agents known to improve protein maturation and modulate the UPR in other disease models [22]. Our experiments

demonstrate that chaperone treatment reduced ER stress and improved viability in cells expressing trafficking defective CNG channel subunits. The chemical chaperone 4PBA increased survival of cells expressing either R563H or Q655X subunits (Fig. 10) and reduced markers of ER stress, with the exception of BiP protein levels (Figs. 5 and 6). This result suggests an adaptive state in which the endogenous chaperone BiP remains elevated while the pro-apoptotic CHOP returns to unstressed levels. Similarly, the chemical chaperone TUDCA rescued cells from the cytotoxicity associated with R563H or Q655X subunit expression (Fig. 10). Although mRNA markers of ER stress were reduced by TUDCA (Fig. 5), reductions of BiP and CHOP protein levels were not observed (Fig. 6). While chemical chaperones can stabilize proteins and hydrophobic aggregates indiscriminately, pharmacological chaperones bind and stabilize a specific structural element in a protein (e.g., ligand-binding pocket) in a quasi-native conformation [22]. Notably, the membrane-permeant ligand analog CPT-cGMP reduced markers of ER stress and cytotoxicity in cells expressing R563H but had no significant effect on cells expressing Q655X (Figs. 5, 6 and 10). The Q655X mutation eliminates a region distal to the cyclic nucleotide-binding domain (CNBD) important for subunit assembly [32, 37], thus it is not surprising that CPT-cGMP had no effect on cells expressing Q655X subunits. In all cases, improvements in cell viability occurred concomitantly with reduction in ER stress markers, providing strong evidence to support the hypothesis that ER stress contributes to the deleterious cellular effects of localization defective CNG channels.

The mechanisms underlying chaperone rescue of cell viability include overt changes in the processing of defective CNG channels. While progressive degeneration of cone photoreceptors in *CNGA3*^{-/-} and *CNGB3*^{-/-} mice demonstrates that CNG channels are necessary for cone photoreceptor function and survival [38, 39], here it is unlikely that chaperone rescue depends only on restoration of channel activity, since expression of wild type CNG channels did not improve cell viability (data not shown). Rather, chaperones likely alleviate ER stress by assisting CNG channel-maturation processes such as folding and trafficking. The relative steady-state expression levels of R563H and Q655X remained statistically equivalent to wild-type subunits after chaperone treatment (Figs. 7A, 8A, and data not shown). However, Q655X subunits degraded more rapidly than wild-type subunits (Fig. 2C), indicating a higher turnover rate. Chemical chaperones decreased the degradation rate of Q655X (Fig. 7) without changing relative steady state levels, thereby reducing the turn-over rate of Q655X subunits. Additionally, TUDCA improved surface localization and reduced ER retention of Q655X subunits (Fig. 9). Thus, decreased processing demand and improved trafficking correlated with reduced ER stress in chaperone-treated cells expressing Q655X subunits. Similarly, TUDCA and CPT-cGMP improved surface localization and reduced ER retention of R563H subunits (Figs. 8 and 9), suggesting that improved trafficking alleviates ER stress in cells expressing R563H. Chaperones may support forward trafficking by facilitating protein-protein interactions that promote ER exit (e.g., endogenous chaperones). Chemical chaperones are expected to operate in a general manner, and indeed surface localization of Q655X and R563H was indistinguishable from wild-type channels in cells treated with 4PBA (data not shown). The pharmacological chaperone CPT-cGMP is expected to aid folding of the CNBD, perhaps improving consequent interactions with proteins promoting forward trafficking. Although trypsin protease sensitivity of R563H was not significantly different from wild-type subunits (Fig. 2F), this method may not reveal subtle, local structural abnormalities. In agreement with this scenario, Matveev and coworkers [40] analyzed a nearby CNBD mutation and found a local effect on CNBD folding that did not alter proteolytic (trypsin) sensitivity. Collectively, these results suggest that improved CNG channel subunit maturation and trafficking may underlie alleviation of ER stress and improvement of viability in chaperone-treated cells.

Taken together, our results suggest that localization defective CNG channel subunits may contribute to the etiology of retinal disease by inducing ER stress. Genetic and functional defects that give rise to human channelopathies have been extensively studied (reviewed in [41]), and the involvement of ER stress in the context of channel trafficking abnormalities is gaining recognition. It has been proposed that cystic fibrosis-related diabetes (CFRD) is a result of ER stress-mediated apoptosis of pancreatic β -cells [42], in which the CFTR has been shown to be highly expressed [43]. CFRD is more than 20-fold more commonly associated with mutations that produce localization-defective rather than localization-competent CFTR [44]. Illustrating this, severe pancreatic insufficiency is common in patients homozygous for the $\Delta F508$ mutation [45]. For a HERG channelopathy, expression of the trafficking defective I593R subunit activates ER stress pathways that are hypothesized to contribute to the characteristic long QT syndrome, in part by altering calcium-handling physiology [16, 46]. Together, these studies imply that disease mechanisms arising from trafficking defective channel proteins are not limited to a loss of channel function, but also may include significant ER stress-mediated changes in cell physiology and viability.

Crosstalk of the UPR with both the intrinsic and extrinsic apoptotic pathways allows the ER to monitor and integrate other stressors in addition to protein accumulation. While oxidative stress, perturbed calcium homeostasis, and metabolic overload have been implicated in photoreceptor degeneration [47], appreciation of the connection with ER stress and the UPR is a more recent development. In a study using ER stress-activated indicator (ERAI) transgenic mice, application of NMDA to stimulate glutamatergic activity in the retina caused intracellular calcium overload, nitric oxide production, and ER stress-dependent apoptosis [23]. In the *rd1* mouse model of RP, the disabled cGMP phosphodiesterase causes increased CNG channel activity, leading to photoreceptor degeneration reported to arise from disrupted calcium homeostasis and oxidative stress [48]. Expression of UPR markers in the *rd1* mouse retina increased at post-natal day 10 and peaked at post-natal day 12, coinciding with the onset and peak of photoreceptor apoptosis [49]. These observations suggest that ER stress and the UPR are intimately involved in photoreceptor survival and may serve as a central processor of multiple sources of cellular insult.

The UPR has evolved as a protective mechanism, responding to internal and external stresses by returning the ER to homeostasis or inducing apoptosis in response to acute stress, and by adapting cellular mechanisms to accommodate chronic stress. Alternatively, successful adaptation allows cells to become resistant to future stresses, including those unrelated to the initial insult, in a process known as “preconditioning” [14, 50]. If the UPR does serve as a central processor of cellular stressors, then it might in some cases explain the phenotypic heterogeneity of retinal degeneration in individuals with similar genotypes, since environmental factors or genetic background would contribute to the stress load sensed by the ER. Photo-receptors have evolved to withstand a lifetime of physiological demands and environmental exposure. Thus it is conceivable that regulation of their UPR is heavily biased for adaptation and a large accumulation of insults is required to initiate apoptosis.

The current study, along with other recent evidence [23, 24, 26], suggests involvement of ER stress in photoreceptor degeneration. We envision a model in which trafficking-defective channels induce ER stress and activate the UPR, ultimately leading to apoptosis. Based on our model, chaperone therapy might intervene in this process to slow photoreceptor death. In addition, elements of the UPR (e.g., translation attenuation) may complicate gene replacement strategies designed to restore function in cases involving misfolded and/or mislocalized endogenous channels. Alternatively, gene therapy targeting elements of the UPR itself may prove beneficial. Recognizing the contribution of ER stress and the UPR to the deleterious consequences of CNG channel-trafficking defects may offer new avenues for treatment or prevention of debilitating retinal dystrophies.

Acknowledgments

The authors thank E. Rich for expert technical support, and J. Harding and L. Orfe for critical comments on the manuscript. The authors are also grateful to Prof. M. R. Al-Ubaidi for providing the 661W cell line, and to Prof. K.-W. Yau for sharing the cDNA clone for human CNGA3.

FUNDING

The work was supported by grants from NIH/NEI (EY12836 to M.D.V. and EY19907 to R.L.B.), the Adler Foundation and the Autzen Endowment to M.D.V., and by a Poncin Award to D.L.D.

Abbreviations

CNG	Cyclic nucleotide-gated
UPR	unfolded protein response
cGMP	cyclic guanosine monophosphate
ACHM	achromatopsia
PCD	progressive cone dystrophy
MD	macular degeneration
RP	retinitis pigmentosa
PM	plasma membrane
ER	endoplasmic reticulum
PERK	protein kinase-like ER kinase
IRE-1	inositol requiring enzyme 1
ATF6	activating transcription factor 6
eIF2a	eukaryotic translation initiating factor 2
BiP	binding Ig Protein
XBP1	X-box binding protein 1
CHOP	C/EBP homologous protein
CFTR	cystic fibrosis transmembrane regulator
HERG	human <i>ether-a-go-go</i> related gene
4PBA	sodium 4-phenylbutyrate
TUDCA	tauroursodeoxycholate sodium salt
CPT-cGMP	8-(4-chlorophenylthio)-cGMP
DTT	1,4-dithiothreitol
Tn	tunicamycin
Tg	thapsigargin
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
LDH	lactate dehydrogenase
Ct	threshold cycle
HPRT	hypoxanthine-guanine phosphoribosyltransferase

CNBD	cyclic nucleotide-binding domain
ERAD	ER-associated degradation
CFRD	cystic fibrosis-related diabetes
ERAI	ER stress-activated indicator
P-PERK	phosphorylated PERK

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Figure 1. Disease-associated mutations R563H and Q655X impair plasma-membrane localization and increase ER retention of CNGA3 channel subunits

(A) CNGA3 subunit topology and location of mutations. (B–G) Expression and localization of human cone CNGA3 and CNGB3 subunits in 661W cells, determined using cell-surface biotinylation with avidin-retrieval (B, C) or confocal microscopy (D–G). (B) Representative immunoblot showing total (1% of input, with actin for normalization) and surface localized CNGA3 subunits. (C) Cell-surface localization of CNGA3 subunits, displayed as avidin pullouts from (B) relative to wild type, is impaired by R563H and Q655X mutations. Data are means $\pm$ SEM of six independent experiments; $*p < 0.05$. (D) Confocal fluorescence microscopy images and graphs showing plasma membrane (PM) marked with wheat germ

agglutinin (red) and FLAG-CNGA3 (green); PM localization (yellow) of R563H and Q655X subunits is impaired. **(E)** Colocalization calculated for regions encompassing cross-sections in **(D)** using Pearson's product-moment correlation coefficient, showing decreased PM localization of R563H and Q655X subunits. Images are 0.4–0.6 micron optical Z sections, representative of > 10 cells each; data are means $\pm$ SEM ($n = 6$ cells); $*p < 0.0001$. **(F)** Confocal images showing CNGA3 subunits (green) and endoplasmic reticulum (ER) marked with DsRed2-ER (blue). **(G)** Colocalization calculated for regions defined in **(F)** using Pearson's product-moment correlation coefficient, showing increased ER localization of R563H and Q655X subunits. Images are 0.4–0.6 micron optical Z sections, representative of > 10 cells each; data are means $\pm$ SEM ($n = 6$ cells); $*p < 0.05$.

Figure 2. Localization-defective channels display altered stability and protease sensitivity
(A–D) Amount of CNGA3 and CNGB3 subunits remaining at indicated time points after arrest of protein synthesis. **(A, B)** Representative immunoblots of CNGA3 or CNGB3 subunits, respectively. **(C)** Time-dependent decrease of CNGA3 subunits expressed as percent of initial amount, showing increased degradation rate for Q655X relative to wild type. **(D)** Percent initial amount of CNGB3, with indicated CNGA3 subunits, showing that trafficking defective CNGA3 subunits increased the degradation rate of coexpressed CNGB3 subunits. Data are means $\pm$ SEM of seven independent experiments. $*p < 0.05$. **(E, F)** Protease-sensitivity assay to probe CNGA3 subunit folding. **(E)** Representative western blot for proteolysis of purified channels. **(F)** Percent initial amount of CNGA3 remaining

after trypsin proteolysis at indicated time points, showing altered trypsin sensitivity. Data are means $\pm$ SEM of three independent experiments; $*p < 0.05$.

Figure 3. Pharmacological stress induces the unfolded protein response in 661W cells
(A–D) RT-qPCR quantitation of XBP1 (spliced and total), BiP, CHOP, and HPRT (internal standard) mRNA from cells treated with DTT, thapsigargin (Tg), or tunicamycin (Tn), relative to untreated cells. Percentage of XBP1 spliced (**A**) determined by percentage of spliced:total mRNA. Fold changes of total XBP1 (**B**), BiP (**C**), and CHOP (**D**). **(E, F)** Immunoblot quantitation of phosphorylated PERK (P-PERK), BiP, CHOP, and actin after treatment with Tg or Tn. **(E)** Representative immunoblot. **(F)** Levels of P-PERK, BiP, and CHOP (relative to actin) after treatments indicated in (E) normalized to levels in untreated cells. Data are means $\pm$ SEM of 3–6 independent experiments; * $p < 0.05$.

Figure 4. Localization defective CNG channels selectively induce the unfolded protein response RT-qPCR and immunoblot quantitation of ER stress markers in cells expressing CNGA3 and CNGB3 subunits. **(A)** Percent of XBP1 spliced and fold changes of BiP and CHOP mRNA were normalized to values in cells expressing wild-type channels. Data are means $\pm$ SEM of three independent experiments; $*p < 0.05$. **(B)** Representative immunoblot showing expression of P-PERK, BiP, CHOP, and actin. **(C)** Amounts of P-PERK, BiP, and CHOP (relative to actin) normalized to levels in cells expressing wild-type channels. Data are means $\pm$ SEM of 4–6 independent experiments; $*p < 0.05$.

Figure 5. Chemical and pharmacological chaperones reduce mRNA indicators of ER stress induced by localization defective CNG channels

RT-qPCR analysis of chaperone-treated cells expressing CNGB3 subunits. Percent of XBP1 spliced (A) and fold changes of BiP (B) and CHOP (C) mRNA normalized to values in untreated cells expressing wild-type channels. Data are means $\pm$ SEM of 4–5 independent experiments; # $p < 0.05$ compared to untreated cells expressing wild-type channels; * $p < 0.05$ compared to untreated cells.

Figure 6. Chaperones selectively reduce BiP and CHOP protein levels induced by localization defective CNG channels

Immunoblot analysis of chaperone-treated cells expressing CNGA3 and CNGB3 subunits. (A) Representative immunoblot. Amounts of BiP (B) and CHOP (C) protein (relative to actin) normalized to levels in untreated cells expressing wild-type channels. Data are means $\pm$ SEM of 4–5 independent experiments. # $p < 0.05$ compared to untreated cells expressing wild-type channels; * $p < 0.05$ compared to untreated cells.

Figure 7. Chaperones selectively enhance stability of localization defective CNGA3-Q655X subunits

Immunoblot analysis of proteins in chaperone-treated cells expressing CNGA3 and CNGB3 subunits after cycloheximide protein synthesis arrest. **(A)** Representative immunoblot. **(B)** Amount of CNGA3 subunit protein remaining after 4 hours relative to initial amount, normalized to values in untreated cells. Data are means $\pm$ SEM of four independent experiments; * $p < 0.05$ compared to untreated cells.

Figure 8. Chaperones selectively enhance cell-surface localization of CNGA3-R563H subunits

Immunoblot analysis of CNGA3 plasma membrane (PM) localization using cell-surface biotinylation with avidin-retrieval. **(A)** Representative immunoblot showing total (1% of input) and surface localized CNGA3 subunits. **(B)** PM localization of CNGA3 subunits, displayed relative to untreated cells expressing wild-type channels. Data are means $\pm$ SEM of three independent experiments; # $p < 0.05$ compared to untreated cells expressing wild-type channels; * $p < 0.05$ compared to untreated cells.

Figure 9. Chaperones selectively enhance plasma-membrane localization and reduce ER retention of localization defective CNGA3-R563H and -Q655X subunits
Colocalization of CNGA3 (green) with PM (red) or ER (blue), determined by confocal fluorescence microscopy. (A, C) Representative confocal images. (B, D) Colocalization calculated for regions defined in (A) or (C) using Pearson's product-moment correlation coefficient, normalized to untreated cells expressing wild-type channels. Images are 0.4–0.6 micron optical Z sections, representative of > 10 cells each; data are means $\pm$ SEM (n = 4 cells); #*p* < 0.05 compared to untreated cells expressing wild-type channels; **p* < 0.05 compared to untreated cells.

Figure 10. Chaperones improve survival of cells expressing localization defective CNG channels
 Quantitation of relative cell death, using lactate dehydrogenase (LDH) release assay. Percent cell death (relative to vector-only transfected cells), normalized to untreated cells expressing wild-type channels, showing rescue of cell viability with chaperone treatment. Data are means $\pm$ SEM of 3–5 independent experiments; [#] $p < 0.02$ compared to untreated cells expressing wild-type channels; ^{*} $p < 0.01$ compared to untreated cells.