Alterations in endoplasmic reticulum (ER) calcium (Ca\(^{2+}\)) levels diminish insulin secretion and reduce β-cell survival in both major forms of diabetes. The mechanisms responsible for ER Ca\(^{2+}\) loss in β cells remain incompletely understood. Moreover, a specific role for either ryanodine receptor (RyR) or inositol 1,4,5-triphosphate receptor (IP\(_3\)R) dysfunction in the pathophysiology of diabetes remains largely untested. To this end, here we applied intracellular and ER Ca\(^{2+}\) imaging techniques in INS-1 cells and isolated islets to determine whether diabetogenic stressors alter RyR or IP\(_3\)R function. Our results revealed that the RyR is sensitive mainly to ER stress–induced inhibition of the RyR with ryanodine and inhibition of the IP\(_3\)R activity. Consistent with this observation, pharmacological targeting glucose-induced Ca\(^{2+}\) oscillations in tunicamycin-treated INS-1 cells and mouse islets and Akita islets. Monitoring at the single-cell level revealed that the RyR is sensitive mainly to ER stress–induced dysfunction, whereas cytokine stress specifically alters IP\(_3\)R activity.

Under normal conditions, the concentration of calcium (Ca\(^{2+}\)) within the β cell endoplasmic reticulum (ER)\(^4\) is estimated to be at least three orders of magnitude higher than that of the cytosol. This steep Ca\(^{2+}\) concentration gradient is maintained by the balance of ER Ca\(^{2+}\) uptake via the sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) pump, buffering by ER luminal Ca\(^{2+}\)–binding proteins such as GRP78/BiP, calnexin, and calreticulin, and ER Ca\(^{2+}\) release through the ryanodine (RyR) and inositol 1,4,5-triphosphate (IP\(_3\)) receptors (IP\(_3\)R) (1–4). ER luminal Ca\(^{2+}\) serves as a required cofactor for insulin production and processing, while also playing a critical role in patterning glucose-induced Ca\(^{2+}\) oscillations (GICOs) and phasic insulin secretion (5–7).

Although alterations in β cell ER Ca\(^{2+}\) homeostasis lead to diminished insulin secretion and reduced β-cell survival in both type 1 and type 2 diabetes (3, 8–10), the underlying pathways responsible for β cell ER Ca\(^{2+}\) loss remain incompletely understood. Reduced β cell SERCA activity and expression have been described in rodent and human models of diabetes, and SERCA2 haploinsufficiency was shown recently to result in reduced insulin secretion and decreased β-cell proliferation under high-fat diet conditions (8–11). Similarly, genetic mouse models expressing mutated forms of the RyR2, leading to increased ER Ca\(^{2+}\) leak, also exhibited reduced insulin secretion, whereas pharmacological antagonists of the RyR and IP\(_3\)R were found to reduce β-cell death in response to thapsigargin treatment (4, 12–14).

Whereas a handful of studies suggest a potential role for RyR and IP\(_3\)R dysfunction in diabetes, the specific mechanisms of how RyR and IP\(_3\)R shape β cell ER Ca\(^{2+}\) dynamics and survival under disease conditions is unclear. To this end, we aimed to define whether RyR and IP\(_3\)R were differentially modulated in response to cytokine treatment and ER stress, two conditions known to contribute to diabetes pathophysiology. Using intra…

\(^4\)The abbreviations used are: ER, endoplasmic reticulum; SERCA, sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase; RyR, ryanodine receptor; IP\(_3\)R, inositol 1,4,5-triphosphate receptor; IP\(_3\), inositol 1,4,5-triphosphate; IP\(_3\)R, IP\(_3\) receptor; GICO, glucose-induced Ca\(^{2+}\) oscillation; TM, tunicamycin; ILHG, interleukin 1β combined with high glucose; Ry, ryanodine; XeC, xestospongin C; FLIM, fluorescence lifetime imaging microscopy; T-M, targeted MS; UPR, unfolded protein response; Ctr, control; AUC, area under the curve; Fura-2 AM, Fura-2-acetoxyethylmethylster; CPVT, catecholaminergic polymorphic ventricular tachycardia; TG, thapsigargin; CFP, cyan fluorescent protein; RT-qPCR, real-time quantitative polymerase chain reaction.
cellular and ER Ca\(^{2+}\) imaging techniques, we found impaired IP\(_3\)R function in response to cytokine treatment, whereas RyR-mediated ER Ca\(^{2+}\) leak was preferentially induced under ER stress conditions. RyR inhibition was distinct in its ability to prevent \(\beta\)-cell death, potentiation of the unfolded protein response, and dysfunctional glucose-induced Ca\(^{2+}\) oscillations in response to tunicamycin-induced ER stress in INS-1 \(\beta\) cells and islets from a genetic model of \(\beta\) cell ER stress. Monitoring at the single cell level revealed that ER stress acutely increased the frequency of spontaneous intracellular Ca\(^{2+}\) transients in INS-1 cells and cadaveric human islets, which depended on both ER Ca\(^{2+}\) leak from the RyR as well as plasma membrane depolarization. In aggregate, these findings suggest efforts to maintain ER Ca\(^{2+}\) levels through stabilization of the RyR may improve \(\beta\)-cell function and survival, and thus represent a potential therapeutic target in diabetes.

**Results**

**ER stress and cytokine-induced stress lead to ER Ca\(^{2+}\) loss**

The pathophysiology of type 1 and type 2 diabetes involves both \(\beta\) cell ER stress and cytokine-induced \(\beta\)-cell dysfunction (8, 15, 16). To define how these stress paradigms specifically influenced ER Ca\(^{2+}\) storage, INS-1 \(\beta\) cells were treated with 300 nM tunicamycin (TM) or 5 ng/ml interleukin 1\(\beta\) combined with 25 mM high glucose (ILHG) in time-course experiments. Cytosolic Ca\(^{2+}\) imaging was performed according to the schematic shown in Fig. 1A. Results revealed a time-dependent loss of ER Ca\(^{2+}\) with both TM (Fig. 1, B and C) and ILHG (Fig. 1, D and E) treatment. In both stress paradigms, significant reductions in ER Ca\(^{2+}\) were seen within 6 h, with further reductions observed throughout the 24-h exposure period. Reductions appeared specific to these stress paradigms as high glucose alone or mannitol (employed as an osmotic control) did not significantly impact ER Ca\(^{2+}\) storage (Fig. S1).

**RyR and IP\(_3\)R functions are differentially altered in response to ER and cytokine-induced stress**

Whereas previous studies have implicated \(\beta\) cell SERCA2 dysfunction in diabetes, a role for either RyR or IP\(_3\)R dysfunction has not been well-characterized (8–11). To test whether RyR and IP\(_3\)R activity were altered in models of ER and cytokine stress, TM- and ILHG-treated INS-1 \(\beta\) cells were loaded with the low-affinity Ca\(^{2+}\) indicator Mag-Fluo-4 AM, followed by membrane permeabilization with saponin to deplete cytosolic Mag-Fluo-4. As shown in Fig. 2A, Mag-Fluo-4 AM was efficiently cleared from the cytosol, but remained sequestered within the ER, as indicated by overlap with RFP-calnexin (Fig. 2A). Next, ATP was added to achieve steady-state ER Ca\(^{2+}\) levels via SERCA activation. Caffeine and IP\(_3\) were added to activate RyRs and IP\(_3\)Rs, respectively, and dose-response curves were generated (Fig. 2B). Our analysis revealed that TM-induced ER stress primarily altered RyR responses (Fig. 2C), whereas IP\(_3\)R function was minimally impacted by TM treatment (Fig. 2D). In the short term, TM increased the maximal RyR response, whereas reductions in RyR activity were observed with chronic TM treatment (Fig. 2C). In contrast, RyR activity remained largely unaffected by ILHG (Fig. 3A). In contrast, chronic ILHG treatment reduced the EC\(_{50}\) levels before the RyR response to agonist (Fig. 3B). Together, these results suggest that TM-induced ER stress preferentially impacted RyR function, whereas ILHG treatment preferentially impaired the IP\(_3\)R response to agonist.
Stress-mediated ER Ca²⁺ loss was reduced by RyR and IP₃R inhibition

To determine whether RyR or IP₃R inhibition was sufficient to prevent ER Ca²⁺ loss under these two stress conditions, we tested the effects of RyR antagonists, dantrolene and ryanodine (Ry), and the IP₃R antagonist, xestospongin C (XeC). Following TM treatment, there was no significant improvement in ER Ca²⁺ storage with dantrolene (Fig. 4A), whereas inhibition of RyR with Ry partially restored ER Ca²⁺ levels compared with TM alone (Fig. 4B). Consistent with data from functional assays shown in Figs. 2 and 3, XeC had no effect on TM-induced loss of ER Ca²⁺ (Fig. S2A). Similarly, Ry was unable to block ER Ca²⁺ loss in response to ILHG (Fig. S2B). In contrast, inhibition of IP₃R with XeC partially rescued ER Ca²⁺ levels following ILHG treatment (Fig. 4C).

To confirm these results, direct monitoring of ER Ca²⁺ levels was performed in D4ER-transduced INS-1 cells using fluorescence lifetime imaging microscopy (FLIM). FLIM analysis revealed an increase in the lifetime of the donor probe with TM treatment, indicating a reduction in ER Ca²⁺ levels. Ry treatment was able to prevent this TM-induced loss of ER Ca²⁺ (Fig. 4, D and E). Next, this was tested in D4ER-transduced mouse islets using fluorescence resonance energy transfer (FRET). Again, ER Ca²⁺ levels were reduced in TM-treated islets (detected as a decrease in FRET), whereas Ry was able to prevent this reduction (Fig. 4, F and G).
**Figure 3. Cytokine stress led to impaired IP₃R function.** A, dose-response curves for RyR activation by caffeine in INS-1 cells pretreated with 5 ng/mg IL + 25 mM glucose (ILHG) for 6, 12, and 24 h. B, dose-response curves for IP₃R activation in INS-1 cells pretreated with ILHG for 6, 12, and 24 h. Values shown are the LogEC₅₀ for INS-1 cells analyzed under control conditions (top) and following ILHG treatment (bottom). Data are from a minimum of three independent experiments for each time point and agonist concentration. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001 compared with control conditions. Error bars indicate ± S.D.

**Figure 4. ER Ca²⁺ loss was prevented by blocking the RyR under ER stress conditions and by IP₃R blockade under cytokine stress conditions.** A and B, INS-1 cells were co-treated with 300 nM TM and 1 μM dantrolene (Dt) (A) or with 100 μM Ry (B) for 24 h. Representative traces for TG-induced Ca²⁺ release (left) and quantified results (right), n = at least three times repeated per condition. C, INS-1 cells were treated with 5 ng/ml IL-1β + 25 mM glucose (ILHG) for 24 h with or without 5 μM XeC. Representative traces for the TG-induced Ca²⁺ release (left), and quantified results (right), n = at least three times repeated per condition. D and E, INS-1 cells were transduced with the D4ER adenovirus and co-treated with 300 nM TM with or without 100 μM Ry for 24 h. FLIM was used to measure ER Ca²⁺ levels. Shown are representative lifetime map images with look-up table indicating donor lifetime in nanoseconds (ns) (D) and quantified results (E), n = at least three times repeated per condition; scale bar = 20 μm. F and G, islets from 8- to 10-week-old C57BL/6J mice were transduced with the D4ER adenovirus and co-treated with 300 nM TM with or without 100 μM Ry for 24 h. Z-stack images were obtained and intensities of CFP and YFP from positively transduced β cells were quantitated and presented as a ratio. F, representative maximum intensity projection images. Scale bar = 50 μm. G, quantitated FRET/CFP ratios, *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001 compared with control conditions. §, p = 0.001 for comparison between TM and TM + Ry in (B), (E), and (G). #, p ≤ 0.05 for comparison between ILHG and ILHG + XeC in (C). Error bars indicate ± S.D. A.U., arbitrary units.
ER stress–induced β cell ryanodine receptor dysfunction

**Figure 5. Ryanodine treatment prevented TM-induced cell death.** A, caspase 3/7 activity was measured in INS-1 cells after co-treating with 300 nM TM and 100 μM Ry for indicated times. n.s., no significance. B, immunoblot analysis was performed in INS-1 cells treated with 300 nM TM with or without 100 μM Ry for indicated times using antibodies against cleaved caspase-3 and actin. Quantitative protein levels are shown graphically. C, caspase 3/7 activity was measured in INS-1 cells treated with 5 ng/ml IL-1β + 25 mM glucose (ILH) for 24 h; **, p ≤ 0.001 compared with control condition. Error bars indicate ± S.D.

ER stress and cytokine stress are known to induce β-cell death (17), so we tested next whether modulation of ER Ca^{2+} loss via RyR or IP_{3}R inhibition were sufficient to protect against β-cell death. TM treatment led to a time-dependent increase in caspase 3 and 7 activity (Fig. 5A) and expression of cleaved caspase 3 protein (Fig. 5B). Interestingly, cell death was partially abrogated by Ry co-treatment (Fig. 5, A and B). This effect on tunicamycin-induced cell death was not recapitulated by either dantrolene or XeC (Fig. S2C). Moreover, despite an observed effect to partially restore ER Ca^{2+} levels (Fig. 4C), XeC was unable to reduce caspase activity in response to ILH (Fig. 5C). As expected, Ry treatment also had no effect on ILH-induced caspase activity (Fig. S2D). In aggregate, these data revealed a unique ability of RyR inhibition to improve cell survival in response to ER stress–induced loss of ER Ca^{2+}.

**RyR dysfunction is not mediated via reduced RyR2 expression**

The presence of RyR in the pancreatic β cell has been debated in published studies (18, 19). To document RyR expression in our own hands, we utilized a combination of RT-qPCR in INS-1 cells and sorted mouse β cells (Fig. S3) and targeted MS analysis. Heart tissue was used as a positive control (Fig. 6, B–E). First, we confirmed RyR mRNA expression in INS-1 cells and found that RyR2 was the most highly expressed isoform in this model system as determined by lowest ΔC_{T} values (Fig. 6A). Similarly, expression of RyR1 and RyR2 was observed in Newport Green sorted mouse β cells (Fig. S3), with RyR2 again expressed at an earlier ΔC_{T} value compared with RyR1 (Fig. 6, B and C). Finally, to confirm RyR2 protein expression in mouse β cells, a targeted MS (T-MS) assay was developed using a peptide specific for RyR2 (891-IELGWQYGPVR-901). T-MS confirmed the presence of RyR2 protein in mouse islets (Fig. 6D) and Newport Green sorted mouse β cells (Fig. 6E). Gel electrophoresis of the PCR product and immunoblot confirming RyR expression are shown in Fig. S4. Moreover, INS-1 cells treated with TM did not exhibit reduced RyR2 expression, as shown by T-MS analysis (Fig. 6, F and G) and RT-qPCR (Fig. 6H). Taken together, these data indicate that RyRs are indeed present in rodent β cells and that TM-induced dysfunction does not result from decreased RyR2 expression.

**Ryandoline and diazoxide suppressed TM-induced Ca^{2+} transients**

Our results thus far suggested a dominant role for RyR dysfunction under ER stress conditions, but primarily focused on bulk analysis of Ca^{2+} dynamics in large cell populations. Ca^{2+} serves as the primary ligand for the RyR, and spontaneous intracellular Ca^{2+} transients attributable to RyR-mediated ER Ca^{2+} leak have been observed in other excitable cells such as neurons and cardiac myocytes (20). However, this process has not been studied in the pancreatic β cell, under either normal or stress conditions. To identify mechanisms of ER Ca^{2+} release through the RyR, spontaneous Ca^{2+} transients were measured at the single-cell level in response to graded Ca^{2+} loading. By increasing the extracellular Ca^{2+} concentration up to 2 mM, oscillating and spontaneous Ca^{2+} transients were induced in 10.40 ± 1.54% (S.D.) of β cells under control conditions. In response to TM-induced ER stress, the percentage of responding cells increased significantly to a maximum of 55.74 ± 6.67% after 12 h of treatment (Fig. 7, A and C). Ry co-treatment significantly decreased TM-induced Ca^{2+} transients (Fig. 7, A and D), indicating the ER Ca^{2+} leak was mediated through the RyR. In addition, the response to caffeine was inhibited in the presence of 100 μM Ry (Fig. 7B), confirming that Ry was indeed acting through inhibition of RyR-mediated Ca^{2+} transients.

To define whether β-cell depolarization contributed to the spontaneous Ca^{2+} transients induced by ER stress, cells were hyperpolarized by diazoxide (Dz) to inhibit activation of voltage-gated Ca^{2+} (CaV) channels. In this context, TM-induced Ca^{2+} transients were completely suppressed (Fig. 7E), suggesting that depolarization may be an essential component of these
spontaneous Ca\(^{2+}\) transients from the RyR under normal and ER stress conditions.

**Reduced RyR-dependent ER Ca\(^{2+}\) leak suppressed TM-induced Ca\(^{2+}\) transients and delayed activation of the UPR**

During ER stress, cells activate an adaptive response known as the unfolded protein response (UPR) to clear unfolded proteins and improve ER protein folding capacity (21). However, prolonged UPR activation eventually leads to apoptosis if cellular homeostasis is not restored (22, 23). Although UPR activation has been linked with ER Ca\(^{2+}\) loss (24), the temporal relationships and causal effects between UPR activation and ER Ca\(^{2+}\) loss have not been fully delineated. To address this, we first measured XBP1 mRNA splicing to validate this as an early indicator of UPR activation. An increase in the spliced to total XBP1 ratio was seen within 2 hours of TM treatment and occurred prior to induction of both ATF4 and CHOP, both of which increased around 6 hours (Fig. 8A). Next, time-course experiments were performed to define how suppression of ER Ca\(^{2+}\) leak from the RyR impacted UPR activation. This analysis revealed that Ry was able to significantly delay TM-induced UPR activation, as measured by quantification of the spliced to total XBP1 ratio (Fig. 8B). To study this further, single-cell Ca\(^{2+}\) transients were measured again at these early time points. Intracellular Ca\(^{2+}\) transients were found to increase within 3 hours of TM treatment. Similar to results obtained with chronic TM treatment, co-treatment with Ry was sufficient to suppress these Ca\(^{2+}\) transients (Fig. 8, C and D), indicating that ER Ca\(^{2+}\) leak is an early response to misfolded protein accumulation and occurs prior to full expression of the ER stress signaling cascade. Moreover, our results suggested that suppression of RyR-mediated Ca\(^{2+}\) leak was sufficient to delay UPR initiation.

**Pharmacological inhibition of the RyR improved intracellular Ca\(^{2+}\) dynamics in TM-treated human islets and islets isolated from Akita mice**

To test whether these findings could be recapitulated in a human model system, dispersed cadaveric human islets were treated with TM and cytosolic Ca\(^{2+}\) transients were recorded. Similar to results observed in INS-1 \(\beta\) cells, spontaneous Ca\(^{2+}\) transients were found to increase within 3 hours of TM treatment. Similar to results obtained with chronic TM treatment, co-treatment with Ry was sufficient to suppress these Ca\(^{2+}\) transients (Fig. 8, C and D), indicating that ER Ca\(^{2+}\) leak is an early response to misfolded protein accumulation and occurs prior to full expression of the ER stress signaling cascade. Moreover, our results suggested that suppression of RyR-mediated Ca\(^{2+}\) leak was sufficient to delay UPR initiation.
alone, or TM + Ry for 48 h. Compared with Ctr islets, TM-treated islets exhibited altered oscillatory patterns under low (G5) and high (G15) glucose, and the area under the curve (AUC) response was significantly reduced by TM under G15 conditions (Fig. 10, A–C). Ry treatment increased the AUC of the oscillatory response compared with Ctr and TM-treated islets under both G5 and G15 glucose conditions (Fig. 10, B and C).

Finally, we tested whether RyR inhibition would show similar benefits in a genetic model of ER stress. To this end, islets were isolated from 6- to 8-week-old Akita and WT littermate mice. Akita mice harbor a spontaneous mutation in one allele of the INS2 gene, resulting in impaired proinsulin folding and severe ER stress (25). Fura-2 AM imaging experiments were performed in Akita islets treated with or without Ry. GICOs were markedly diminished in Akita islets under control conditions, whereas treatment with Ry improved the oscillation frequency and AUC of the glucose-induced Ca2+ responses (Fig. 11, A–D). Moreover, Ry treatment significantly decreased cell death in islets from Akita mice. (Fig. 11, E and F).

Discussion

Reduced β cell ER Ca2+ levels have been shown to impair insulin secretion and lead to activation of cell-intrinsic stress responses including ER, mitochondrial, and oxidative stress, ultimately resulting in reduced β-cell survival (3, 5–7, 26). The RyR and IP3R are cation-selective and ligand-gated Ca2+ channels.
release channels that exist as macromolecular complexes within the ER or sarcoplasmic reticulum membranes. The goal of our study was to test whether RyR or IP3R dysfunction contributed to altered β cell ER Ca\(^{2+}\) storage under diabetic conditions. To this end, we applied intracellular and ER Ca\(^{2+}\) imaging techniques to measure activity of both receptors in response to two distinct stress paradigms. ER stress was induced chemically in INS-1 cells, mouse islets, and cadaveric human islets using tunicamycin, a compound that inhibits protein glycosylation (8, 15, 27). In addition, aspects of our model were evaluated in islets from Akita mice, which is a genetic model of ER stress. To recapitulate cytokine-induced diabetogenic stress, INS-1 cells were treated with a combination of high glucose and IL-1β. This specific cytokine was selected because it is known to be systemically elevated in diabetes and prediabetes (28). Moreover, IL-1β has been shown to induce β-cell death, whereas IL-1β antagonism in humans yielded beneficial effects in the treatment of type 2 diabetes (29–31).

Our results revealed a preferential sensitivity of the RyR to ER stress–induced dysfunction, whereas cytokine stress was found to primarily impact IP3R activity. Pharmacological inhibition of the RyR with ryanodine and inhibition of the IP3R with xestospongin C were able to prevent ER Ca\(^{2+}\) loss under these respective stress conditions. However, inhibition of RyR-mediated Ca\(^{2+}\) loss was distinct in its ability to prevent β-cell death. Additional analysis showed that RyR inhibition also delayed initiation of the UPR, while leading to improvements in glucose-induced Ca\(^{2+}\) oscillations under ER stress conditions. These findings are noteworthy because several groups are actively involved in drug discovery efforts aimed at identifying small molecule RyR stabilizers (32, 33).

Others have investigated a functional role for the RyR in the pancreatic β cell under normal conditions. Several reports have shown that β cell RyRs regulate classical Ca\(^{2+}\)-induced Ca\(^{2+}\) release from intracellular stores (26, 34) as well as mitochondrial ATP synthesis in response to GLP-1 stimulation (35).
RyRs have also been identified on the surface of \(H_9252\) cell dense core secretory vesicles, where they have been implicated in secretory vesicle Ca\(^{2+}\) release and regulation of localized Ca\(^{2+}\) signals responsible for granule exocytosis (36). Johnson et al. also identified RyR expression in the \(H_9252\) cell endosomal compartment and showed the inhibition of RyR with micromolar doses of Ry decreased insulin secretion from human \(H_9252\) cells (37). Taken together, this background suggests a role for RyR in the modulation of \(\beta\)-cell calcium signaling and insulin secretion under normal conditions.

Despite this existing literature, the topic of RyR expression in the pancreatic \(H_9252\) cell has been controversial. There are three RyR isoforms encoded by three distinct genes (38). At least one group has been unable to detect RyR mRNA expression in intact islets and purified mouse \(H_9252\) cells (5, 18). However, multiple other groups have documented RyR expression in human and rodent islets (37, 39, 40). Similar to other groups (39), we identified RyR2 as the most abundant isoform in mouse and rat \(H_9252\) cells. To address lingering concerns regarding expression of RyR2 protein in the \(H_9252\) cell, we developed a targeted MS assay. Using this assay, we confirmed expression of RyR2 protein in intact mouse islets, sorted mouse \(H_9252\) cells, and INS-1 cells. Our confirmation that RyR2 is the most highly expressed isoform is notable because dantrolene was shown to have lesser effects on RyR2 activity when compared with the other isoforms (38). This could explain some of the differences we observed in the ability of dantrolene and Ry to prevent ER stress–induced ER Ca\(^{2+}\) loss.

Ryanodine receptor dysfunction has been documented in other disease states, including cancer-associated muscle weakness (41), Alzheimer’s disease (42), and cardiac arrhythmias (43). A handful of molecular pathways have been implicated as potential contributors to \(\beta\) cell RyR dysfunction. Mice with a mutated form of the RyR2 leading to constitutive CaMKII-mediated phosphorylation and chronic RyR2 activation exhibited impaired glucose-induced insulin and Ca\(^{2+}\) responses as well as glucose intolerance (14). RyR2 mutations leading to dissociation of the interacting protein calstabin2 result in RyR gain of function and a condition known as catecholaminergic polymorphic ventricular tachycardia (CPVT) in humans (44, 45). Mice expressing two mutated forms of the RyR2 associated with CPVT were found to be glucose intolerant, whereas islets isolated from these mice exhibited decreased glucose-stimulated insulin secretion and impaired mitochondrial metabo-
lism. Intriguingly, humans with CPVT were found to have higher glucose levels and lower insulin levels during an oral glucose tolerance test compared with age- and BMI-matched controls (19).

Oxidative stress has been shown to contribute to both calstabin dissociation from the RyR as well CaMKII-mediated RyR phosphorylation (46). Indeed, alterations in calstabin and RyR association were demonstrated in islets from donors with type 2 diabetes (19). More recently, loss of sorcin, a Ca\(^{2+}\) sensor protein that inhibits RyR activity, was shown to lead to glucose intolerance, whereas sorcin overexpression improved glucose-stimulated insulin secretion and ER Ca\(^{2+}\) storage. Interestingly, palmitate-induced lipotoxicity was also shown to decrease sorcin expression in human and mouse islets (47). In aggregate, genetic models support a role for RyR activity in the maintenance of normal \(\beta\)-cell function. In addition, published studies hint at a potential role for impaired regulation of \(\beta\) cell RyR activity in models of diabetes through either

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**Figure 11.** Ca\(^{2+}\) signaling and cell death were rescued by ryanodine treatment in islets from Akita mice

A and B, glucose-stimulated calcium oscillations were measured in islets isolated from Akita mice treated with DMSO or Ry for 24 h and WT littermate mice treated with DMSO for 24 h. Shown are representative recordings from four individual islets for Akita (A) and WT (B) mice. C and D, the frequency of oscillations (C) and baseline corrected area under curve for calcium responses (D) were quantified from three biological replicates per conditions. E, representative pictures of live (green) and dead (red) staining performed in Akita islets treated with DMSO or Ry for 48 h. Scale bar = 100 \(\mu\)m. F, quantification of the \% of dead cells from three repeated experiments. *, \(p \leq 0.05\); **, \(p \leq 0.01\); ***, \(p \leq 0.001\) for comparisons between indicated groups. G and H, overall model. G, our data indicate that under ER stress conditions, RyR function is disrupted, leading to increased ER Ca\(^{2+}\) leak, decreased ER Ca\(^{2+}\) storage, and altered ER Ca\(^{2+}\) dynamics. As a consequence, cellular excitability and GICOs are disrupted and activation of the UPR is increased, eventually leading to cell death. H, inhibition of RyR-mediated loss of ER Ca\(^{2+}\) leads to a partial rescue of ER Ca\(^{2+}\) dynamics under ER stress conditions, which improved cellular excitability and GICOs, delayed initiation of the UPR, and decreased \(\beta\)-cell death. Error bars indicate \(\pm\) S.D.
impaired activity of channel-stabilizing proteins or via loss of inhibitory proteins. Our results indicate that RyR dysfunction was uniquely induced by misfolded protein accumulation, whereas ILHG treatment had little impact on RyR function. Our data from time-course experiments further indicate that TM-induced ER Ca\(^{2+}\) release through RyR began even before full expression of the unfolded protein response. Thus, it is possible that RyR dysregulation could be the result of a direct interaction of misfolded or unfolded proteins with RyRs in a manner that increases channel opening. In this regard, unfolded proteins directly bind the ER luminal GRP78/BiP to initiate the UPR, although unfolded proteins have also been shown to bind and activate IRE1 (48). Consistent with this notion, prions as well as \(\beta\)-amylloid protein accumulation in cortical neurons induced RyR-mediated ER Ca\(^{2+}\) release and ER stress in neuronal tissues (49). Still another possibility is that ER stress changes the status of the ER microenvironment in a manner that favors deleterious posttranslational modifications of the RyR. TM treatment has been shown to increase ER hydrogen peroxide levels in endothelial cells (50). TM has also been shown to increase expression of the major superoxide-producing enzyme Nox4 in as little as 4 h in smooth muscle cells (51). Interestingly, Nox4 binds to RyR1 in skeletal muscle, leading to oxidization of the unfolded protein response. Thus, it is possible that RyR dysfunction may improve \(\beta\)-cell function and survival, and thus represent a potential therapeutic target in diabetes (Fig. 11, G and H).

**Experimental procedures**

**Materials**

Tunicamycin and thapsigargin were purchased from Cayman Chemical Co. (Ann Arbor, MI). Caffeine, d-myo-Inositol-1,4,5-triphosphate hexapotassium salt (IP\(_3\)), and Xestospongic C were from Santa Cruz Biotechnology (Dallas, TX). Fura-2-acetoxymethylester (Fura-2 AM), Mag-Fluo-4 AM, and recombinant mouse IL-1\(\beta\) were from Thermo Fisher Scientific. Diazoxide and ATP magnesium salt were from Sigma-Aldrich. Ryanodine and carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) were from Tocris Bioscience (Minneapolis, MN).

**Animals, islets, and cell culture**

Male C57BL/6j mice and heterozygous Ins2\(^{Akiita}\) (Akita) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained under protocols approved by the Indiana University Institutional Animal Care and Use Committee. Mice were kept in a standard light-dark cycle with ad libitum access to food and water. Pancreatic islets were isolated by collagenase digestion, handpicked, and allowed to recover overnight as described previously (57). INS-1 832/13 cells were cultured in RPMI 1640 with 11.1 mM glucose (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 \(\mu\)M/ml \(\beta\)-mercaptoethanol (58, 59). Human islets were obtained from the Integrated Islet Distribution Program and cultured as described previously (59). Donor characteristics are shown in Table S1.

**\(\beta\)-cell purification using flow cytometry**

Mouse islets were gently dissociated using Accutase (EMD Millipore, Billerica, MA) at 37 °C for 10 min. Dissociated cells were washed once with 0.1% BSA in PBS and cultured with RPMI 1640 supplemented with 10% FBS and 100 units/ml penicillin and 100 \(\mu\)g/ml streptomycin. Newport Green (25 \(\mu\)M/ml) was then added to the culture media and incubated with dissociated cells for 90 min. Next, Newport Green–stained cells were washed twice with 0.1% PBS and filtered using 5-ml tubes attached to a cell strainer cap. Cells positive and negative for Newport Green (excitation: 485 and emission: 530 nm) were sorted using a BD FACSaria Flow Cytometer (BD Biosciences). The purity of sorted cell populations was verified by immunofluorescent staining for insulin and glucagon (Fig. S3).
**Targeted mass spectrometry**

Relative Ryr2 levels in mouse islets and INS-1 cells were measured using a parallel reaction monitoring (PRM)–based targeted MS methodology. In brief, protein extraction was performed by treating with 8 M urea in 50 mM Tris-HCl, followed by sonication. Samples were further processed and digested with Trypsin Gold (Promega, Madison, WI) before Tandem Mass Tag–based labeling of the digested peptides as well as the Ryr2 (891-IELGWQYGPVR-901) synthetic trigger peptide. PRM-based nano-LC/MS/MS analyses were performed on a Q Exactive Plus coupled to an Easy-nLC 1200 (Thermo Fisher Scientific). Data were analyzed using SEQUEST-HT as the database search algorithm within Proteome Discoverer (Version 2.2, Thermo Fisher Scientific). Complete methodology can be found in the supporting information.

**Immunoblot and quantitative RT-PCR**

Immunoblot experiments were performed as described (60) using either the Cell Signaling Caspase-3 Antibody (no. 9662; Danvers, MA) or the Merck Millipore MAB1501 actin antibody (Billerica, MA). Images were analyzed using LI-COR Biosciences Image Studio (Lincoln, NE) and ImageJ software (National Institutes of Health). Cultured cells or isolated islets were processed for total RNA using the Qiagen RNeasy Mini Plus Kit (Valencia, CA), and quantitative RT-PCR was performed using SYBR Green I dye and previously published methods (58). The primer sequences employed are detailed in Table S2.

**Calcium imaging and IP3R and RyR functional assays**

Intracellular Ca2+ was measured using the FLIPR Calcium 6 Assay Kit and a Molecular Devices FlexStation 3 system (Sunnyvale, CA). In brief, INS-1 832/13 cells were plated on black wall, clear bottom, 96-multiwell plates from Costar (Tewksbury, MA) and cultured for 2 days. Following drug or stress treatment, cells were transfected to Ca2+-free Hanks’ balanced salt solution (Thermo Fisher Scientific) supplemented with 0.2% BSA and EGTA. Calcium 6 reagent was added directly to cells, and cells were incubated for an additional 2 h at 37 °C and 5% CO2. ER Ca2+ was estimated by measuring the increase of cytosolic Ca2+ upon application of 10 μM thapsigargin (TG). Data acquisition on the FlexStation 3 system was performed at 37 °C using a 1.52-s reading interval with an excitation wavelength of 485 nm and an emission wavelength at 525 nm. For data analysis, values derived from the TG response were processed for total RNA using the Qiagen RNeasy Mini Plus Kit (Valencia, CA), and quantitative RT-PCR was performed using SYBR Green I dye and previously published methods (58). The primer sequences employed are detailed in Table S2.

**Cell death assays and insulin secretion**

To measure caspase 3/7 activity, INS-1 cells were cultured in black wall, clear bottom, 96-multiwell plates for 2 days. Following drug or stress treatment, Caspase-Glo reagent (Promega, Madison, WI) was added directly to cells, and cells were incubated for an additional 30 min at room temperature. The luminescence of each sample was measured by using a Zeiss LSM 800 affixed with an Ibidi stage top incubator and intensities of CFP and YFP from positively transduced β cells were quantitated with ImageJ (National Institutes of Health) and presented as a ratio. Representative images are shown as maximum intensity projections and were generated using CellProfiler 3.0 (Broad Institute) (62).

**Statistical analysis**

Unless indicated, results were displayed as the mean ± S.D. and differences between groups were analyzed for significance using GraphPad Prism Software. When comparing two groups, unpaired Student’s t tests were utilized, and differences between two or more groups were compared using one-way analysis of variance (ANOVA) with Tukey-Kramer post hoc
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test. A p value < 0.05 was used to indicate a significant difference between groups.

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