ENDOPLASMIC RETICULUM CALCIUM DYNAMICS AND INSULIN SECRETION IN PANCREATIC β CELLS

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Under normal conditions, ER Ca^{2+} levels are estimated to be at least three orders of magnitude higher than intracellular Ca^{2+}. This steep Ca^{2+} concentration gradient is maintained by the balance of Ca^{2+} uptake into the ER via the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump and ER Ca^{2+} release through Ryanodine receptors (RyR) and Inositol 1,4,5-triphosphate (IP_3) receptors (IP_3R). Emerging data suggest that alterations in β cell ER Ca^{2+} levels lead to diminished insulin secretion and reduced β cell survival in both type 1 and type 2 diabetes. However, the mechanisms leading to β cell ER Ca^{2+} loss remain incompletely understood, and a specific role for either RyR or IP_3R dysfunction in diabetes has been largely untested. To this end, we applied intracellular and ER-Ca^{2+} imaging techniques in INS-1 β cells and isolated mouse and human islets to define whether RyR or IP_3R activity were altered under diabetogenic conditions. Results revealed preferential alterations in RyR function in response to ER stress, while pro-inflammatory cytokine stress primarily impacted IP_3R activity. Consistent with this, pharmacological inhibition of RyR and IP_3Rs prevented ER Ca^{2+} loss under ER and pro-inflammatory stress, respectively. However, RyR inhibition was unique in its ability to prevent β cell death, delayed initiation of the unfolded protein response (UPR), and dysfunctional glucose-induced Ca^{2+} oscillations in tunicamycin-treated INS-1 β cells and islets from Akita mice. Monitoring at the single cell level revealed that ER stress acutely increased intracellular Ca^{2+} transients and this was dependent on both ER Ca^{2+} leak from the RyR and plasma membrane depolarization, suggesting ER Ca^{2+} dynamics regulate cellular excitability. Collectively, our findings

suggest that ER-stress induced RyR dysfunction regulates β cell ER Ca^{2+} dynamics, propagation of the UPR, insulin secretion, and cell survival. These data indicate that RyR-mediated loss of ER Ca^{2+} and β cell hyperexcitability may be early pathogenic events in diabetes.

Carmella Evans-Molina, M.D., Ph.D, Chair

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LIST OF ABBREVIATIONS

ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
APCs	Antigen presenting cells
BABYDIAB	Baby Diabetes
Bim	Bcl-2-interacting mediator of cell death
Ca^{2+}	Calcium
CaMKII	Ca^{2+}/calmodulin dependent protein kinase II
cAMP	Cyclic AMP
CICR	Ca^{2+} induced Ca^{2+} release
CPVT	Catecholaminergic polymorphic ventricular tachycardia
Cx36	Connexin 36
DIPP	Diabetes Prediction and Prevention Project
DPP4	Dipeptideyl peptidese-4
EC	Electrical – contraction
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
GWAS	Genome-wide association studies
GAD	Glutamic acid decarboxylase
GICOs	Glucose-induced Ca^{2+} oscillations
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
Glut	Glucose transporters

GPDH	Glycerophosphate dehydrogenase
GPCR	G protein coupled-receptor
IA2	Islet antigen 2
IGF2	Insulin-like growth factor 2
IL-1β + HG	Interleukin 1β combined with high glucose
Ins	Islet proteins
IP_3	Inositol 1,4,5-triphosphate
IP_3R	Inositol 1,4,5-triphosphate receptors
IRE1	Inositol-requiring enzyme 1
JNK	c-Jun N-terminal kinase
K_{ATP} channels	ATP-sensitive K^+ channels
LETM1	Leucine- zipper EF-hand-containing transmembrane protein 1
MCU	Mitochondrial Ca^{2+} uniporter
NCLX	$Na^+/K^+/Ca^{2+}$-exchange protein 6
NCX	Na^+/Ca^{2+} exchanger
Ngn3	Neurogenin-3
Pdx-1	Pancreatic and duodenal homeobox 1
PERK	PKR-like ER kinase
PKA	Protein kinase A
PKG	cGMP-dependent kinase
PMCA	Plasma membrane Ca^{2+} ATPase
PPAR-γ	Peroxisome proliferator-activated receptor gamma
RyR	Ryanodine receptors
SERCA	Sarco-endoplasmic reticulum Ca^{2+} ATPase
SNS	Sympathetic nervous system

SOICR	Store overload induced Ca^{2+} release
SR	Sarcoplasmic reticulum
SSTR2	Somatostatin receptor 2
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TEDDY	the Environmental Determinants of Diabetes in the Young
T_{eff}	Effector T cell
TM	Tunicamycin
T_{reg}	Regulatory T cell
UPR	Unfolded protein response
VDCCs	Voltage-dependent Ca^{2+} channels
Vm	Membrane potential
XBP1	X-box binding protein 1
ZnT8	Zinc transporter 8

Chapter 1. Introduction

1.1 Diabetes Mellitus and Pancreatic β Cells

Diabetes Mellitus is an important public health concern that affects the health and prosperity of individuals throughout the world. According to data from the International Diabetes Federation, 415 million adults aged 20-79 were affected by diabetes in 2015, whereas another 318 million adults had impaired glucose tolerance with an associated increased risk of developing diabetes (1). Moreover, the number of adults with diabetes is expected to increase to approximately 642 million in 2040, accounting for nearly 1 in 10 individuals. Annually, diabetes is estimated to cause over 5 million deaths, and the financial burden of this disease is enormous. It is estimated that diabetes accounts for 5 – 20% of the total health care expenditures of an individual country. In the US, 29.3 million adults had diabetes in 2015, and diabetes-related expenditures were estimated at 320 millions US dollars. To reverse these trends, improvements are needed in the prevention, diagnosis, management, and treatment of diabetes. Given the close association between obesity and Type 2 diabetes, continued efforts focused on lifestyle and improvements in nutrition are required. To stem the tide of Type 1 diabetes, an improved understanding of disease triggers and the identification of agents that both prevent immune activation and improve the health of the β cell will be needed.

The research in this thesis will explore the mechanisms underlying failure of pancreatic β cells in diabetes with an emphasis on dysregulated calcium storage in the endoplasmic reticulum, an intracellular organelle that is crucial for insulin biosynthesis by the pancreatic β cell and normal control of blood glucose.

1.1.1 The main forms of Diabetes: Type 1 and Type 2 Diabetes Mellitus

The hallmark of diabetes mellitus is an elevated blood glucose level (hyperglycemia) that occurs secondary to either a complete or relative deficiency of insulin secretion and/or insulin action. Insulin is a hormone produced solely by the pancreatic β cells that activates glucose transport into cells via glucose transporters, where it is utilized as a source of energy. In the case of insulin deficiency or impaired insulin action, peripheral tissues cannot efficiently take up glucose. The resulting hyperglycemia damages a number of tissues, resulting in diabetic macrovascular complications (i.e heart disease and stroke) and several microvascular complications, including, kidney failure, retinopathy, and neuropathy.

Diabetes is a heterogenous disease, rarely caused by single gene mutations (2) and is typically caused by a diverse input of many modest influences dictated by environment, lifestyle, aging, and genetics. The two main subtypes of diabetes are phenotypically categorized as Type 1 and Type 2 diabetes mellitus (3,4). Type 1 diabetes (T1DM) is an autoimmune disease characterized by auto-immune mediated β cell destruction and a nearly complete absence of insulin production. T1DM accounts for 5 – 10% of all cases of diabetes. To regulate blood glucose and decrease diabetes-associated complications, persons with T1DM must administer multiple daily injections of exogenous insulin or wear an insulin pump that delivers continuous insulin infusions subcutaneously (5).

T1DM is characterized by β cell destruction that is mediated by islet infiltrating autoreactive immune cells (6). The mechanism of T1DM is not fully understood. However, it is generally accepted that insufficient T regulatory cells (Treg) suppression of effector T cells (Teff) that target the β cells is an early pathogenic event in T1DM (7). More recently it has been appreciated that β cell failure also contributes to T1DM pathogenesis (8). Clinically, T1DM is typically first detected by the onset of severe

hyperglycemia that may also be accompanied by ketoacidosis. A hallmark of T1DM is the presence of circulating autoantibodies against key autoantigens including insulin, islet antigen 2 (IA2), glutamic acid decarboxylase (GAD), and zinc transporter 8 (ZnT8). Individuals at risk of developing T1DM are identified by the presence of these autoantibodies at a time prior to the onset of clinically significant hyperglycemia (9).

What triggers the autoimmune attack against the β cells in T1DM remains unclear. However, T1DM is thought to arise in genetically susceptible individuals in response to an environmental or viral triggering event that initiates the autoimmune response. A family history of T1DM plays an important role in determining risk. For first-degree relatives, the risk of T1DM is estimated to be approximately 3 – 8%, depending on whether an individual's mother, father, or a sibling has T1DM (10,11). In addition, human leukocyte antigen serotype *HLA-DR3-DQ2* and *HLA-DR4-DQ8* class II haplotypes are known to be robustly associated with T1DM, and 90% of children with T1DM have one or both of these haplotypes (12,13). Other than the *HLA* Class II locus, other genetic variants have been associated with T1DM risk. These include differences in the variable number of tandem repeat regions in the insulin gene (14) and polymorphisms in the *CTLA-4* gene that negatively regulate immune responses (15), variants in protein tyrosine phosphatase, non-receptor type 22, *PTPN22*, encoding the lymphoid protein tyrosine phosphatase that negatively regulates the T-cell receptor (16), and *IL2RA* that forms the IL-2 receptor complex with CD25 expressed on activated T-cells and on regulatory T-cells (Tregs) (17).

The pathogenesis of T1DM begins with the interactions of the innate and adaptive immune system with β cells. Initially, islet resident antigen presenting cells (APCs) such as macrophages and dendritic cells receive and process islet antigens. As a consequence, APCs migrate to pancreatic lymph nodes (15). Within the lymph nodes, islet antigens are presented to and activate naïve circulating autoreactive T cells. This

causes T cell activation and initiates immune cell infiltration into the islet (18). Increased levels of pro-inflammatory and toxic cytokines and chemokines are released from resident APCs, infiltrating T cells, and the β cells themselves, causing additional immune cell recruitment into the islet (19). These cytokines indirectly cause β cell death, while further β cell death occurs through direct interactions between $CD8^+$ T cells and the β cell (20,21).

Currently a number of prevention efforts are being tested in individuals who already have established autoimmunity and autoantibody positivity. Prevention efforts are also being tested in birth cohorts identified on the basis of genetic risk, who are then followed longitudinally to identify both environmental contributors as well as patterns of immune activation before and after antibody seroconversion (5). Studies include the Environmental Determinants of Diabetes in the Young (TEDDY) (22), Baby Diabetes (BABYDIAB) (23), Diabetes Prediction and Prevention Project (DIPP) (24), and the double-blind, placebo-controlled, dose-escalation, phase 1/2 clinical pilot study (Pre-POINT studies) (25).

A number of recent studies have also tested immunomodulatory therapies to either prevent T1DM. These include efforts focused on restoration of tolerance (26,27), T cell or B cell inhibition (28), T_{reg} induction (29), suppression of innate immunity and inflammation (4,30), immune system reset (31), and islet transplantation (32). Trials have tested antigen-specific therapies to induce immune tolerance (33), monoclonal antibodies (28,34,35) and fusion proteins (36,37) that target specific immune cells, and strategies to alter the effector T cell (T_{eff})/T_{reg} balance. Overall, each treatment has limited duration of effect, and no single therapy has stopped the autoimmune attack on β cells.

Given these disappointing outcomes, there has been a recent emphasis on testing a combination of therapies at T1D onset. (6). Additionally, there is an emphasis

on testing prevention efforts in individuals who already have established autoimmunity and autoantibody positivity, but lack clinical hyperglycemia. Prevention efforts are also being tested in birth cohorts identified on the basis of genetic risk, who are then followed longitudinally to identify both environmental contributors as well as patterns of immune activation before and after antibody seroconversion (5).

Type 2 diabetes (T2DM) is the most common form of diabetes and accounts for 90 – 95% of all diabetes cases. Similar to T1DM, the exact cause of this disease is still unknown but risk factors include obesity, poor nutrition, physical inactivity, smoking, (38) and a variety of genetic factors (3,38). Classically, T2DM begins with peripheral insulin resistance, in which glucose uptake and the metabolic effects of insulin are decreased in all tissues in body (39). In the setting of obesity, insulin resistance, and high fat diet lead to increased release of free fatty acids from the adipose tissue, which further exacerbate glucose uptake into muscle. A second key contributing factor is increased gluconeogenesis at the level of the liver that also worsens hyperglycemia (40).

Initially, β cells are able to overcome insulin resistance through increased insulin production and secretion. However, as T2DM progresses, β cell dysfunction ensues and insulin secretion from the β cells begins to diminish (41). In addition to gluco-lipotoxicity as a primary cause of β cell failure (42), emerging areas of research have focused on pathways leading to pro-inflammatory stress or ER stress (43), and oxidative stress in the β cells (44). Recently, dedifferentiation, which is defined as loss of expression of key β cell specific genes has been proposed as a cause of T2DM (45,46).

Genome-wide association studies (GWAS) have identified a number of risk variants associated with T2DM risk. Susceptibility loci identified to date have primarily been linked to the regulation of β cell function (38). For example, polymorphisms have been identified in the *KCNJ11* gene that encodes the potassium inwardly rectifying

channel subfamily J, member 11 (38,45-47). In addition, variants in *TCF7L2* have been identified. This gene encodes the ubiquitous transcription factor 7-like 2 (48), a mediator of Wnt signaling (49) and inhibitor of insulin secretion (50) Polymorphisms have also been identified in the IRS1 gene that encodes insulin receptor substrate 1. *IRS1* plays a role in β cell health and function as wells as peripheral insulin signaling (51). Islets from *IRS1* knock-out mice and *IRS1* deficient β cells exhibited decreased in insulin content and reduced glucose stimulated insulin secretion (52). Variants in *MTNR1B* gene, which encodes the melatonin receptor 1B, is thought to increase the risk of T2DM by inducing impaired early insulin secretion (53).

Polymorphisms have also been identified in genes with more pleiotropic metabolic effects. The *PPARG2* gene encodes peroxisome proliferator-activated receptor gamma (PPAR-γ) 2, which is a member of the nuclear hormone receptor subfamily. PPAR-γ is thought to regulate insulin action, but has also been shown to be expressed in the β cell. Agonists of PPAR-γ improve insulin sensitivity and have been associated with improved β cell function. Our lab has previously shown that PPAR-γ regulates transcription of the ER Ca^{2+} transporter Sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) 2 level in the β cell, leading to improved β cell Ca^{2+} signaling and ER Ca^{2+} storage (54), which will be discussed later. Additional risk variants have been identified in the *IGF2BP2* gene that encodes the insulin-like growth factor two binding protein 2 and regulates translation of the insulin-like growth factor 2 (IGF2), and the fat mass and obesity-associated gene, FTO, which is associated with obesity and weight gain (55).

1.1.2 Therapy for Type 1 and Type 2 Diabetes Mellitus

The treatment of T2DM is complicated and often requires multiple agents to achieve glycemic control. Treatment is typically initiated with an oral agent, and the first line agent for most newly diagnosed individuals with T2DM is the biguanide metformin

(56). The precise molecular mechanisms of metformin action are not fully understood. However, this drug is thought to primarily lower blood glucose levels by reducing hepatic glucose output through reduced gluconeogenesis. The use of metformin has also been associated with increased insulin induced glucose uptake and increased glycogenesis in skeletal muscle (57). Metformin has been shown to regulate the expression of genes related to hepatic gluconeogenesis through activation of AMP-activated protein kinase (AMPK) (58,59). Additionally, recent data have shown that metformin inhibits mitochondrial glycerophosphate dehydrogenase (GPDH) to alter the hepatocellular redox state, which result in reduced gluconeogenesis (60). Other advantages using metformin include reduced cardiovascular events, decreased mortality (61,62), and lower risk of hypoglycemia using this drug (63).

Other commonly utilized oral agents include sulfonylureas, which close the ATP-sensitive potassium channel of the pancreatic β cells to stimulate insulin secretion (64). However, the effect of sulfonylureas depends on the presence of enough functional β cells to increase insulin secretion, and diabetes progression is typically associated with failure to respond to sulfonylureas. Adverse effects of these medications include an increased risk of hypoglycemia (65) and weight gain. The risk of hypoglycemia is higher for older persons with impaired renal and hepatic function (66).

Incretins are a group of hormones that decrease blood glucose levels by promoting insulin secretion. Incretin-based therapies have been introduced in recent years. The incretin hormones include glucagon-like peptide-1 (GLP-1), primarily arise from the intestinal enteroendocrine L cells (67), and glucose-dependent insulinotropic polypeptide (GIP), which are released in response to a meal, leading to increased insulin secretion. Incretin secretion is dramatically diminished in T2DM (68,69). In T2DM, administration of spraphysiological amounts of GLP-1 can lead to enhanced glucose-stimulated insulin secretion, whereas administration of GIP does not induce insulin

secretion regardless of the concentration utilized (70). Therefore, GLP-1 is therapeutically more favored than GIP, and inhibitors of dipeptideyl peptidase-4 (DPP-4), the enzyme that degrades GLP-1, have been developed for clinical use (71,72).

Plasma levels of GLP-1 are relatively low compared to other hormones, and GLP-1 has a very short half-life due to DPP4 activity. GLP-1 is metabolized within 1 minute around the L cells (73,74), which generated considerable controversy as to the source of GLP-1 that acts on distant organs such as the pancreatic β cells. Recently, an updated model was proposed whereby GLP-1 derived from the L cells activates neurons to regulate insulin secretion, and GLP-1 derived from islet α cells serves as paracrine factor to induce insulin secretion in β cells (75).

As described, GLP-1 has a short half-life due to DPP-4 activity, so the effects of GLP-1 by subcutaneous injections were very short-lasting (76). That led to the development of inhibitors of DPP-4, or stable DPP-4 resistant GLP-1 analogs (77). Exendin 4, isolated from *Heloderma suspectum*, is a peptide that has 50% sequence homology to GLP-1. This peptide was found to be resistant to DPP-4 activity and is eliminated only by glomerular filtration by the kidney (78), thereby increasing the plasma half-life of Exendin 4 to as long as 30 minutes (79).

Other orally active agents include α-glucosidase inhibitors, which inhibit breakdown and digestion of carbohydrates in the intestine, and thiazolidinediones (TZD), which activate PPARs, including PPAR gamma. These agents are less widely utilized and their clinical effects are summarized in Table 1. A large percentage of individuals with T2DM will eventually fail oral agents and incretin-based therapies and require insulin injections as β cell failure ensues (3).

A recent option to treat diabetes in morbidly obese patients is bariatric surgery, which either reduces the size of stomach to decrease food intake or re-routes the small intestine to cause malabsorption (80). Clinical studies show that blood glucose is often

normalized within a week of surgery, even prior to significant weight loss. At present, the exact mechanisms of improved glycemia in the short-term are not well understood but it is hypothesized that surgery leads to either increased release of GLP-1, alterations in gut microbiota, and the anticipated metabolic effects of acute and severe caloric restriction peri-operatively (3).

Table 1. Drug Treatment for Type 2 Diabetes

Class Name	Drug Name	Target	Action	
α-glucosidase inhibitors (AGIs)	Acarbose Miglitol Voglibose	α-glucosidase	Carbohydrate absorption↓ No weight gain	
Biguanides	Metformin	AMPK GPDH (Still debated)	Blood glucose↓ Insulin action↑ Hepatic output↓ Cardiac risk↓ Hypoglycemia Risk↓ No weight gain	(60,61)
GLP-1 Analog	Exenatide Liraglutide	GLP-1R	Insulin secretion↑ Glucagon secretion↓ Satiety↑ Hypoglycemia Risk↓ No weight gain	(81)
Sulfonylurea	Glyburide Glipizide Gliclazide Glimepiride	K_{ATP} channels	Insulin secretion↑ Weight ↑	(65)
Thiazolidinedione (TZDs)	Troglitazone Roziglitazone Pioglitazone	PPAR-γ	Insulin action↑ Hypoglycemia Risk↓ Weight ↑ Glycemic control↑	(82)
Insulin	Lispro Aspart Glulisine	Direct target	Blood glucose↓ Weight ↑	

1.1.3 Pancreatic islet β cells, and other cell types

The endocrine pancreas consists of the islets of Langerhans that make up about ~5% of the total pancreatic mass. The endocrine islets were named for the German physician Paul Langerhans, who discovered the cells that secrete insulin. Islets consist of aggregates of ~1000 different cells. The insulin producing β cells make up ~75% of cells found in the islet. Additional cell types found in the islet include α cells, δ cells, PP cells and ε cells. The α cells consist of ~20% of total islet cells (83), and secrete glucagon. Glucagon is described as a counter-regulatory hormone to the effects of insulin and acts to raise blood glucose levels. Glucagon promotes gluconeogenesis in the liver and induces fatty acid oxidation and ketogenesis in the liver. The δ cells consist of less than 10% of islet cells (83), and these cells secrete somatostatin. Somatostatin receptors are expressed on both α and β cells, and the somatostatin receptor 2 (SSTR2) is the functionally dominant form of the receptor in humans and mice (84). The SSTR2 is a G_i-coupled G protein coupled-receptor (GPCR) that inhibits adenylyl cyclase activity and cyclic AMP (cAMP) generation. Activation of SSTR induces hyperpolarization of cells and thus suppression of glucagon and insulin secretion (84). In response to elevated blood glucose levels, insulin and somatostatin levels rise. Both insulin and somatostatin act on α cells to lower cAMP production and suppress glucagon secretion (85). Precisely how exactly these cells communicate *in vivo* remain to be completely understood.

Other endocrine cells in islet include the polypeptide-producing PP cells and ghrelin-producing ε cells, which consist of less than 5% and 1% of islet cells, respectively (83). The function of these cells is less understood in terms of regulation of β cell function. Ghrelin is known to inhibit insulin secretion (86) and also has activity as a "hunger hormone" by acting on hypothalamus (87).

The development and differentiation of the endocrine cell in the islet has been extensively studied using a variety of knockout mouse models. Neurogenin-3 (Ngn3) serves as the key marker for the endocrine pancreatic progenitor cell and is first expressed in the pancreatic buds around E9.5 in the mouse. Ngn3 expression is crucial for commitment to the endocrine cell lineage (88). Lineage-tracing studies have shown that Ngn3 positive cells serve as the precursor for all five endocrine cell subtypes (89). During this differentiation process, Ngn3 coordinates with the pancreatic and duodenal homeobox 1 (Pdx-1) transcription factor in a time specific manner (90). Additional research is needed to define the differentiation pathway to establish β cells from stem cells, elucidate transcriptional micro RNA and epigenetic factors that regulate β cell differentiation, establish signature of β cell "specific" gene expression, and delineate pathological changes in these areas that are causative or associated with diabetes.

1.1.4 Mechanisms of insulin secretion

A striking feature of the pancreatic β cell is its ability to monitor blood glucose on a minute to minute basis and to precisely match ambient glucose levels with regulated levels of insulin secretion to maintain euglycemia in the range of 3.9 – 5.5 mM. To accomplish this task, the β cell relies on several unique pathways to monitor blood glucose concentrations and regulate insulin release (Fig. 1) (91). Glucose transporters (Glut), mitochondrial ATP synthesis, closure of ATP-sensitive K^+ channels (K_{ATP} channels), Ca^{2+} influx from plasma membrane channels are all involved in this process and any disruption of any of these steps will lead to impaired insulin secretion.

β cells are electrically excitable, and they possess a variety of channels that modulate membrane potential (Vm) and Ca^{2+} influx. In response to an increase in plasma glucose, glucose first enters the β cell through Glut transporters. Glucose

metabolism in the mitochondria leads to an increase in the ATP/ADP ratio (92). The main regulators of β cell electrical activity are the K_{ATP} channels. Under low glucose conditions, K_{ATP} channels are open to induce a K^+ outward current to maintain plasma membrane hyperpolarization at ~ -70mV (91). In β cells at rest, the constitutive hyperpolarizing K^+ efflux and the constitutive unknown background-depolarizing current are balanced to achieve the resting membrane potential. Under high glucose concentrations, an increase in the ATP/ADP ratio leads to K_{ATP} channel closure. The reduced hyperpolarizing current by the closure of K_{ATP} channels will be exceeded by background-depolarizing current to depolarize cells. The mechanism that induces this background-depolarizing current and whether that current is modulated by glucose per se are not well understood. It has been speculated that forward operation of the Na^+/Ca^{2+} exchanger (93), Cl^- efflux, or Na^+ (94) or Ca^{2+} influx (95) through unknown channels are involved in this background-depolarizing current. This depolarization activates voltage-dependent Ca^{2+} channels (VDCCs), and the resulting Ca^{2+} influx triggers exocytosis of insulin vesicles and insulin secretion. Action potentials generated by this process are primarily result from Ca^{2+} influx in mice. However, a Na^+ current is also involved in human and rat β cells.

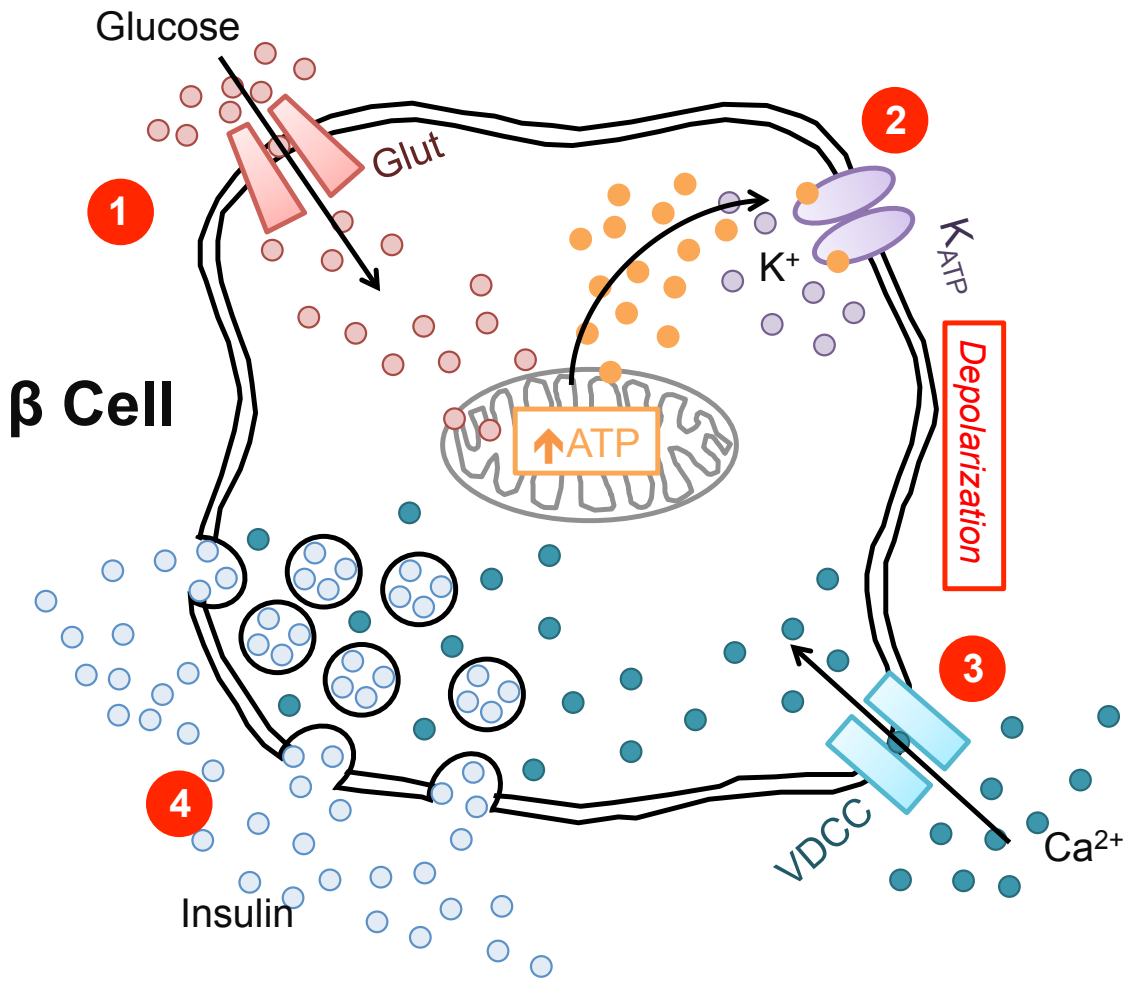

Figure 1. Molecular mechanisms of insulin secretion in the pancreatic β cells.

(1) Glucose uptake occurs through the Glut glucose transporters, and glucose is metabolized to produce adenosine triphosphate (ATP). (2) The increase in ATP will close ATP-sensitive K^+ channels (K_{ATP}) to trigger depolarization. (3) VDCCs open and Ca^{2+} influx occurs. (4) Ca^{2+} induces exocytosis of insulin vesicles. The colored circles indicate glucose (pink), ATP (orange), K^+ (purple), Ca^+ (green), and insulin (blue).

1.1.5 ER stress and various stresses are present in diabetes

During the evolution of both Type 1 and 2 diabetes, β cells are exposed to multiple stressors, including elevated levels of pro-inflammatory cytokines, glucose, and free fatty acids, hypoxia, and misfolded protein accumulation, leading to activation of a number cell intrinsic stress pathways such as oxidative and ER stress, and maladaptive changes in autophagy (96). These multiple stressors activate the stress pathways and contribute to β cell dysfunction and death and may contribute to β cell de-differentiation (97,98).

Pancreatic β cells possess a highly developed endoplasmic reticulum in order to meet the high demands of insulin protein synthesis and secretion. As a result, β cells are particularly susceptible to endoplasmic reticulum stress, which has been a focus of my thesis studies. Specifically, my studies have investigated novel pathways that lead to dysregulation of ER Ca^{2+} homeostasis and how loss of ER Ca^{2+} leads to ER stress.

The endoplasmic reticulum (ER) is an organelle found in all eukaryotic cells. The structure of ER is a continuous membranous network surrounding a series of sacs known as cisternae. The ER is divided into a ribosome-rich “rough” ER and a ribosome-free “smooth” ER (99). Rough ER is abundant in cells that secrete large amount of proteins including insulin-producing pancreatic β cells (100). The ER membrane constitutes more than half of the total cellular membrane structure and it functions as the site of (a) synthesis, folding, modification and transport of proteins; (b) quality control for newly synthesized proteins; and (c) Ca^{2+} storage that plays a role both in the maintenance of normal ER functions and as a source of released Ca^{2+} that serves a cellular second messenger to regulate a variety of signaling pathways (101). Under normal conditions, Ca^{2+}- dependent chaperone proteins assist in the folding of proteins. These folded proteins are transported to the Golgi apparatus for further maturation. Proteins that are improperly folded or “misfolded” are removed by a quality control

system called ER-associated degradation (ERAD) (102). ERAD involves protein ubiquitination that promotes retro-translocation from the ER to the cytosol, where misfolded proteins undergo proteasome-induced degradation. On the contrary to the effect favor to help the ER functions, once the demand of the protein synthesis surpasses the ER capacity of protein folding and quality control, misfolded proteins can accumulate within the ER lumen, leading to ER stress. Accumulation of misfolded proteins triggers an adaptive cascade aimed at restoring normal ER function, which is referred to as the unfolded-protein response (UPR) (103,104). This adaptive response induces downregulation of overall protein translation, while chaperone proteins and ERAD-associated proteins are preferentially translated (103,105). If the UPR's adaptive response cannot restore normal ER function, prolonged activation of UPR will eventually initiate cellular apoptosis (106). ER stress can impact a variety of different cell types and contributes to a number of disease pathologies, including cardiac and neurodegenerative diseases as well as diabetes.

The molecular mechanisms of the UPR have been well studied (Fig. 2). There are three main branches of this signaling pathway and the initiation of each branch is regulated by three different ER stress sensors located on ER membrane. These include the inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and PKR-like ER kinase (PERK). Upon binding of unfolded proteins to these three sensor proteins, the dissociation of Bip protein from the sensor proteins occurs. Although the exact mechanisms of how Bip protein attaches and dissociate from each sensor is s unknown (107), this is thought to initiate the unfolded proteins response, UPR. In addition, the direct interaction of misfolded proteins with the luminal portion of IRE1 has also been shown to activate the UPR (108). Following Bip dissociation, IRE1 undergoes oligomerization and autophosphorylation, initiating this branch of the UPR. IRE1 has

dual functions as endoribonulease and kinase. To reduce the overall protein load, IRE1 degrades a large number of mRNAs (109), while X-box binding protein 1 (XBP1) mRNA is spliced by IRE1, allowing translation of XBP1 protein. XBP1 is a transcription factor that increases the expression of genes related to both UPR and ERAD (110). Included in these targets are chaperone proteins, which play a central role in the folding of client proteins in the ER, ATF6 that is the second of these ER stress sensors with which activation of this branch is initiated by translocation of ATF6 to the Golgi apparatus under ER stress conditions. ATF6 is subsequently cleaved to give rise to a cytosolic fragment known as p50 that forms either a homodimer or heterodimer with XBP1 to promote transcription of additional UPR effectors including ATF6 itself (105,111). Finally, the PERK branch of the UPR is thought to be initiated BiP dissociation, followed by the oligomerization and autophosphorylation of PERK. Phosphorylated PERK phosphorylates eukaryotic initiation factor eIF2α to inhibit activity of this translation factor. This results in a global translational block to prevent further accumulation of misfolded protein. However, selective translations of transcription factor, such as transcription factor 4 (ATF4), will be taken place. ATF4 expression is repressed in the unstressed state, but the phosphorylation of eIF2 under ER stress will initiate the translation of ATF4 (112).

In contrast to this adaptive effect, prolonged activation of the UPR results in apoptosis. Key transcriptional regulators in this pathway, ATF4, ATF6, and XBP1, also initiate transcription of genes encoding proapoptotic proteins, such as C/EBP homologous protein (CHOP). The function of CHOP is to decrease expression of the anti-apoptotic protein Bcl-2 and activate the pro-apoptic factor Bcl-2-interacting mediator of cell death (Bim) (113,114). Similarly, phosphorylated IRE1 can activate the apoptotic pathway via activation of c-Jun N-terminal kinase (JNK), which leads to inactivation of

anti-apoptotic Bcl-2 and activation of pro-apoptotic Bim (115). As a consequence of Bcl-2 inhibition, the pro-apoptotic proteins Bax and Bak undergo oligomerization that permeabilizes the outer mitochondrial membrane, resulting in Ca^{2+} overload in the mitochondria. Cytochrome C is subsequently released from mitochondria, leading the initiation of caspase-mediated cell death pathways.

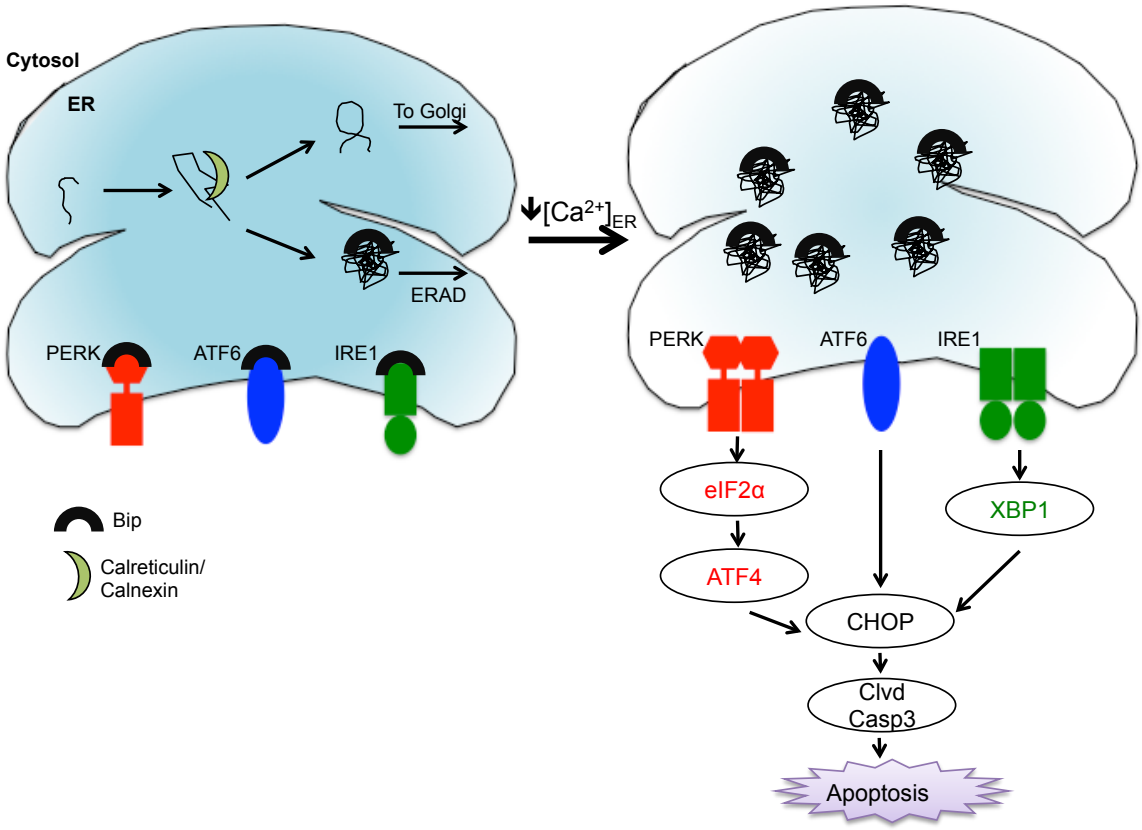

Figure 2. Unfolded protein response and ER Ca^{2+}

Under ER stress conditions, ER Ca^{2+} homeostasis is disrupted and Ca^{2+} -dependent chaperone proteins cannot meet the excess demand for protein folding leading to the accumulation of unfolded proteins. As a result, dissociation of chaperone protein Bip from the ER stress sensor proteins PERK, ATF6, and IRE1 occur. This triggers the activation of unfolded protein response (UPR). Prolonged activation of the UPR eventually causes apoptosis.

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1.2 ER Ca^{2+} dynamics and diseases

A focus of my thesis work has been on the identification of pathways that maintain ER Ca^{2+} levels. Cells possess a variety of systems and safeguards to maintain distinct Ca^{2+} stores and concentration gradients that are crucial for cellular function and survival. Specifically, a distinct Ca^{2+} gradient exists between the membranes of the extracellular space, cytosol, and intracellular organelles. These gradients allow cells to maintain organelle function, while also contributing to spatiotemporal calcium signaling. Disruption of gradients can contribute to disease pathophysiology. In the pancreatic β cell, disruption of these gradients leads to impaired insulin production and secretion as well as apoptosis. Under resting conditions, intracellular Ca^{2+} is maintained at a level at least four orders of magnitude lower than extracellular Ca^{2+}. In addition, free ER Ca^{2+} is maintained at approximately two orders of magnitude higher than the intracellular Ca^{2+} (116), while mitochondrial Ca^{2+} levels are comparable to that of intracellular Ca^{2+} (117,118). The rank order of Ca^{2+} concentration in each compartment at rest is summarized as: Extracellular Ca^{2+} (~2 mM)> ER Ca^{2+} (~100 μM) > Mitochondria Ca^{2+} (~200 nM) ≥ Intracellular Ca^{2+} (~100 nM).

Meticulous coordination of ion transporters including channels on the plasma and organelle membranes establish these Ca^{2+} gradients. On the β cell plasma membrane, the Na^{+}/Ca^{2+} exchanger (NCX) and the plasma membrane Ca^{2+} ATPase (PMCA) transfer excess intracellular Ca^{2+} into the extracellular space to maintain low cytosolic calcium levels. Among the many cellular organelles, the ER is the largest Ca^{2+} store. As is the case for NCX and PMCA, the Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) also works to eliminate excess intracellular Ca^{2+} by pumping calcium into the ER. This process is regulated by SERCA, PMCA, and NCX and is called Ca^{2+} clearance. The process of calcium clearance is particularly important following a β cell secretory burst. Mathematical modeling based on the experiments performed in

dispersed mouse β cells suggests 50-64% of intracellular Ca^{2+} transients induced by KCl, which causes depolarization, are removed by the SERCA pump (119). After that, 21-30% of the excess Ca^{2+} will be cleared by NCX when intracellular Ca^{2+} is high ~>1 μM, and 21-27% of Ca^{2+} will be removed by PMCA when intracellular Ca^{2+} is low at around ~0.5 μM (119). Other factors such as the mitochondrial calcium uniporter (MCU) and the gap junction channels, connexin 36 (Cx36), are anticipated to play a role, but have been less well studied (91).

Like the PMCA pump, the SERCA pump uses ATP to transport Ca^{2+} against the steep calcium concentration gradient between the cytosol and ER. In contrast to SERCA, inositol 1,4,5-trisphosphate and ryanodine receptors (IP_3R and RyR) are Ca^{2+} releasing channels that passively release Ca^{2+} from the ER lumen into the cytosol. Another important organelle for cellular Ca^{2+} homeostasis is the mitochondria, which has been less characterized in β cells, but may function as a transient Ca^{2+} buffer. The MCU and leucine- zipper EF-hand-containing transmembrane protein 1 (LETM1) are localized to the mitochondrial inner membrane and drive Ca^{2+} uptake into mitochondria (120). In contrast, $Na^+/K^+/Ca^{2+}$-exchange protein 6 (NCLX) is the transporter that releases Ca^{2+} from mitochondria into the cytosol (96,121).

1.2.1 The role of the SERCA in health and disease

ER Ca^{2+} levels represent the balance of calcium uptake via the SERCA pump and calcium release via the RyR and the IP_3R. SERCA is an active transporter that senses and pumps excess intracellular Ca^{2+} into the ER. Since this process is also ATP dependent, SERCA functions as a metabolic sensor (122,123).

SERCA protein is encoded by three distinct genes: *ATP2A1*, *ATP2A2*, *ATP2A3* (SERCA1, SERCA2 and SERCA3 respectively). There is a high level of conservation among the different isoforms, with more than 75% structural homology observed

between the isoforms (124). Each gene encodes 2-6 alternatively spliced transcripts. SERCA1 is expressed exclusively in fast-twitch skeletal muscle. SERCA2 splice variants are expressed in a tissue-specific manner. SERCA2a is expressed mainly in cardiac and slow-twitch skeletal muscle, whereas SERCA2b is ubiquitously expressed. SERCA3 is expressed only in particular tissues including β cells and is usually co-expressed with SERCA2b (125).

Acquired SERCA deficiencies have been implicated in a variety of diseases. Darier-White disease is an inherited skin disease that arises as a result of SERCA2 haploinsufficiency and is characterized by the loss of keratinocyte adhesion, which is termed acantholysis, occurring as a result of disruption of ER Ca^{2+} levels in the keratinocytes (126,127). Persons with Darier-White disease also have higher rates of epilepsy and depression (126,128). Whether these individuals are more suspectible to metabolic disease has not been tested. SERCA2a expression and Ca^{2+} affinity are decreased in heart failure (129) and have been associated with cardiac myopathies (130). Moreover, cardiac function was improved by overexpression of SERCA2a in a rat model of heart failure (131,132). In addition, the Ca^{2+} affinity of SERCA2 was increased by suppression of the phosphorylated form of the membrane-localized inhibitory protein, phospholamban in a hamster model of heart failure (133). Also, ablation of phospholamban in cardiomyopathic mice (134) has been shown to improve contractility in heart failure (135). Therefore, improving SERCA function may represent a potential therapeutic strategy for a variety of diseases.

The dominant form of SERCA in the pancreatic β-cells is SERCA2b (54). The structural difference between SERCA2a and SERCA2b is limited to the COOH-terminal (C-terminal) of each protein. SERCA2b contains a unique 49-amino acid C-terminal extension that creates an 11th transmembrane α-helix in this protein with the C-terminus

tail projecting into the lumen of the ER. This structural difference grants this isoform the highest affinity to Ca^{2+} (2b>2a>3) and the lower ATPase turnover rate (3>2a>2b) (136).

Accumulating evidence suggests that altered SERCA expression and activity contributes to the pathophysiology of diabetes, leading to both impaired insulin secretion and β cell death (137-139). The contribution of SERCA to Ca^{2+} clearance following glucose exposure has been experimentally assessed using dispersed mouse islet β cells. Chen et al. demonstrated that about two-thirds of the depolarization-induced Ca^{2+} influx was removed by SERCA (119). This suggests that SERCA can indirectly regulate insulin secretion by controlling intracellular Ca^{2+}. Moreover, SERCA may play a significant role in the amplitude and the downward phase of the glucose-induced Ca^{2+} oscillations (GICOs) that additionally regulate insulin secretion. Functional studies of SERCA have been enabled due by the SERCA inhibitor, thapsigargin (TG). Inhibition of SERCA leaves only Ca^{2+} releasing channels, RyR and IP_3R functional, which results in the depletion of ER Ca^{2+} across the concentration gradient. Prolonged TG treatment leads to ER stress and apoptosis (140), again emphasizing that ER Ca^{2+} is required for cell survival and function.

1.2.2 The role of the Ryanodine Receptor in health and disease

Whereas SERCA functions to pump Ca^{2+} into ER to maintain ER Ca^{2+} levels, ER Ca^{2+} releasing channels, IP_3R and RyR, are both expressed in the β-cell (141-143) and coordinately function to maintain ER Ca^{2+} homeostasis and ER function. For instance, excess ER Ca^{2+} is released by IP_3R and RyR to maintain Ca^{2+} balance and homeostasis. In addition, acute release from these channels also contributes to a variety of intracellular signaling pathways. Mammalian RyR has three different isoforms. RyR1 is mainly expressed in skeletal muscles and is localized in junctional terminals between the plasma membrane and sarcoplasmic reticulum (SR), while RyR2 is predominantly

expressed in cardiac muscles. RyR3 was found to be ubiquitously expressed (144-146). Functional RyRs consist of homotetramers (147). RyRs are also expressed in other nonmamalian vertebrates such as birds and fish with the isoforms RyRa and RyRb (148). Furthermore, RyR expression has been found in other metazoans, including C. elegans and Drosophila melanogaster (149,150).

RyRs are associated with many different human diseases, with the majority of diseases arising as a result of alterations in channel gating properties (147). Diseases caused by mutations in the RyR1 gene include malignant hyperthermia, leading to increased sensitivity to inhalation anesthetics that can lead to sustained muscle contraction (151). RyR1 mutations may also lead to exertional rhabdomyolysis, characterized by the breakdown of striated muscles in response to heat or exercise (152); central core disease, which is a mild congenital myopathy characterized by hypotonia and muscle weakness of the upper legs and hips (153); and multiminicore disease that is autosomal recessive myopathy with degeneration of muscle fibers causing weak limb muscles (154).

Diseases caused by mutations in the RyR2 include catecholaminergic polymorphic ventricular tachycardia (CPVT), which is characterized by stress- or exercise-induced ventricular tachycardia that can lead to sudden death (155). Calstabin1 and 2 (also called FKBP12 and FKBP12.6) are expressed in most tissues and function to bind and stabilize the RyR1 and RyR2 in the closed state (156-159). Mutation of the V2461 in RyR1 abolished calstabin1 binding (160). Inhibition or loss of calstabin1 binding greatly increased the open probability and open duration for the channel (161,162) Calstabin1 displacement in skeletal muscle alters EC coupling between RyR1 and Cav1.1 (163), whereas loss of calstabin1 in cardiac muscle results in cardiomyopathy as well as developmental congenital heart defects (164).

In vitro and animal studies have shown that the CPVT associated mutation in RyR2 results in Ca^{2+} leak through the receptor by lowering affinity for FKBP12.6, also known as calstabin2 (165) or by increasing sensitivity to β-adrenergic receptors to induce prolonged Ca^{2+} transients (166). In CPVT, protein kinase A (PKA) and Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) phosphorylation of RyR2 has been also been documented to change RyR2 function (167,168). Thus, in persons with CPVT, β-adrenergic stress and exercise induced PKA phosphorylation along with a leaky RyR2 are thought to contribute to arrhythmias. Mutations in RyR2 are also responsible for arrhythmogenic right ventricular dysplasia type 2 (ARDV2), which is an autosomal dominant disease characterized by degeneration of myocardium, leading to ventricular arrhythmias and sudden death (169). The mutation associated with ARDV2 also leads to dysfunction in RyR2 via Ca^{2+} leak from myocardial SR (170). In contrast to RyR1 and RyR2, RyR3 is the isoform that has been least studied. One study suggested that upregulation of RyR3 in cortical neurons may play a protective role in Alzheimer's disease (171).

The RyRs exhibit approximately 65% sequence homology, but each RyR isoform has different modes of regulation. RyRs form a macromolecular complex with a variety of proteins including L-type voltage dependent channels, Cav1.1/Cav1.2, PKA, Calstabin1 and 2, CaM, CaMKII and calsequestrin. Therefore, receptor function can be modulated by proteins within these larger complexes. The regulation of these complexes have been extensively studied in electrical – contraction (EC) coupling, which is regulated by Ca^{2+} release from SR in skeletal and cardiac muscles. In skeletal muscle, multiple regions of Cav1.1 and RyR1 physically interact to regulate EC coupling (172-174). Due to this physical interaction rather than Ca^{2+} induced activation, EC coupling in skeletal muscle is prolonged even in the absence of extracellular Ca^{2+} (175,176). In contrast, RyR2 in cardiac muscle is activated by Ca^{2+} influx through Cav1.2, and this

mode of RyR2-mediated ER Ca^{2+} release is called Ca^{2+} induced Ca^{2+} release (CICR) (177). Ca^{2+} influx through Cav1.2 does not directly induce muscle contraction, but much larger Ca^{2+} release from RyR2 by CICR mediates muscle contraction (178).

Single channel studies have shown that RyRs exhibit biphasic activation by Ca^{2+}. Low intracellular Ca^{2+} concentrations (<0.01-20μM) can activate RyRs, whereas higher intracellular Ca^{2+} concentration (~100 μM – 10 mM) can inhibit RyRs (179). In addition to the activation from the cytosolic side, single channel recordings have shown that Ca^{2+} can activate RyR from ER luminal side in response to increased ER Ca^{2+} (180,181). This is referred to as store overload induced Ca^{2+} release (SOICR). Structural studies indicate that highly electronegative luminal loop domains are responsible for RyR activation in SOICR (182). Spontaneous SOICR in cardiac cells are capable of inducing Ca^{2+} waves and delayed after depolarizations resulting in ventricular tachyarrhythmias (183). This is caused by the abnormal RyR2 activation due to RyR2 mutations associated with CPVT, which lowered the threshold of the SOICR (22 23).

Ca^{2+} may also regulate RyR function through calmodulin (CaM), which is a ubiquitously expressed Ca^{2+} binding protein. CaM contains four EF-hand Ca^{2+} binding sites. CaM is known to bind to each RyR monomer (184) and also plays a role in regulating Cav1.1 and Cav1.2 (185-187). Studies have shown that CaM is capable of suppressing RyR2 function by elevating the threshold of intracellular Ca^{2+} for RyR2 activation in cardiac muscle (188). Decreased CaM binding affinity for RyR2 was observed in the mouse model of CPVT that has a RyR2 R2474S mutation, resulting in lethal arrhythmias (189).

Post-translational modification of RyRs also regulate their activity. RyRs possess multiple phosphorylation sites in the cytoplasmic domains including sites for phosphorylation by PKA, CaMKII and cGMP-dependent kinase (PKG) (190-193). During the “fight or flight” stress response, the sympathetic nervous system (SNS) is activated

resulting in increased calcium transients and faster muscle contractions (194). This pathway involves β adrenergic receptor and PKA activation leading to RyR phosphorylation and altered gating properties (167,195). In addition hyperphosphorylation of RyR2 serine residues at the calstabin 2 binding site and concomitant dissociation of calstabins and resulting RyR2-mediated Ca^{2+} leak are suggested to play a role in both heart failure and during severe stress (168). CaMKII dependent phosphorylation of RyR2 is also suggested to enhance SR Ca^{2+} leak and decrease of SR Ca^{2+} load in heart failure (196).

In addition, alterations in redox state of RyRs can both activate (197,198) or inhibit channels (199). Cysteines in RyR1 and RyR2 are covalently bound by NO (S-nitrosylation) (200), and high level of ROS/RNS can irreversibly modify RyR function (201). For example, S-nitrosylation of Cys3635 in RyR1 has been found to reverse the CaM mediated inhibition leading to channel activation (202), which can facilitate muscle contraction (203). S-nitrosylation of Cys3635 in RyR1 has also been observed in muscular dystrophy leading to elevated SR Ca^{2+} leak (204). On the contrary, RyR2 cannot S-nitrosylated by NO, and RyR2 has to go through S-nitrosoglutathione, endogenous NO carriers and donors, to undergo S-nitrosylation (205). In addition to Ca^{2+}, Mg^{2+} and ATP also regulate RyRs. Mg^{2+} is suggested to reduce the open probability of RyR by competing with Ca^{2+} for the Ca^{2+} activation site (206,207). ATP may also activate RyRs in absence of Ca^{2+}, but will have a maximum effect in the presence of Ca^{2+} (206,208).

While the association of abnormal RyR function has been well-described in heart disease, how RyRs function and dysfunction affects the pancreatic β cell and the development of diabetes is not well understood. Since only Cav1.2 and Cav1.3 are detected in rodent and human β cells (209), depolarization induced RyR activation mediated by Cav1.1 does not exist in β cells. Rather, CICR mediated by Cav1.2 and

RyR2 is thought to be the major form of RyR regulation in the β cell. Santulli et al. reported the metabolic phenotype of mice with RyR2 mutation in R2474S and N2386I and, humans with CPVT mutation in RyR2. Collectively, these mutations induced dysregulated ER Ca^{2+} release through RyRs and led to impaired insulin secretion (210). This group found that these mutations caused dissociation of calstabin2 from RyR2, and thus pharmacologically stabilizing interactions between calstabin2 and RyR2 by S107. S107 functioned to enhance binding of calstabin2 to RyR2, and therefore partially rescued the ER Ca^{2+} release through RyR and abnormal insulin secretion (210). Furthermore, it was found that Dantrolene, which is an inhibitor of RyR1 and RyR3, reduced cell death under TG-induced ER stress in Min6 β cells (211). Similarly, Lu et al. reported that treating with Dantrolene protected from apoptosis induced by TG using INS-1 cells where the WFS1 gene was knocked down (212). However, these studies were performed by inducing ER Ca^{2+} loss through SERCA inhibition, and they provide limited insight into how physiological stressors may change RyR function and ER Ca dyanamics. Thus, a goal of my project has been to study β cell RyR function in the context of pro-inflammatory stress and ER stress.

1.2.3 The role of the IP_3R in health and disease

Inositol 1,4,5-triphosphate receptors (IP_3R) are expressed in most eukaryotes and play an important role in regulating ER calcium release. In contrast to RyRs, functional IP_3Rs consist of tetramers assembled by either identical subunits or by mixture of the three subtypes IP_3R1, IP_3R2 and IP_3R3 and each subtype has many splice variants (213-215) . IP_3R are activated by IP_3, which is produced by the $G\alpha_q$ G-protein coupled receptor (GPCR) pathway and acts to stimulate Ca^{2+} release from the major IP_3-sensitive Ca^{2+} stores, including the nucleus, ER (216), Golgi, and likely the secretory vesicles. (217-219). In response to various endogenous ligands, $G\alpha_q$ subunit

activates phospholipase C (PLC) to catalyze hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce IP_3 and diacyl glycerol (220). IP_3 interacts with α- and β-domains of IP_3R, pulling the two domains closer (221). IP_3Rs play a central role in the propagation of Ca^{2+} waves, which is regenerative increased intracellular Ca^{2+}. The increased intracellular Ca^{2+} is sensed by RyR or IP_3R to induce ER Ca^{2+} release called Ca^{2+}-induced Ca^{2+} release (CICR) (222). The Ca^{2+} wave can propagate throughout the entire cell or even between cells to induce various biological effects. This was first observed during fertilization, which was found to occur through an IP_3R dependent process (223). IP_3 can hierarchically induce different forms of IP_3R activation, ranging from activation of a single IP_3R, to activation of a cluster of several IP_3Rs, and finally to activation of all cellular IP_3R to induce a Ca^{2+} wave (224,225). Activation of IP_3Rs requires both IP_3R and intracellular Ca^{2+} (226,227). However, whether each subunit of IP_3R tetramer requires the occupancy of an IP_3 molecule is still not known. In parallel to RyRs, the effects of Ca^{2+} are biphasic. Modest increases in intracellular Ca^{2+} (~μM) enhance IP_3 responses, whereas higher intracellular Ca^{2+} (μM-mM) can inhibit the IP_3R response (226,228). Experiments performed using purified IP_3Rs reconstructed in a lipid bilayer have confirmed the activation of IP_3R by Ca^{2+}, but not its inhibition (229,230). These results suggest that proteins might be responsible for inhibition mediated by high Ca^{2+} levels, rather than direct Ca^{2+} binding to the IP_3R (231). CaM has been suggested to be such a protein responsible for this effect based on the presence of CaM binding domains in the IP_3R. However, this as yet to be proven (232). Other IP_3R regulators include intracellular ATP (233) and cyclic AMP (cAMP), which is an intermediate product of $G\alpha_s$-GPCR pathway. Cyclic AMP can regulate IP_3R through multiple mechanisms. First, cAMP induces production of IP_3 through EPAC-mediated activation of PLC and IP_3 production (232,234). In addition, all three IP_3R subtypes are phosphorylated by PKA, which is in turn activated by cAMP. Thus, cAMP-mediated phosphorylation potentiates

IP_3-induced Ca^{2+} release by IP_3R1 and IP_3R2 (235). Finally, high cAMP concentrations induce a direct interaction between cAMP and IP_3Rs to sensitize their activities (236).

Structurally speaking, very little is known about how Ca^{2+} regulates IP_3R. Unlike the RyR, there are no EF Ca^{2+}-binding motifs within IP_3R. However, there are clusters of negatively charged residues that could serve as Ca^{2+} binding sites (237). A study that mutates the Glu2100 residue to a aspartic acid or glutamine reduced the Ca^{2+} sensitivity of the IP_3R by 5 – 10 fold, suggesting might serve as the site of IP_3R regulation by Ca^{2+} (238,239). Two studies have suggested that an increased ER Ca^{2+} load increased the sensitivity of IP_3R to IP_3 (240,241). Thus, regulation of the IP_3R by luminal ER Ca^{2+} regulation is possible, but has yet to be definitively proven. A high-affinity Ca^{2+}-binding site exists within the luminal loop of the transmembrane domain 5 and 6 contains conserved acidic residues that could mediate luminal Ca^{2+} regulation (242).

While altered IP_3R function has been suggested in a variety of diseases, including cardiac and neurodegenerative diseases, overall this topic has been less studied than RyR dysfunction. The expression level of IP_3Rs have been shown to be increased in cardiac hypertrophy, atrial fibrillation and hypertension (243-245). Among the three subtypes, IP_3R1 is the dominant form in brain (214), and impaired IP_3R1 function has been associated with Huntington's disease (246) and Alzheimer's disease (247). Mutations in the gene encoding IP_3R1 leads to spinocerebellar ataxia, a disorder characterized by progressive loss of muscle coordination (248,249). Compared to studies investigating RyR dysfunction, even fewer number of studies have linked IP_3R dysfunction to diabetes pathophysiology. So far, it has been shown that TG induced cell death was partially protected by inhibiting IP_3Rs in the Min6 β cell line (211). Further investigation will be necessary to fully define a role for the IP_3R in diabetes pathophysiology.

1.2.4. ER Ca^{2+} dynamics and cellular excitability

As with other secretory cells, pancreatic β cells are electrically excitable and capable of firing action potentials. Action potentials exist in both metazoan and plant cells, and they serve as a means to facilitate rapid communication between cells or tissues. In this regard, action potentials are used in β cells within islets to achieve rapid and synchronous insulin secretion, thus minimizing the time gap between glucose sensing and integrated insulin secretion at a whole-islet level (250).

Action potentials in β cells are generated mostly via Ca^{2+} influx through the activation of voltage-gated Ca^{2+} channels (47). Unlike mouse β cells, Na^{+} current also contributes to action potentials in human β cells (47,251). The pattern of action potential firing can increase the toxi Hyperexcitation is described as a state of excess action potential generation and has been shown to contribute to neurological disorders such as Alzheimer's disease (252,253), Parkinson's disease (254), epilepsy (255), and neuropathic pain (256). Hyperexcitation is also known to be toxic to cells and is referred to by the term "excitotoxicity". In neurons, cells undergo excitotoxicity due to overstimulation by excitatory amino acids, such as glutamate, resulting in excessive depolarization and action potential generation leading to cell death (257). In this process, glutamate receptors and Ca^{2+} channels induce Ca^{2+} overload, which triggers mitochondrial production of reactive oxygen species and induction of apoptosis (257,258). Another mechanism that induce neural excitotoxicity is hypoglycemia, because glucose is required for cells to take up a glutamate in the brain (259). In addition to neuronal diseases, hyperexcitability of cells may also contribute to cardiac arrhythmias (260,261).

Limited studies have been performed to investigate the relationship between β cell hyperexcitability and diabetes. In this regard, studies have investigated the relationship between sulfonylureas, which block K_{ATP} channels to induce β depolarization

and hyperexcitability. Clinical studies have suggested that sulfonylureas may hasten β cell failure (262), but the mechanisms underlying loss of insulin secretion by sulfonylureas remains poorly understood and is controversial. However, a leading hypothesis is that chronic hyperexcitation of β cells (263) eventually causes excitotoxic response, leading to loss of β cell function (264-266) and eventual cell death (267) and. Chronic glucotoxicity has been similarly implicated. In fact, in the presence of high glucose for extended periods, glucose-stimulated insulin secretion was impaired and islet insulin content was depleted in rat and human islets (268-270). This was prevented by co-treatment with the K_{ATP} channel opener diazoxide, as opening of K_{ATP} channel resulted in hyperpolarization, which inhibits Ca^{2+} influx from Cav (271-273). Furthermore, gain-of-function mutation of K_{ATP} channels, which increases excitability of β cells, caused high insulin secretion independent of blood glucose level and caused congenital hyperinsulinism of infancy (274). The loss-of-function mutation of K_{ATP} channels, which decreases excitability of β cells, caused neonatal diabetes mellitus (275). Therefore, K_{ATP} channels plays a critical roles of the excitability of β cells in health and disease. The exact molecular mechanisms for excitotoxicity, including the channels responsible for this phenomenon are yet to be found.

1.2.5. Ca^{2+} oscillation and ER Ca^{2+} dynamics

In response to elevated blood glucose levels, β-cells begin to oscillate intracellular Ca^{2+} levels synchronously in waves that propagate throughout the entire islet, with a frequency ranging from one per second to minutes (250). This phenomenon is referred to as glucose-induced Ca^{2+} oscillations (GICOs) and result from synchronous oscillation of V_m leading to Ca^{2+} influx through voltage-gated Ca^{2+} channels (250,276). The synchrony of oscillations among all islet β-cells are due to gap junction channels, which are composed of connexin 36 (Cx36) and mediate diffusion of ions and

small molecules between neighboring β cells (277). Cx36 has high affinity for K^+, and K^+ influx from K_{ATP} channels is uniformly diffused throughout β cells establishing a uniform V_m that is a key basis for synchronous Ca^{2+} oscillation (278). Ca^{2+} triggers insulin exocytosis, GICOs are coupled with the rhythms of pulsatile insulin secretion. Therefore, studying the mechanisms of Ca^{2+} oscillation in health and in diabetes has clinical significance. The pulsatile insulin secretion in humans is crucial for maintaining normal glycemic control (279). Loss of oscillations are observed in diabetes, and disruptions in the normal patterns of oscillatory insulin secretion is thought to contribute to insulin secretion secondary to desensitization of receptors in peripheral tissues (280,281). Despite this background knowledge, the exact mechanisms of how GICOs occur and how loss of GICOs occurs under diabetic stress have not been fully established. Since it is known that ER Ca^{2+} dynamics modulates V_m and action potential firing, dysfunction of ER Ca^{2+} dynamics may contribute to impaired GICOs and altered insulin secretion in diabetes.

Chapter 2. Results

2.1 ER stress and pro-inflammatory cytokine stress caused ER Ca^{2+} loss and disruption of glucose-induced Ca^{2+} oscillations.

The pathophysiology of type 1 and type 2 diabetes involves both β cell ER stress and pro-inflammatory cytokine-induced β cell dysfunction. To define how these stress paradigms influenced the ER Ca^{2+} store, we treated with compounds and factors that induce ER stress and pro-inflammatory cytokine stress. INS-1 β cells were treated with 300 nM Tunicamycin (TM) or 5 ng/ml Interleukin 1β combined with 25 mM high glucose (IL-1β + HG) in time-course experiments, and Ca^{2+} imaging was performed according to the schematic shown in Figure 3A. Results revealed a time-dependent loss of ER Ca^{2+} with TM (Fig. 3B-C) and IL-1β + HG (Fig. 3D-E), where significant reductions in ER Ca^{2+} were observed within 6 hrs and further reductions were observed throughout the 24 hr exposure period. We next studied the effect of TM and IL-1β + HG on glucose-induced Ca^{2+} oscillations (GICOs) in islets from 8 wk old C57BL6/J mice. Under control conditions, regular intracellular Ca^{2+} oscillations were observed in response to glucose stimulation (Fig. 3F). In contrast, both chemically induced ER stress and pro-inflammatory cytokine stress abolished normal patterns of GICOs in mouse islets (Fig. 3G). To compare these findings in analogous mouse models, islets were isolated from 6-8-wk old Akita mice, which harbor a spontaneous mutation in one allele of the *INS2* gene, resulting in impaired proinsulin folding and severe ER stress (282,283). To recapitulate pro-inflammatory cytokine stress, islets were isolated from 9-12-wk old hyperglycemic db/db mice. The strain has a mutated form of the leptin receptor resulting in hyperglycemia, obesity, and elevated systemic levels of pro-inflammatory cytokines (283,284). Similar to results observed with *ex vivo* TM and IL-1β+HG treatment, GICOs were significantly impaired in islets isolated from Akita and db/db mice (Fig. 3H). Taken together, these data indicate that ER and pro-inflammatory stress are sufficient to disrupt

ER Ca^{2+} storage as well as GICOs in pancreatic β cells.

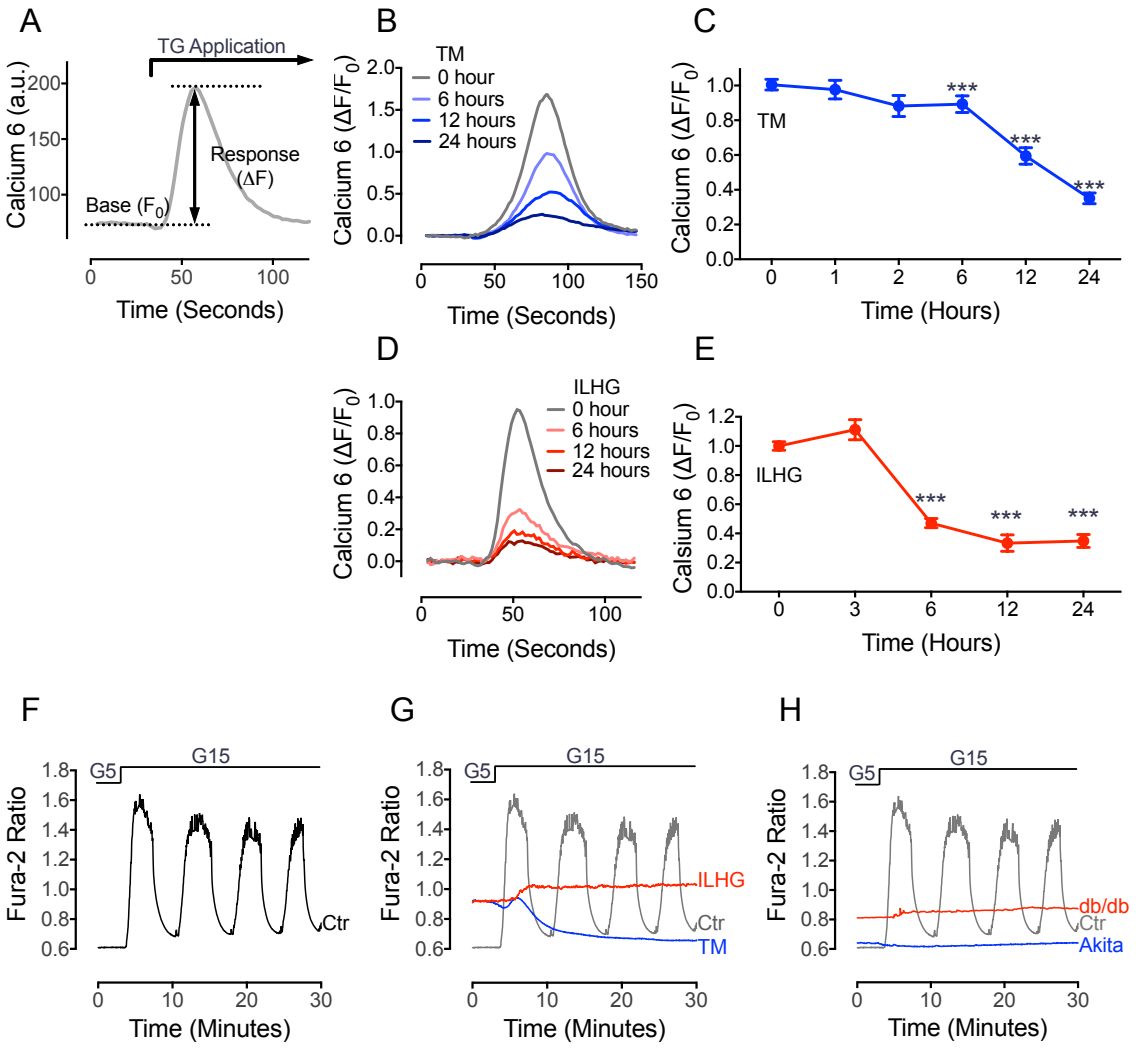

Figure 3. ER stress and pro-inflammatory cytokine-induced stress led to ER Ca^{2+} loss and disruption of glucose-induced Ca^{2+} oscillations

Experiments were conducted using INS-1 cells (A-E) and mouse islets (F-G). (A) To estimate ER calcium storage, Calcium 6 was used to measure intracellular Ca^{2+} levels before and after application of 10 μM thapsigargin (TG), a potent inhibitor of SERCA pump activity. (B) Representative traces for TG-induced Ca^{2+} release following treatment with 300 μM TM for indicated times. (C) TM-treatment led to a time-dependent reduction in ER Ca^{2+} levels; n = at least 7 replicates for each timepoint. (D) Representative traces for TG-induced Ca^{2+} release following treatment with 300 μM IL-

1β + 25 mM glucose (ILHG) for indicated times. (E) ILHG-treatment led to a time-dependent reduction in ER Ca^{2+} levels; n = at least 3 replicates for each time point. (F) Representative trace of changes in Fura2 intensity in islets from 8-12 week old wild-type C57BL6/J mice. Islets were incubated in 5 mM glucose (G5) and then stimulated with G15 to induce GICOs; n = 3 biological replicates. (G) Representative traces of GICOs in C57BL6/J islets under control conditions (Ctr) or following treatment with either 5 ng/ml IL-1β+25mM glucose for 24 hours (ILHG) or 10 μM TM for 48 hrs (TM); n = at least three biological replicates for each condition. (H) Representative GICO traces in islets from 9-12 week old db/db mice or 6-7 wk old Akita mice overlaid with GICOs of islets from C57BL6/J (Ctr) mice; n = at least three biological replicates for each genotype. For (C) and (E); ***$p \leq 0.001$ compared to Time 0.

2.2 RyR and IP_3R function are differentially altered in response to ER and cytokine-induced stress

Whereas previous studies have implicated β cell SERCA2 dysfunction in diabetes, a role for either RyR and IP_3R dysfunction remains incompletely characterized (285). To define whether RyR and IP_3R activity were altered in models of ER stress and pro-inflammatory stress, TM and IL-1β+HG-treated INS-1 β cells were loaded with the low-affinity Ca^{2+} indicator Mag-Fluo-4 AM, followed by membrane permeabilization with saponin. As shown in Figure 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_3Rs, respectively, and dose-response curves were generated (Fig. 4B). Interestingly, this analysis revealed that TM-induced ER stress primarily altered RyR responses (Fig. 4C), while IP_3R function was minimally impacted by TM treatment (Fig 4D). In the short-term, TM increased the maximal RyR response, while reductions in RyR activity were observed with chronic TM treatment (Fig. 4C). In contrast to TM treatment, RyR activity remained largely unaffected by IL-1β + HG (Fig. 5A). Longer-term IL-1β + HG treatment reduced the EC_{50} of the IP_3R response to agonist (Fig. 5B). Together, these results suggest that ER stress preferentially impacted RyR function, while chronic pro-inflammatory stress preferentially impaired the IP_3R response to agonist.

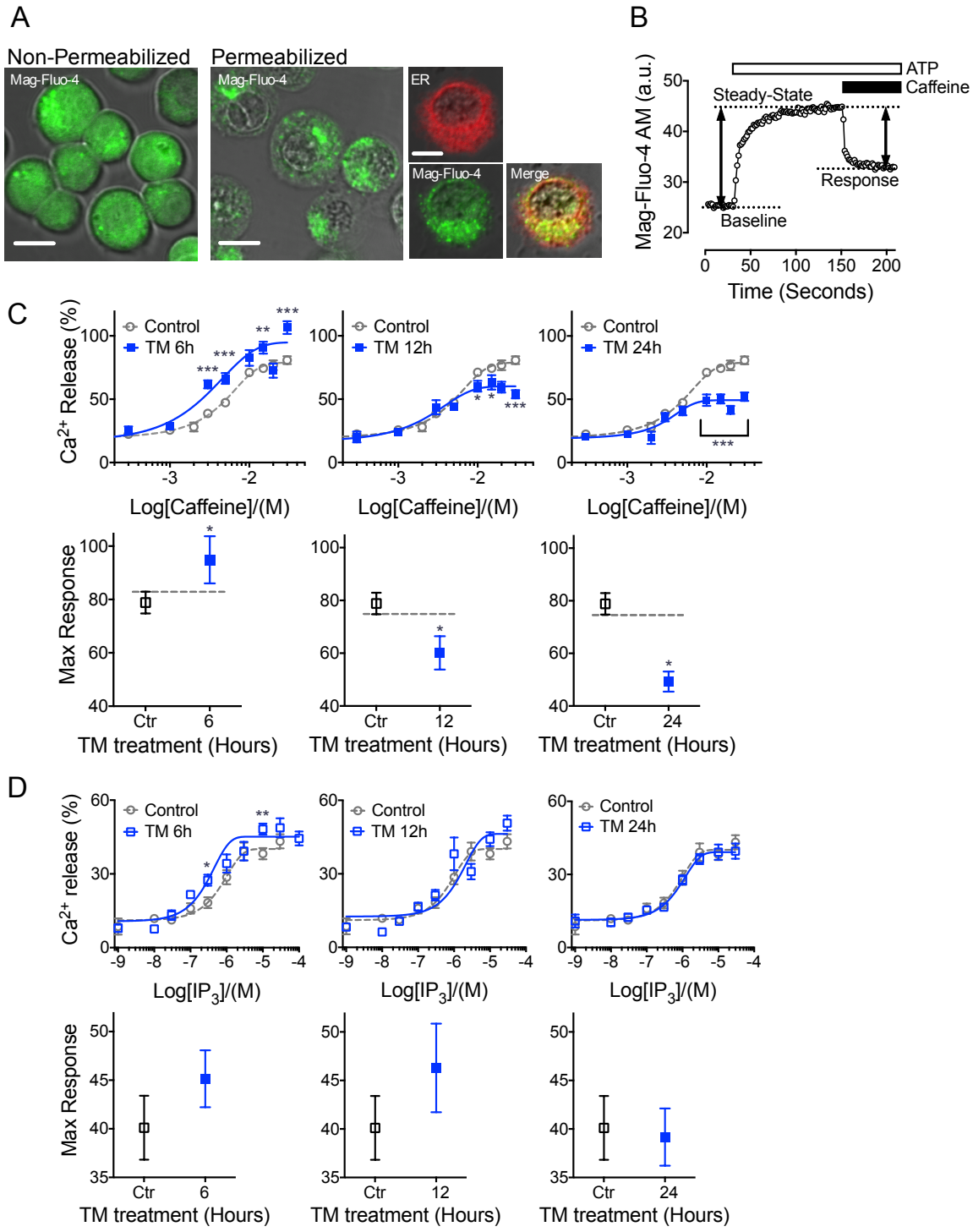

Figure 4. RyR function was preferentially altered by TM-induced ER stress

(A) INS-1 cells were loaded with the low affinity Ca^{2+} indicator Mag-Fluo-4 AM (Green). Following plasma membrane permeabilization, Mag-Fluo-4 AM was retained in the ER as demonstrated by co-localization with calnexin (Red). To estimate RyR and IP_3R

activity, calcium imaging was performed according to the schematic shown in Panel (B). First, 1.5 mM Mg-ATP was added to establish steady-state ER Ca^{2+} levels. Caffeine is an agonist for RyR, while IP_3 is an agonist for IP_3R. Caffeine or IP_3 was applied in the indicated concentrations to generate-dose response curves of RyR and IP_3R activation, respectively. Decreases in Mag-Fluo-4 AM intensity were used to calculate relative ER Ca^{2+} release, and GraphPad Prism Software was used to fit data from IP_3R and RyR functional assays to sigmoidal dose-response curves, which were analyzed by two-way ANOVA with Tukey-Kramer post-test. (C) Upper panels show the dose-response curves for RyR activation by caffeine in INS-1 cells pre-treated with 300 nM TM or DMSO for 6, 12, and 24 hrs. Lower panels show the maximal response and 95% confidence intervals for each timepoint; n = at least 3 replicates for each timepoint in different caffeine concentration. (D) Upper panels show the dose-response curves for IP_3R activation by IP_3 in INS-1 cells pre-treated with 300nM TM for 6, 12, and 24 hrs. Lower panels show the maximal response and 95% confidence intervals; n = at least 3 replicates for each timepoint and IP_3 concentration. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ compared to control conditions.

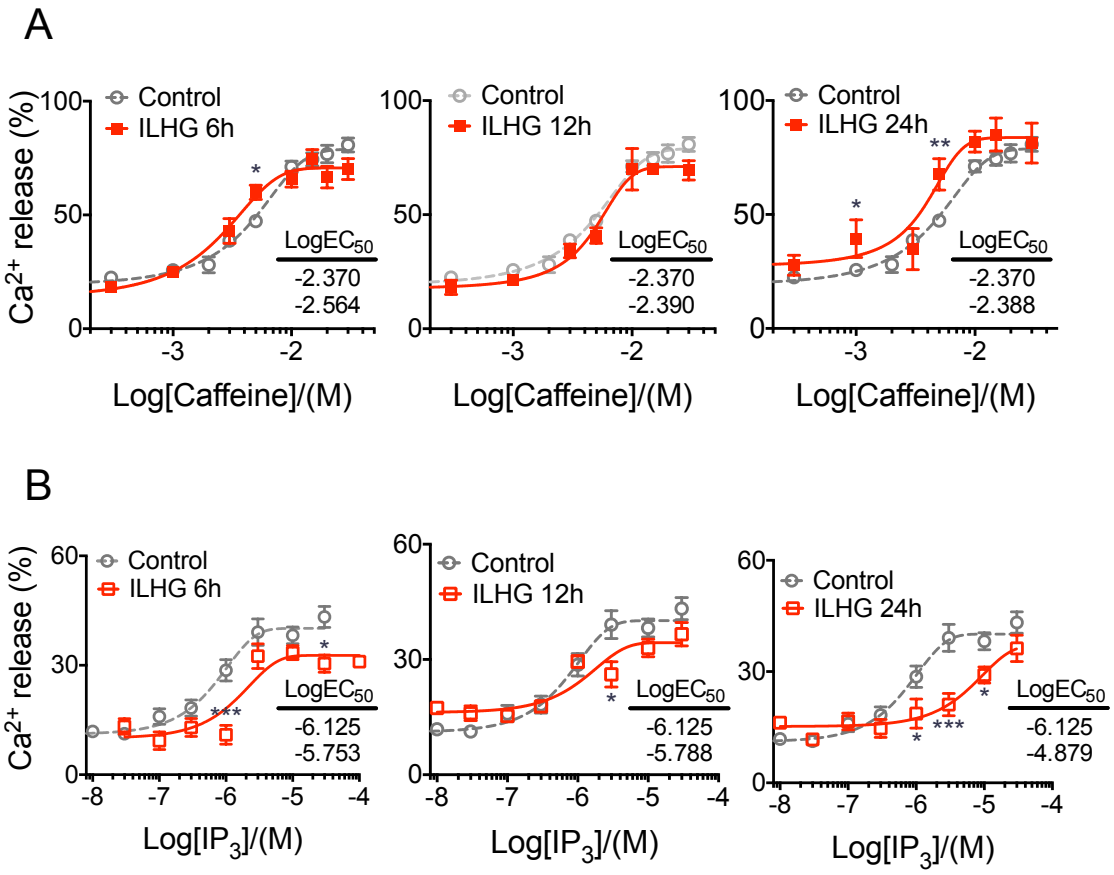

Figure 5. Pro-inflammatory cytokine stress impaired IP_3R function

(A) Dose-response curves for RyR activation by caffeine in INS-1 cells pre-treated with 5 ng/mg IL + 25 mM glucose (ILHG) for 6, 12, and 24 hrs; n = at least 3 replicates for each timepoint and caffeine concentration. (B) Dose-response curves for IP_3R activation in INS-1 cells pre-treated with pretreated with ILHG for 6, 12, and 24 hrs. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 compared to control conditions. Values shown are the $LogEC_{50}$ for INS-1 cells analyzed under control conditions (top) and following ILHG-treatment (bottom).

2.3 Stress-mediated ER Ca^{2+} loss was reduced by RyR and IP_3R inhibition

To test whether RyR or IP_3R inhibition was sufficient to prevent ER Ca^{2+} loss under diabetogenic stress conditions, we tested the effects of RyR antagonists, dantrolene and ryanodine (Ry), and the IP_3R antagonist, xestospongin C (XeC) using INS-1 cells. Since Ry can activate RyR at low concentration (~ 1 nM) Ry 100 μM was used to inhibit RyR. Following tunicamycin treatment, there was a trend towards improved ER Ca^{2+} levels with dantrolene (Fig. 6A), while inhibition of RyR with Ry (Fig 6B) significantly prevented ER Ca^{2+} loss compared to TM alone. Consistent with results from the functional assays, Ry was unable to block ER Ca^{2+} loss in response to IL-1β+HG (Fig. 6D), whereas inhibition of IP_3R with XeC partially rescued ER Ca^{2+} levels following IL-1β+HG treatment (Fig. 6C).

ER stress and cytokines are known to induce β cell death (286), so we next tested whether modulation of ER Ca^{2+} loss via RyR or IP_3R inhibition were sufficient to protect against β cell death. TM-treatment resulted in a time-dependent increase in caspase 3- and 7 activity (Fig. 7A) and led to increased expression of cleaved-caspase 3 protein (Fig. 7B). Interestingly, cell death was partially abrogated by Ry co-treatment (Fig. 7A-B), suggesting that cell survival was improved through prevention of RyR-mediated ER Ca^{2+} leak in response to TM-induced ER stress. In contrast, despite an observed effect to partially restore ER Ca^{2+} levels (Fig. 5C), XeC was unable to reduce caspase activity in response to IL-1β+HG (Fig. 7C).

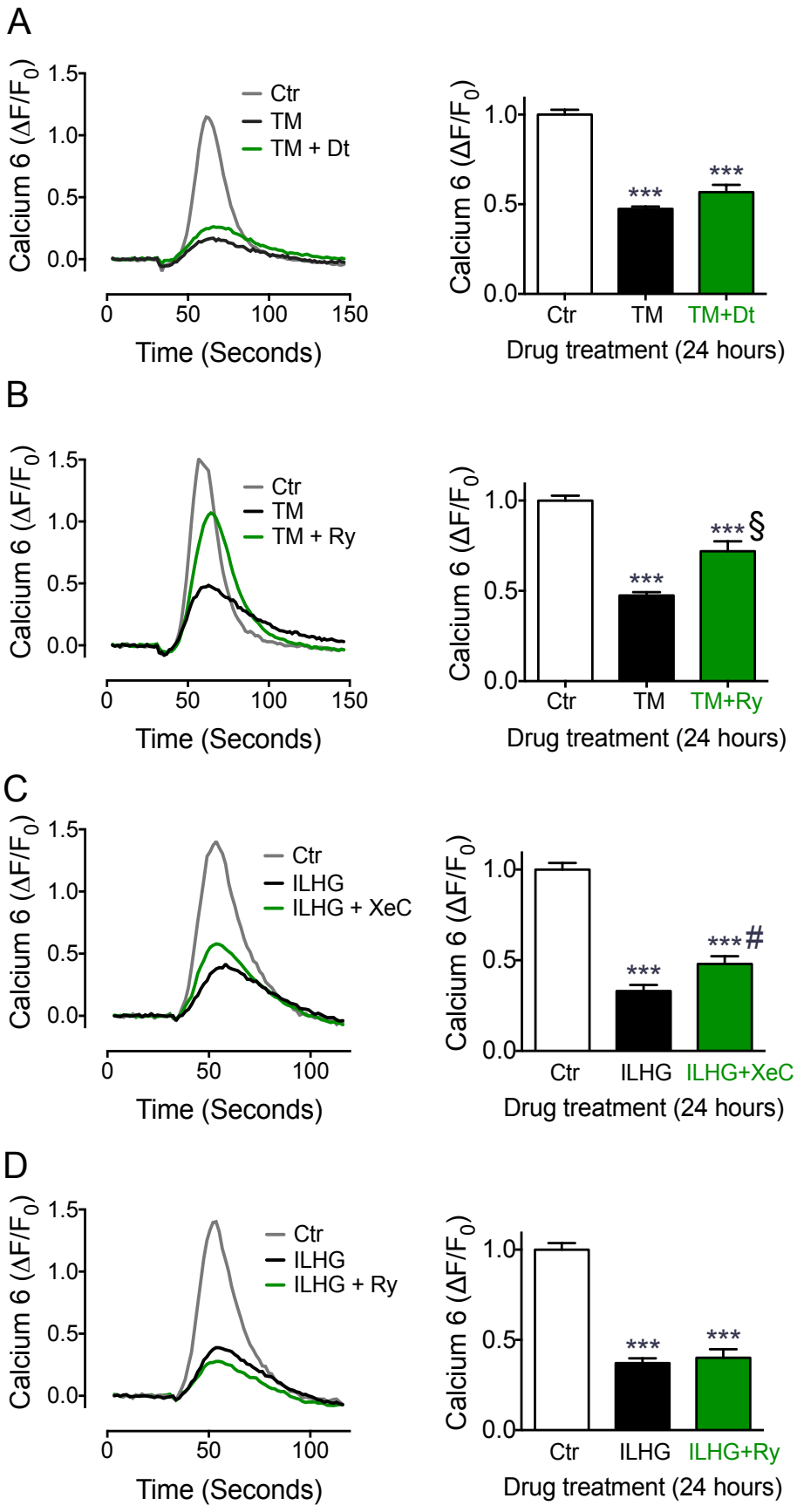
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Figure 6. ER Ca^{2+} loss was prevented by blocking RyR under ER stress conditions and by IP_3R blockade under pro-inflammatory cytokine stress conditions

INS-1 cells were treated with 300nM TM for 24 hours with or without 1 μM Dantrolene (Dt) (A) or 100 μM Ryanodine (Ry) (B). (A-B) Representative traces for TG-induced Ca^{2+} release (left), and quantified results (right); n = at least 11 replicates per condition. (C-D) INS-1 cells were treated with 5 ng/ml IL1β+25 mM glucose (ILHG) for 24 hrs with or without 5 μM XeC (C) or 100 μM Ry (D). (C-D) Representative traces for the TG-induced Ca^{2+} release (left), and quantified results (right); n = at least 15 readings per condition; *p ≤ 0.05; **p≤ 0.01; ***p≤ 0.001 compared to control conditions. §p ≤ 0.001 for comparison between TM and TM + Ry. #p ≤ 0.05 for comparison between ILHG and ILHG + XeC.

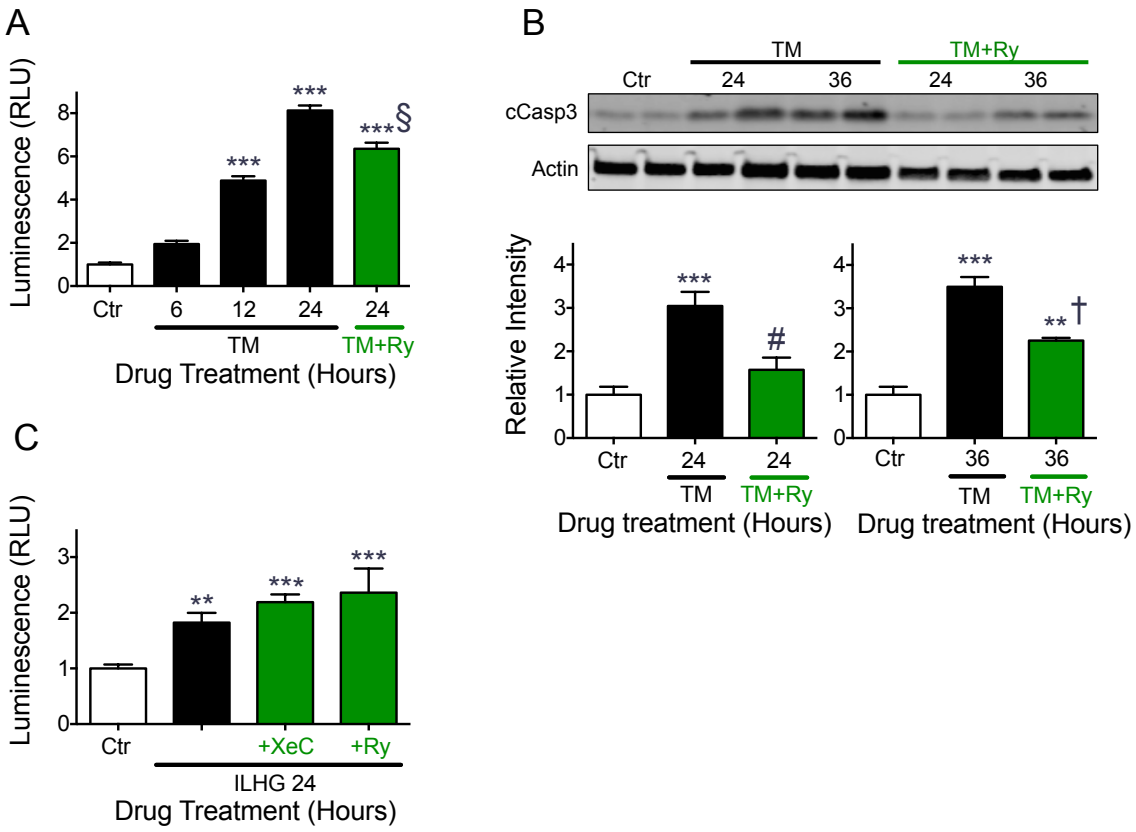

Figure 7. Ryanodine treatment prevented TM-induced cell death

(A) Caspase 3/7 activity was measured in INS-1 cells treated with 300 nM TM with or without 100 μM uM Ryanodine for indicated times; n = 4 replicates for each timepoint and condition. (B) Immunoblot analysis was performed in INS-1 cells treated with 300 nM TM with or without 100 μM Ryanodine for indicated times using antibodies against cleaved caspase-3, and Actin. Quantitative protein levels from three independent experiments are shown graphically. (C) Caspase 3/7 activity was measured in INS-1 cells treated with 5 ng/ml IL1β + 25 mM glucose (ILHG) for 24 hours; n = 4 replicates for each condition. **p ≤ 0.01 and ***p ≤ 0.001 compared to control conditions. In (A), §p ≤ 0.001 for the comparison between TM for 24 hrs and TM+Ry for 24 hrs. In (B), #p ≤ 0.05 and †p ≤ 0.01 for the comparisons between TM and TM + Ry for 24 and 36 hrs, respectively.

2.4 Reduction of ER Ca^{2+} leak via the RyR suppressed TM-induced Ca^{2+} transients and activation of the UPR

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. To define patterns of ER Ca^{2+} leak through the RyR in real-time at the single-cell level, spontaneous Ca^{2+} transients were measured in response to graded Ca^{2+} loading. Ca^{2+} serves as the primary ligand of the RyR. Spontaneous Ca^{2+} transients are observed in excitable cells, such as neurons and cardiac myocytes and have been shown to be coupled with ER Ca^{2+} leak through the RyR (287). To date, this process has not been studied in the pancreatic β cell, either under normal or stress conditions. By increasing extracellular Ca^{2+} concentration up to 2 mM, oscillating and spontaneous Ca^{2+} transients were induced in 10.40 % ±1.54 % (S.E.M) 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 % of cells after 12 hrs of treatment (Fig. 8A, C). Ryanodine co-treatment was found to significantly decrease TM-induced Ca^{2+} transients (Fig. 8A, D), suggesting the ER Ca^{2+} leak was mediated through the RyR. Notably, the response to caffeine, a pharmacological agonist of the RyR, was inhibited in the presence of 100 μM Ry (Fig. 8B), confirming that Ry was indeed acting through inhibition of RyR-mediated Ca^{2+} transients and cellular hyperexcitability.

To define whether β cell depolarization contributed to these ER-stress induced Ca^{2+} transients, cells were hyperpolarized by diazoxide (Dz) to inhibit activation of voltage-dependent Ca^{2+} channels (VDCCs). In this context, spontaneous Ca^{2+} transients induced by tunicamycin were similarly reduced (Fig. 8E), suggesting that ER-stress induced Ca^{2+} transients originate from ER Ca^{2+} leak from the RyR as well as through secondary signals generated by Ca^{2+} influx through VDCCs.

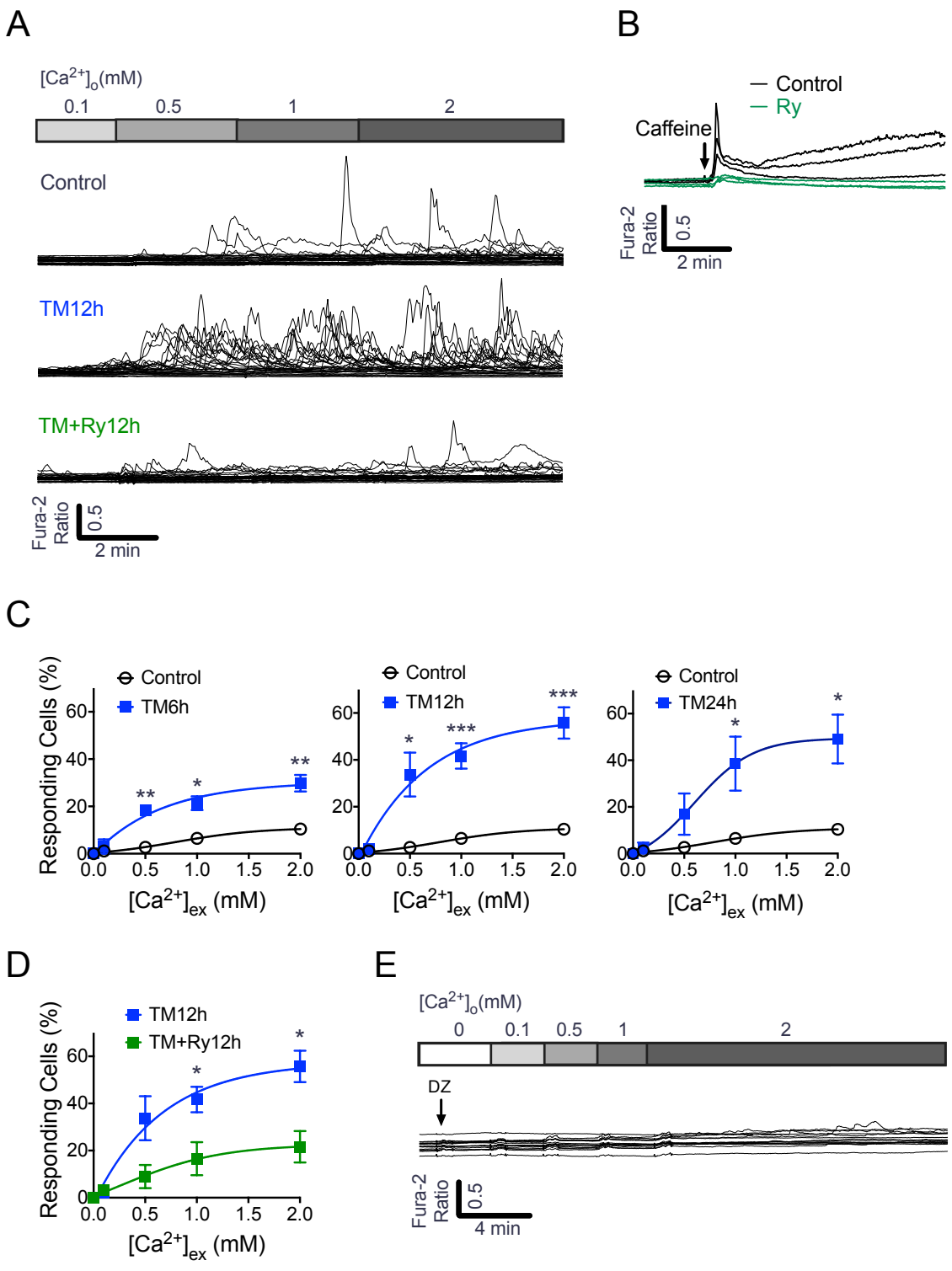

Figure 8. ER Ca^{2+} dynamics regulated electrical activity of β cells

(A) Intracellular Ca^{2+} transients were measured in response to graded Ca^{2+} loading in INS-1 cells treated under control conditions (top), following treatment with TM for 12 hrs

(middle), or following co-treatment with TM + Ry for 12 hrs (both). Data shown are representative of 30 single cell recordings from at least three independent experiments. (B) Traces show intracellular Ca^{2+} levels in INS-1 cells in response to caffeine treatment under control conditions and with ryanodine pre-treatment. (C) Percentages (%) of responding cells in INS-1 cells treated with TM for 6 hrs (n= 2 replicates); 12 hrs (n = 5 replicates); and 24 hrs (n = 4 replicates). (C) The % of responding cells was significantly reduced by Ry co-treatment (n = 3 replicates per conditions). (D) Representative trace indicates that spontaneous TM-induced Ca^{2+} transients were reduced by diazoxide (Dz). Data shown are representative of 15 single cell recordings. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ for indicated comparisons.

During ER stress, cells activate an adaptive response known as the UPR in order to both clear unfolded proteins and increase the folding capacity of the ER (288). However, prolonged UPR activation eventually leads to apoptosis if cellular homeostasis is not restored (289,290). While UPR activation has been linked with ER Ca^{2+} loss (138), the temporal relationships and causal effects between UPR activation and ER Ca^{2+} loss remain poorly understood. To address, we first measured *XBP1* mRNA splicing to validate this marker as an early upstream indicator of UPR activation. An increase in the spliced to total *XBP1* ratio was observed within 2 hrs of TM treatment and occurred prior to induction of both AFT4 and CHOP expression, which both increased around 6 hrs (Fig. 9A). Next, time-course experiments were performed to define how suppression of ER Ca^{2+} leak from the RyR impacted UPR activation. Our results revealed that ryanodine was able to significantly delay TM-induced UPR activation (Fig. 9B). To study this further, single-cell Ca^{2+} transients were measured at these early timepoints. Intracellular Ca^{2+} transients were found to increase within 3 hrs of TM treatment. Similar to results obtained with chronic TM treatment, co-treatment with Ry was sufficient to suppress these Ca^{2+} transients (Fig. 9C-D), indicating that ER Ca^{2+} leak is an early response to misfolded protein accumulation that occurs prior to full expression of the ER stress signaling cascade. Moreover, our results indicated that suppression of RyR-mediated Ca^{2+} leak was sufficient to reduce propagation of the UPR.

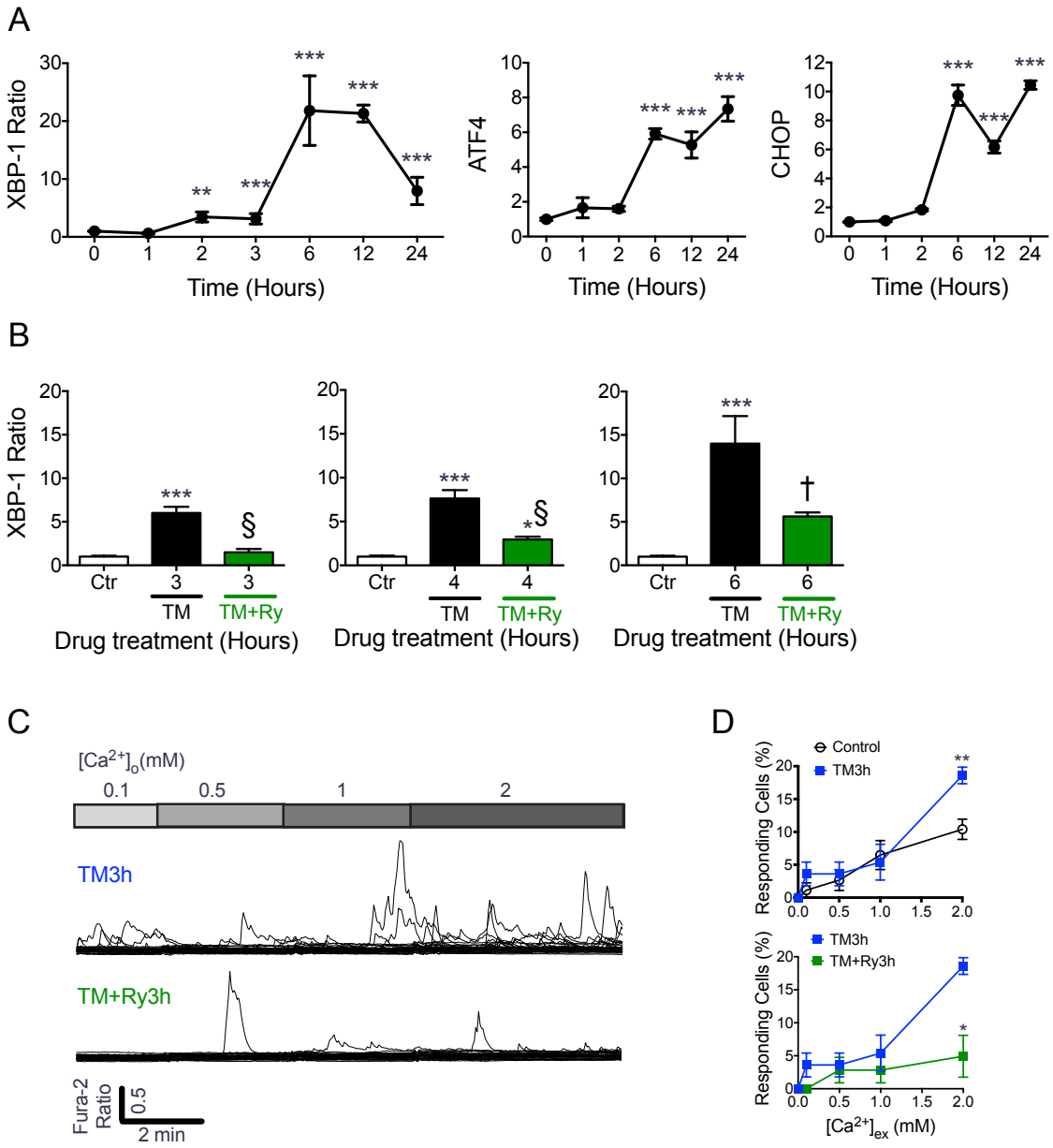

Figure 9. Inhibition of RyR-mediated ER Ca^{2+} leak attenuated activation of the unfolded protein response

(A) INS-1 cells were treated with 300 nM TM for indicated times, and mRNA levels of the spliced/unspliced XBP-1 ratio, ATF4, and CHOP were measured by RT-PCR. (B) The spliced/unspliced XBP-1 ratio was measured by RT-PCR in INS-1 cells treated with TM or TM+Ry for 3 hrs (left panel), 4 hrs (center), and 6 hrs (right panel). **p ≤ 0.01; ***p ≤ 0.001 compared to time 0 or control conditions; †p ≤ 0.01 and §p ≤ 0.001 for

comparisons between TM and TM + Ry groups. (C) Intracellular Ca^{2+} transients were measured in response to graded Ca^{2+} loading in INS-1 cells treated with TM for 3 hrs (top) or following co-treatment with TM+Ry for 3 hrs (bottom). Data shown are representative of 30 single cell recordings with n=at least 3 replicates for each condition. (D) Quantification of the % of responding cells in INS-1 cells treated under controls conditions, with TM or with TM + Ry for 3 hrs; n = at least 3 replicates for each condition. *$p \leq 0.05$; **$p \leq 0.01$ for comparison between indicated groups.

2.5 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 tunicamycin and intracellular Ca^{2+} transients were measured. Similar to results observed in INS-1 β cells, spontaneous Ca^{2+} transients were induced under ER stress evoked by TM, and ryanodine co-treatment decreased TM-induced Ca^{2+} transients (Fig. 10A-B),

Finally, we tested whether RyR inhibition would show similar result in an islet model of misfolded protein accumulation and ER stress (283,284). To this end, islets were isolated from 6-week old Akita mice and incubated with ryanodine or DMSO for 24 hours. Fura-2 AM imaging was performed to measure glucose-induced Ca^{2+} oscillations (GICOs). GICOs were markedly diminished in Akita islets under control conditions, while treatment with ryanodine improved the oscillation frequency and area under the curve of the glucose-induced Ca^{2+} responses (Fig. 10C-E). Moreover, Ry treatment significantly decreased the area of dead cells in islets from Akita mice (Fig. 10F-G). Finally, glucose stimulated insulin secretion (GSIS) was measured, the fold-increase of insulin secretion was the same between DMSO and Ry treated Akita islets.

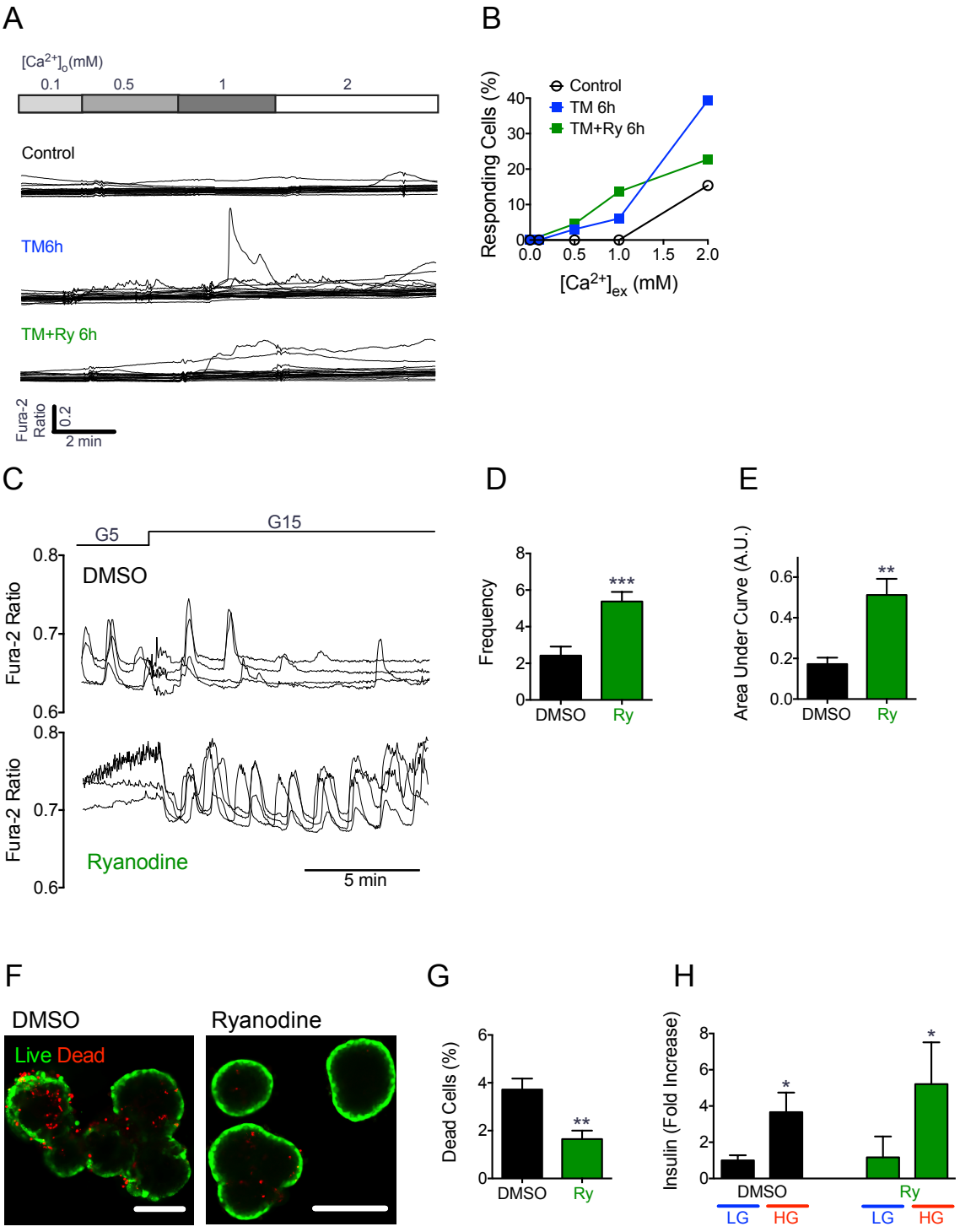

Figure 10. Ca^{2+} signaling and cell death were rescued by ryanodine treatment in tunicamycin-treated human islets and islets from Akita mice

(A) Representative data from 20 single cell calcium recordings performed in dispersed cadaveric human islets from a single biological donor analyzed under control conditions (top), following treatment with TM for 6 hrs (middle), and following co-treatment with TM + Ry for 6 hrs (bottom). (B) The % of responding cells was quantified for each condition. (C) Glucose-stimulated calcium oscillations were measured in islets isolated from Akita mice treated with DMSO or Ryanodine for 24 hrs. Shown are representative recordings from 4 individual islets. (D) The frequency of oscillations and area under curve for calcium responses (E) were quantified from 3 biological replicates per conditions. (F) Representative pictures of Live (green) and Dead (red) staining performed in Akita islets treated with DMSO or Ryanodine for 48 hrs. (G) Quantification of the % of dead cells from n=3 replicates per condition. (H) Using Akita islets GSIS was measured after treating DMSO or 100 μM Ry for 48 hrs. LG indicates low-glucose treated group and HG indicates high-glucose treated group with n=3 replicates; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ for comparisons between indicated groups.

Chapter 3. Discussion

3.1 Summary and significance

The Ca^{2+} concentration within the lumen of the β cell ER is estimated to be three orders of magnitude higher than intracellular Ca^{2+} levels. Ca^{2+} within this cellular compartment serves as an essential cofactor for protein chaperones and foldases required for insulin processing, while Ca^{2+} release from the ER regulates glucose-stimulated Ca^{2+} oscillations and phasic insulin secretion. Loss of ER Ca^{2+} has been shown to lead to impaired insulin secretion as well as activation of cell-intrinsic stress pathways including ER and oxidative stress and reduced β cell survival. Whereas a prominent role for reduced β cell SERCA pump activity has been identified in diabetes, the question remains if alterations in RyR or IP_3 function, which regulate ER Ca^{2+} release, similarly contribute to these processes.

To test this, we applied intracellular and ER- Ca^{2+} imaging techniques to measure ryanodine receptor (RyR) and inositol 1,4,5-triphosphate receptor (IP_3R) activity in response to two distinct stress paradigms known to contribute to the pathogenesis of both type 1 and type 2 diabetes. The stress paradigms tested were ER stress, which was induced chemically using tunicamcyin in INS-1 β cells and in cadaveric human islets. Aspects of our model were also evaluated in islets from Akita mice, a strain harboring a spontaneous mutation in one allele of the INS2 gene, resulting in impaired proinsulin folding and severe ER stress (283,284). To recapitulate pro-inflammatory cytokine induced dysfunction, INS-1 cells were treated with high glucose (25 mM glucose) in combination with IL-1β, a cytokine known to be systemically elevated in diabetes. In aggregate, our results revealed a preferential sensitivity of the RyR to ER stress, while pro-inflammatory cytokine stress was found to primarily impact IP_3R activity. Pharmacological inhibition of the RyR with ryanodine and inhibition of the IP_3R

with xestospongin C were both 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 the initiation of the UPR and an improvement of glucose-induced Ca^{2+} oscillations and insulin secretion.

The RyR is a large macromolecular complex that is primarily localized to the ER or sarcoplasmic reticulum membranes. The RyR remains closed at low intracellular Ca^{2+} concentrations. However, as intracellular Ca^{2+} levels increase in the range of 0.01-20 uM, Ca^{2+} binds the receptor, leading to channel opening and ER Ca^{2+} release (179). . In addition to ligand binding, channel activity is modulated by a number of interacting proteins contained within this macromolecular complex (156-159). RyR was found to be expressed on the surface of β cell dense core secretory vesicles (291), and two independent groups have demonstrated secretory vesicle Ca^{2+} release in response to RyR activation, (291) indicating the RyR may also regulate localized Ca^{2+} signals responsible for granule exocytosis.

RyR dysfunction has been well-documented in a number of other disease states including cancer-associated muscle weakness (153), Alzheimer's disease (171), and cardiomyopathy (164). In contrast, only a handful of studies have investigated whether β cell RyR dysfunction contributes to the diabetes pathophysiology. Luciani and colleagues found that both RyR and IP_3R inhibition were able to attenuate thapsigargin-induced ER stress in MIN6 β cells (211). This approach caused ER stress by inducing ER Ca^{2+} loss, therefore this does not likely show how ER stress affect ER Ca^{2+} dyanamics and cell death. To address this, we have induced ER stress directly by using TM, and our analysis was uniquely able to distinguish the impact of two different stress paradigms on RyR and IP_3R activity and function, a topic unaddressed in previous studies. Our results indicate that RyR dysfunction is uniquely induced by misfolded

protein accumulation, while pro-inflammatory cytokine stress had little impact on RyR function. In support of our findings, the RyR antagonist, dantrolene, was previously shown to reduce cell death in INS-1 β cells lacking WSF1 protein, another model of severe ER stress (212).

To document the mode of RyR dysfunction in response to ER stress, we measured spontaneous intracellular Ca^{2+} transients at the single cell level in response to graded increase in extracellular Ca^{2+}. Our results indicate that ER-stress lowers the threshold and frequency of RyR-mediated ER Ca^{2+} release in both INS-1 cells and cadaveric human islets. We also found these ER-stress induced Ca^{2+} transients were composed of both ER Ca^{2+} release and Ca^{2+} influx through VDCCs, thus contributing to an overall state of β cell hyperexcitability. These results are consistent with findings in other excitable cells and pathologic states. For example, prion as well as amyloid-beta protein accumulation in cortical neurons has been shown to lead to ER stress, ER Ca^{2+} release from the RyR, and increased neuronal excitability (292).

A handful of molecular pathways have been implicated as potential contributors to β cell RyR dysfunction. Mice with a mutated form of the RyR2 leading to constitutive CaMKII-mediated phosphorylation and chronic RyR2 gain of function exhibited impaired glucose-induced insulin and Ca^{2+} responses as well as glucose intolerance (293). Similarly, RyR mutations that lead to dissociation of calstabin2 have been shown to lead to RyR gain of function, resulting in a condition known as catecholaminergic polymorphic ventricular tachycardia (CVPT) in humans. Mice expressing two mutated forms of the RyR2 associated with CVPT were shown to be glucose intolerant. Isolated islets exhibited decreased glucose-stimulated insulin secretion and impaired mitochondrial metabolism. Intriguingly, humans with CPVT were found to have higher glucose levels and lower insulin levels during an oral glucose tolerance test compared to age and BMI-matched controls (210). Oxidative stress has been shown to contribute to both calstabin

dissociation from the RyR as well CaMKII-mediated RyR phosphorylation. Indeed, alterations in calstabin and RyR association have been demonstrated in islets from donors with Type 2 diabetes (210). Recently, loss of sorcin, a Ca^{2+} sensor protein that inhibits RyR activity, was similarly shown to lead to glucose intolerance, while sorcin overexpression improved GSIS and ER Ca^{2+} storage. Palmitate-induced lipotoxicity decreased sorcin expression in human and mouse islets (294). Interestingly, in a model of Parkinson's disease, ER stress itself led to aberrant accumulation of RyR, resulting in increased ER Ca^{2+} release (295). Thus, whether RyR dysfunction occurs secondary to loss of stabilizing or inhibitory protein, through impaired receptor degradation, or through yet another distinct mechanism in the pancreatic β cell will be explored in future studies. Notwithstanding this unknown, our data suggests that targeting the RyR receptor has the potential to improve β cell survival through attenuation of ER stress as well and β cell function through restoration of glucose-induced Ca^{2+} oscillations and improved insulin secretion (Fig. 11). Drug discovery efforts are actively done aimed at identifying small molecular RyR-stabilizers (296). In aggregate, our data suggest such compounds may have utility in improving the health of the diabetic β cell.

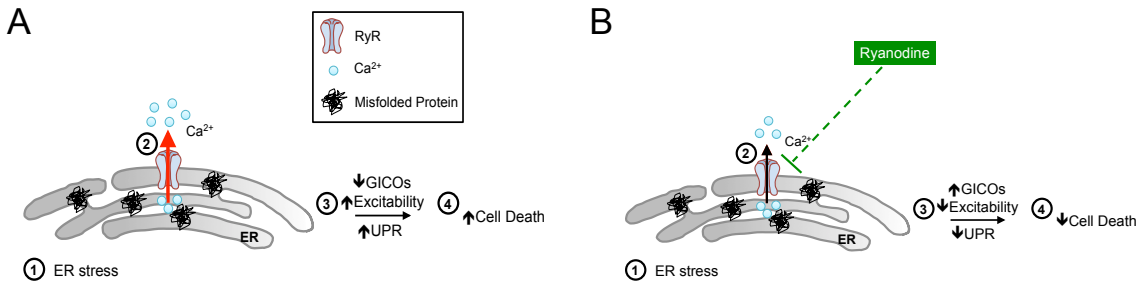

Figure 11. Model for protecting ER Ca^{2+} dynamics by RyR inhibition under ER stress

ER stress leads to RyR-mediated ER Ca^{2+} leak, while inhibition of RyR-mediated ER calcium loss restores ER Ca^{2+} dynamics, glucose-stimulated calcium oscillations and attenuates UPR activation and cell death. (A) Our data indicate that under ER stress conditions, RyR function is disrupted, leading to 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. (B) Inhibition of RyR-mediated loss of ER Ca^{2} leads to a partial rescue of ER Ca^{2+} dynamics under ER stress conditions. This leads to improved cellular excitability, improved GICOs, delayed initiation of the UPR, and improved cellular survival.

3.2 Limitations and Future Study

3.2.1 Techniques to measure ER Ca^{2+} dynamics

In the current research, we have estimated free ER Ca^{2+} concentration in populations of β cells under stress conditions by measuring the increase in intracellular Ca^{2+} following pharmacological inhibition of SERCA in Ca^{2+} free extracellular solutions using the calcium indicator, Calcium 6. SERCA inhibition results in passive ER Ca^{2+} release from RyR and IP_3R until ER Ca^{2+} is depleted (Fig. 3). The kinetics of ER Ca^{2+} release induced by TG is slow process due to the passive release from RyR and IP_3R and reaches a peak fluorescent intensity in tens of seconds to minutes. The kinetics of this process is also affected by the function of plasma membrane ion transporters such as PMCA and NCX whose function may also have been altered by the stress conditions (297). While this technique is widely accepted, inhibition of plasma membrane and organellar Ca^{2+} transporters would have given better estimation especially when it comes to the small change induced by weaker stress.

Furthermore, TG induced minimal elevations in intracellular Ca^{2+} in intact islets, so estimation of ER Ca^{2+} levels in islets was not successfully using this approach. Within the islets, β cells are known to be physically connected through gap junction channels (278) and the increase in intracellular Ca^{2+} is likely buffered by neighboring cells. In addition, TG simply may not penetrate cells inside the islet. Therefore, techniques to directly measure ER Ca^{2+} concentration more reliably should be considered.

We also measured directly ER Ca^{2+} release by using low affinity Ca^{2+} indicator, Mag-fluor 4 AM (Fig. 4 and 5). This was a technique that enabled a high-throughput format and was beneficial in making dose-response curves with agonists. Although this directly measured Ca^{2+} in ER, imaging was done after permeabilization of plasma membrane and by studying population of cells.

The approaches taken above can capture only one aspect of ER Ca^{2+} such as ER Ca^{2+} concentration or ER Ca^{2+} release. These approaches do not capture ER Ca^{2+} dynamics, which are more relevant to physiological activities. Our approach to measure spontaneous intracellular Ca^{2+} transients provides some insight into ER Ca^{2+} dynamics, and showed that bursts of ER Ca^{2+} release increased under ER stress conditions (Fig. 8). Nevertheless, the techniques that can reliably detect ER Ca^{2+} dynamics by directly measuring ER Ca^{2+} will be beneficial, which will be discussed in the future direction.

3.2.2 Basis of RyR and IP_3R dysfunction

Our results from functional assays showed that RyR function was altered under ER stress, while IP_3R function was preferentially altered under pro-inflammatory stress conditions (Fig. 4 and 5). This data was supported by results showing that ER Ca^{2+} loss induced by these respective stress conditions were rescued by inhibiting RyR under ER stress conditions and IP_3R in response to pro-inflammatory stress (Fig. 6 and 7). This approach enabled us to document receptors dysfunction. However, the basis of the dysfunction was not addressed in the current study. This information will be crucial in order to identify more specific drug target.

Possible molecular mechanisms that may cause changes in overall receptor function include changes in expression levels and post-translational modifications. The known regulation of RyR and IP_3R by post-translational modification was summarized in detail in the Introduction section. For instance, RyR undergoes phosphorylation and oxidation, and nitrosylation that can cause dissociation of RyR stabilizing protein calstabin resulting in Ca^{2+} leak from ER (298-300). It is likely that ER stress may have induced one of these modifications, leading to ER Ca^{2+} release and increased intracellular Ca^{2+} transients that exacerbate UPR activation and lead to cell death.

Inhibition of RyR by Ry under ER stress conditions may have mitigated the deleterious effects of these post-translational modifications. Our result showed that S107, which stabilizes the RyR and calstabin interaction did not restore the ER Ca^{2+} under ER stress (Fig. 12). This indicates either ER stress was robust enough to nullify the effects of S107 or that other post-translational modifications were involved in this process. These possibilities could be tested in future studies.

Likewise, IP_3R can be regulated by phosphorylation or by the binding of associated proteins to its regulatory domain. For instance, PKA activation leads to an opening of IP_3R (301,302). Therefore, a more thorough understanding of the effects of post-translational modifications will be necessary. In addition, these results may need to be studied within the context of ER Ca^{2+} dynamics, because efforts to stabilize the calstabin interaction or prevent oxidative stress, for example, may impact spontaneous ER Ca^{2+} release without being able to restore ER Ca^{2+}.

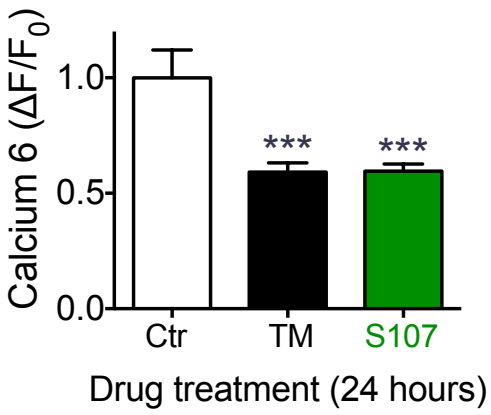

Figure 12. ER Ca^{2+} loss was not prevented by the RyR stabilizer S107 under ER stress conditions

INS-1 cells were treated with 300nM TM for 24 hours with or without 10 μM S107; n = at least 3 replicates per condition; ***p≤ 0.001 compared to control conditions.

3.2.3 Limitations arising from the use of rodent models

The current study was performed using cell lines and through ex vivo analysis of islets from rodent models. These models may not necessarily recapitulate *in vivo* findings. Therefore, key aspects of our model need to be confirmed using *in vivo* models and in human islets and humans. There are considerable difference even in islets between mouse and humans. In a mouse islet, α cells reside mostly on the surface of an islet, while β cells tend to be more in the center of the islet (303). In contrast, α cells and β cells are equally distributed throughout the human islet (303). In addition to localization within islets, channels regulating to Ca^{2+} dynamics and action potentials in β cells are different between mice and human. For instance, human and mouse β cells both express Na^{+} channels. Na^{+} channels in human β cells amplify action potentials (47), but the Na^{+} channels in mouse β cells has minimal effects (251). P/Q-type Ca^{2+} channels and L-type Ca^{2+} channels (Cav 1.2 and 1.3) elicit about 40% of Ca^{2+} current each in human β cells (47), but L-type Ca^{2+} channels elicits more than 60% of total Ca^{2+} current in mouse β cells and P/Q-type Ca^{2+} channels have minimal effects (251).

In addition, *in vivo* plasma glucose levels are continuously fluctuating, and β cells will be exposed to a countless number of molecules such as hormones, lipids or cytokines that can regulate channel activities and as well as cellular activities. To make matters more complicated, there are neuronal innervations to islets and β cells. These include efferent neurons coming from brain as well as inter and intra-islet neuronal networks (304). Therefore, studying β cell function *in vivo* is crucial.

Finally, our apoptosis or cell death assay was difficult to apply to islets. This may be because islets the expression levels of proteins related to apoptosis are low, or macrophages clear dead cells fairy quickly (305). Therefore studying *in vivo* with all these things could tell you more about the relevance of human diseases.

3.2.4 Future directions

Studying ER Ca^{2+} dynamics in islets or β cells is challenging due to the limitations with currently available techniques mentioned above. The genetically-encoded Ca^{2+} indicator D4ER maybe able to resolve the problem, however islet cells exhibits autofluorescence when excited at blue or green light (~430-490 nm) (306), which will cross contaminate and mask the fluorescence of D4ER (Ex. 435 nm, Em. 540 and 475 nm), which is the fluorescent Förster resonance energy transfer (FRET)- based ER Ca^{2+} indicator widely used. To solve the issue with autofluorescence, a Ca^{2+} indicator with other wavelengthes such as mCherry CEPIA (307), or bioluminescence resonance energy transfer (BRET)- based Ca^{2+} indicator (308) would be worthwhile options. BRET is advantageous because it does not require any excitation light, and does not undergo photobleaching because of illumination.

To overcome the limitations with *in vitro* studies, it will be beneficial to perform experiments *in vivo*. To conduct Ca^{2+} imaging *in vivo*, our lab has recently generated an insulin Cre-induced GCaMP6s mouse line. Using this line, we can express GCaMP6s, which are genetically encoded high affinity Ca^{2+} indicator, in β cells. Our approach could be to apply intravital microscopic techniques to image the mouse pancreas (309,310). With this approach, pancreata will be surgically exposed outside the body and the pancreas can be imaged using two-photon microscopy. With this technique, Ca^{2+} oscillation *in vivo* could be studied and this will greatly advance the field to study hormonal, neuronal, and pharmacological regulation of Ca^{2+} in β cells. To follow up the current study, mouse with inducible RyR knockout model under stress conditions could be used to replicate what we found in β cell line and mouse pancreatic islets.

This intravital microscopy will be beneficial not only for the Ca^{2+} imaging, but can also be applied to visualize many other biological assays. For instance, the apoptosis and cell death assay could be tested *in vivo*, and the interactions of immune cells could

be clarified by also visualizing macrophages. Furthermore, UPR marker such as Xbp1 or Chop could be visualized *in vivo* as well.

Chapter 4. Materials and Methods

4.1 Materials

4.1.1. Animals, islet isolation and human islet preparation

a. Mice

C57BL/6J (WT) mice, Akita mice, are db/db mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 5 - 10 weeks of age, and maintained under protocols approved by the Indiana University Institutional Animal Care and Use Committee, the U.S. Department of Agriculture's Animal Welfare Act (9 CFR Parts 1, 2, and 3), and the Guide for the Care and Use of Laboratory Animals

b. Human islets

Human cadaveric islets isolated from non-diabetic donors were obtained from the Integrated Islet Distribution Program or the National Disease Research Interchange. Upon receipt, human islets were hand-picked and allowed to recover overnight in Dulbecco's modified essential medium (DMEM) medium containing 5.5mM glucose, 10% fetal bovine serum (FBS), 100U/ml penicillin, and 100µg/ml streptomycin.

4.1.2 Cell culture and islet isolation

INS-1 832/13 rat insulinoma cells were cultured in regular 11mM glucose Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 1mM sodium pyruvate, 50µM β-macaptoethanol and, 10% FBS, penicillin and streptomycin.

Mice islets were isolated by collagenase digestion as described previously (311). The islets were hand-picked, and allowed to recover overnight in regular RPMI supplemented with 10% FBS, 100U/ml penicillin, and 100µg/ml streptomycin.

4.1.3. Primers, antibodies and reagents

Standard polymerase chain reaction (PCR) reactions for quantitative PCR (qPCR) for gene expressions were performed using oligonucleotides synthesized by Invitrogen (**Table 3**).

Antibodies used in western blotting, immunofluorescent and immunohistochemistry staining are listed in **Table 2**. Chemicals and reagents used in the research are listed in **Table 4**.

Table 2. Antibodies for Western Blotting and Immuno-staining

Targeted protein	Host Species	Manufacturer	Dilution
Total caspase 3	Rabbit	Cell Signaling	1:1000
Mouse IgG (IRDye 800CW)	Donkey	Li-cor	1:10000

Table 3. Quantitative RT-PCR Primers

Gene	Species	Forward (5'-3') Reverse (5'-3')
Total Xbp-1	*Rattus rattus*	AGCACTCAGACTACGTGCGCCTC CCAGAATGCCCAAAAGGATATCAG
Spliced Xbp-1	*Rattus rattus*	CTGAGTCCGCAGCAGGT TGTCAGAGTCCATGGGAAGA
ATF4	*Rattus rattus*	GTTGGTCAGTGCCTCAGACA CATTCGAAACAGAGCATCGA
CHOP	*Rattus rattus*	CCAGCAGAGGTCACAAGCAC CGCACTACCACTCTGTTTC

Table 4. Chemicals and Reagents

Name	Manufacturer
Caffeine	Santa Cruz
D-Glucose	Sigma-Aldrich
Hanks' Balanced Salt Solution (HBSS)	Lonza
D-myo-Inositol-1,4,5-triphosphate hexapotassium salt (IP_3)	Santa Cruz
Xestospongin C	Santa Cruz
Tunicamycin	Cayman Chemicals
Thapsigargin	Cayman Chemicals
Fura-2-acetoxymethylester (Fura-2 AM)	Thermo Fisher
Mag-Fluo-4 AM	Thermo Fisher
Mouse IL-1 beta Recombinant Protein (IL-1β)	Thermo Fisher
Diazoxide	Sigma-Aldrich
Dantrolene	Sigma-Aldrich
Adenosine 5’-triphosphate magnesium salt (ATP)	Sigma-Aldrich
Ryanodine	Tocris
Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP)	Tocris
Calcium 6	Molecular Devices

Table 5. Ca^{2+} indicators and Wavelengths

Name	Excitation and Emission Wavelength	Manufacturer
Calcium 6	Ex. 490 nm, Em. 525 nm	Molecular Devices
Fura 2AM	Ex. 340 and 380 nm, Em. 510 nm	Thermo Fisher
Mag-fluor 4AM	Ex. 490 nm, Em. 525 nm	Thermo Fisher

4.2 Methods

4.2.1 Animal studies and cell culture

Male C57BL/6J, heterozygous $Ins2^{Akita}$ (Akita), and C57BLKS-homozygous $Lepr^{db}$ (db/db) mice were obtained from Jackson Laboratories (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, hand-picked, and allowed to recover overnight as described previously (311). INS-1 832/13 rat insulinoma cells were maintained according to previously published protocols (312,313).

4.2.2 Immunoblot and quantitative PCR

Immunoblot experiments were performed as described (314). In brief, cells were lysed in 1% IGEPAL reagent supplemented with 10% glycerol, 16 mM NaCl, 25 mM HEPES, Sigma-Aldrich, St. Louis, MO), 60 mM n-octylglucoside (Research Products International Corp.), phosphatase inhibitor cocktails (PhosSTOP tablets, Roche) and phosphatase inhibitor cocktails (EDTA-free cOmplete tablets, Roche). Protein concentration was measured using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) and a SpectraMax M5 multiwell plate reader (Molecular Devices, Sunnyvale, CA). Equal concentrations of proteins were suspended in 10% SDS solution. Protein lysates were electrophoresed and transferred to methanol-activated PVDF membrane (Immobilon-FL Transfer Membrane from Millipore). Membranes were then blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE) prior to incubation with primary antibodies listed in **Table 2**. Subsequently, membranes were incubated with IRDye 800 or 680 fluorophore-labeled secondary antibodies from LI-COR. The total protein levels

were normalized to actin protein levels. Images were analyzed using LI-COR Image Studio and Image-J software (National Institutes of Health, Bethesda, MD, USA).

For qPCR, Cultured cells or isolated islets were processed for total RNA using the Qiagen RNeasy Mini Plus kit (Valencia, CA, USA). For reverse transcription-PCR experiments, total RNA was processed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Grand Island, NY). Subsequently, qPCR was performed using JumpStart™ Taq DNA Polymerase (Sigma-Aldrich, St. Louis, MO), and using SYBR Green I dye and previously published methods (312). Primers used for qPCR is listed in **Table 3**.

4.2.3 Stress simulation

To mimic the pro-inflammatory stress milieu of diabetes, cells and mouse islets were treated in RPMI media containing 5 ng/mL of mouse IL-1β for indicated times. To mimic the ER stress in diabetic milieu, INS-1 cells or mouse islets were cultured in RPMI containing 300 nM TM with indicated times.

4.2.4 Intracellular calcium (Ca^{2+}) imaging

Intracellular Ca^{2+} was measured using the FLIPR Calcium 6 Assay Kit and the Molecular Devices FlexStation 3 system (Sunnyvale, CA, USA) or using the ratiometric Ca^{2+} indicator Fura-2 AM as previously outlined (315). In brief, INS-1 832/13 cells were plated in black wall/clear bottom 96-multiwell plates from Costar (Tewksbury, MA, USA) and cultured for 2 days. Following drug or stress treatment, cells were transferred to Ca^{2+} free Hanks' balanced salt solution from Lonza (Basel, Switzerland) supplemented with 0.2% BSA and EGTA. Calcium 6 reagent was added directly to cells, and cells were incubated for an additional 2 hours at 37 °C and 5% CO_2. ER Ca^{2+} was estimated by

measuring the increase of intracellular Ca^{2+} 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 emission wavelength of 525 nm. For data analysis, values derived from the TG response (ΔF) were divided by resting intracellular calcium $[Ca^{2+}]_i$ (F_0), using the formula $\Delta F/ F_0$. Fura-2 AM fluorescence intensity was measured using an excitation wavelength of 340 nm and 380 nm and an emission wavelength of 510 nm. Images were captured using a Zeiss Z1 microscope with a 10x or 20x objective. Spontaneous intracellular Ca^{2+} transients were measured using the method described by Tang et al (287). Briefly, INS-1 cells or dispersed mouse islets were imaged under Ca^{2+} free conditions using Fura-2AM. Extracellular Ca^{2+} was increased in a step-wise fashion to evoke Ca^{2+} transients until a physiological extracellular Ca^{2+} concentration of 2mM was reached. Data were analyzed using Zeiss Zen Blue software (Oberkochen, Germany). The wavelength used for this experiment is summarized in **Table 5**.

4.2.5 IP_3R and RyR functional assays

IP_3R and RyR activation were evaluated in response to IP_3 and caffeine, respectively using modifications to the protocol described by Tovey and Taylor (285). INS-1 cells were loaded with the low-affinity Ca^{2+} indicator, Mag-Fluo-4 AM followed by permeabilization of the plasma membrane with 10 μg/ml saponin, leaving Mag-Fluo-4 AM in the lumen of cellular organelles. Data acquisition on the FlexStation 3 system was performed at 37°C using a 1.52- s reading interval, with an excitation wavelength of 490 nm and an emission wavelength at 525 nm. To establish steady-state ER Ca^{2+} levels, 1.5mM Mg-ATP was added; then IP_3 or caffeine were applied at the indicated concentrations to activate IP_3R and RyR respectively.

4.2.6 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 minutes at room temperature. The luminescence of each sample was measured by using a SpectraMax M5 microplate reader (Molecular Devices). Cell viability in mouse islets was quantitated using the Live/Dead Cell Viability Assay from Thermo Fisher (Waltham, MA), according to the manufacturer's instructions. Images were acquired using a Zeiss LSM 510 confocal microscope, and the area of dead cells was calculated by the ratio of ethidium homodimer-1 positive red area (Dead) and calcein-AM positive green area (Live). Glucose-stimulated insulin secretion was measured in isolated mouse islets as previously described. Insulin secretion into the supernatant was normalized to DNA concentration, and insulin was measured using a mouse insulin ELISA from Mercodia (Uppsala, Sweden) (54,316).

4.2.7 Statistical analysis

Results are displayed as the means ± S.E.M. GraphPad Prism Software (La Jolla, CA, USA) was used to fit data from IP_3R and RyR functional assays to sigmoidal dose-response curves. Differences between groups were analyzed for significance using GraphPad Prism Software and an unpaired Student's t-Test or one-way ANOVA with Tukey-Kramer post-test. A p value < 0.05 was used to indicate a significant difference between groups.

REFERENCES

1. Ogurtsova, K., da Rocha Fernandes, J. D., Huang, Y., Linnenkamp, U., Guariguata, L., Cho, N. H., Cavan, D., Shaw, J. E., and Makaroff, L. E. (2017) IDF Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040. *Diabetes Res Clin Pract* **128**, 40-50
2. Yang, Y., and Chan, L. (2016) Monogenic Diabetes: What It Teaches Us on the Common Forms of Type 1 and Type 2 Diabetes. *Endocr Rev* **37**, 190-222
3. Ashcroft, F. M., and Rorsman, P. (2012) Diabetes mellitus and the beta cell: the last ten years. *Cell* **148**, 1160-1171
4. Prasad, S., Kohm, A. P., McMahon, J. S., Luo, X., and Miller, S. D. (2012) Pathogenesis of NOD diabetes is initiated by reactivity to the insulin B chain 9-23 epitope and involves functional epitope spreading. *J Autoimmun* **39**, 347-353
5. Pociot, F., and Lernmark, A. (2016) Genetic risk factors for type 1 diabetes. *Lancet* **387**, 2331-2339
6. Davis, I. C., Randell, J., and Davis, S. N. (2015) Immunotherapies currently in development for the treatment of type 1 diabetes. *Expert Opin Investig Drugs* **24**, 1331-1341
7. Roep, B. O. (2003) The role of T-cells in the pathogenesis of Type 1 diabetes: from cause to cure. *Diabetologia* **46**, 305-321
8. Gottlieb, P. A., Quinlan, S., Krause-Steinrauf, H., Greenbaum, C. J., Wilson, D. M., Rodriguez, H., Schatz, D. A., Moran, A. M., Lachin, J. M., Skyler, J. S., and Type 1 Diabetes TrialNet, M. M. F. D. Z. B. S. G. (2010) Failure to preserve beta-cell function with mycophenolate mofetil and daclizumab combined therapy in patients with new- onset type 1 diabetes. *Diabetes Care* **33**, 826-832
9. Raab, J., Haupt, F., Scholz, M., Matzke, C., Warncke, K., Lange, K., Assfalg, R., Weininger, K., Wittich, S., Lobner, S., Beyerlein, A., Nennstiel-Ratzel, U., Lang, M., Laub, O., Dunstheimer, D., Bonifacio, E., Achenbach, P., Winkler, C., Ziegler, A. G., and Fr1da Study, G. (2016) Capillary blood islet autoantibody screening for identifying pre-type 1 diabetes in the general population: design and initial results of the Fr1da study. *BMJ Open* **6**, e011144
10. Couper, J. J. (2001) Environmental triggers of type 1 diabetes. *J Paediatr Child Health* **37**, 218-220
11. Ziegler, A. G., Hillebrand, B., Rabl, W., Mayrhofer, M., Hummel, M., Mollenhauer, U., Vordemann, J., Lenz, A., and Standl, E. (1993) On the appearance of islet associated autoimmunity in offspring of diabetic mothers: a prospective study from birth. *Diabetologia* **36**, 402-408
12. Graham, J., Hagopian, W. A., Kockum, I., Li, L. S., Sanjeevi, C. B., Lowe, R. M., Schaefer, J. B., Zarghami, M., Day, H. L., Landin-Olsson, M., Palmer, J. P., Janer-Villanueva, M., Hood, L., Sundkvist, G., Lernmark, A., Breslow, N., Dahlquist, G., Blohme, G., Diabetes Incidence in Sweden Study, G., and Swedish Childhood Diabetes Study, G. (2002) Genetic effects on age-dependent onset and islet cell autoantibody markers in type 1 diabetes. *Diabetes* **51**, 1346-1355
13. Sanjeevi, C. B., Lybrand, T. P., DeWeese, C., Landin-Olsson, M., Kockum, I., Dahlquist, G., Sundkvist, G., Stenger, D., and Lernmark, A. (1995) Polymorphic amino acid variations in HLA-DQ are associated with systematic physical property changes and occurrence of IDDM. Members of the Swedish Childhood Diabetes Study. *Diabetes* **44**, 125-131
14. Cerrone, G. E., Caputo, M., Lopez, A. P., Gonzalez, C., Massa, C., Cedola, N., Targovnik, H. M., and Frechtel, G. D. (2004) Variable number of tandem repeats

of the insulin gene determines susceptibility to latent autoimmune diabetes in adults. *Mol Diagn* **8**, 43-49
15. Wolchok, J. D., and Saenger, Y. M. (2007) Current topics in melanoma. *Curr Opin Oncol* **19**, 116-120
16. Fousteri, G., Jofra, T., Debernardis, I., Stanford, S. M., Laurenzi, A., Bottini, N., and Battaglia, M. (2014) The protein tyrosine phosphatase PTPN22 controls forkhead box protein 3 T regulatory cell induction but is dispensable for T helper type 1 cell polarization. *Clin Exp Immunol* **178**, 178-189
17. Bensinger, S. J., Walsh, P. T., Zhang, J., Carroll, M., Parsons, R., Rathmell, J. C., Thompson, C. B., Burchill, M. A., Farrar, M. A., and Turka, L. A. (2004) Distinct IL-2 receptor signaling pattern in CD4+CD25+ regulatory T cells. *J Immunol* **172**, 5287-5296
18. Hoglund, P., Mintern, J., Waltzinger, C., Heath, W., Benoist, C., and Mathis, D. (1999) Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. *J Exp Med* **189**, 331-339
19. Roep, B. O., and Peakman, M. (2012) Antigen targets of type 1 diabetes autoimmunity. *Cold Spring Harb Perspect Med* **2**, a007781
20. Eizirik, D. L., and Mandrup-Poulsen, T. (2001) A choice of death--the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia* **44**, 2115-2133
21. Lehuen, A., Diana, J., Zaccone, P., and Cooke, A. (2010) Immune cell crosstalk in type 1 diabetes. *Nat Rev Immunol* **10**, 501-513
22. Krischer, J. P., Lynch, K. F., Schatz, D. A., Ilonen, J., Lernmark, A., Hagopian, W. A., Rewers, M. J., She, J. X., Simell, O. G., Toppari, J., Ziegler, A. G., Akolkar, B., Bonifacio, E., and Group, T. S. (2015) The 6 year incidence of diabetes-associated autoantibodies in genetically at-risk children: the TEDDY study. *Diabetologia* **58**, 980-987
23. Ziegler, A. G., Hummel, M., Schenker, M., and Bonifacio, E. (1999) Autoantibody appearance and risk for development of childhood diabetes in offspring of parents with type 1 diabetes: the 2-year analysis of the German BABYDIAB Study. *Diabetes* **48**, 460-468
24. Nejentsev, S., Sjoroos, M., Soukka, T., Knip, M., Simell, O., Lovgren, T., and Ilonen, J. (1999) Population-based genetic screening for the estimation of Type 1 diabetes mellitus risk in Finland: selective genotyping of markers in the HLA-DQB1, HLA-DQA1 and HLA-DRB1 loci. *Diabet Med* **16**, 985-992
25. Bonifacio, E., Ziegler, A. G., Klingensmith, G., Schober, E., Bingley, P. J., Rottenkolber, M., Theil, A., Eugster, A., Puff, R., Peplow, C., Buettner, F., Lange, K., Hasford, J., Achenbach, P., and Pre, P. S. G. (2015) Effects of high-dose oral insulin on immune responses in children at high risk for type 1 diabetes: the Pre-POINT randomized clinical trial. *JAMA* **313**, 1541-1549
26. Bluestone, J. A., and Bour-Jordan, H. (2012) Current and future immunomodulation strategies to restore tolerance in autoimmune diseases. *Cold Spring Harb Perspect Biol* **4**
27. Waldron-Lynch, F., and Herold, K. C. (2011) Immunomodulatory therapy to preserve pancreatic beta-cell function in type 1 diabetes. *Nat Rev Drug Discov* **10**, 439-452
28. Liu, J. K. (2014) The history of monoclonal antibody development - Progress, remaining challenges and future innovations. *Ann Med Surg (Lond)* **3**, 113-116
29. Masharani, U. B., and Becker, J. (2010) Teplizumab therapy for type 1 diabetes. *Expert Opin Biol Ther* **10**, 459-465

30. Luo, X., Herold, K. C., and Miller, S. D. (2010) Immunotherapy of type 1 diabetes: where are we and where should we be going? *Immunity* **32**, 488-499
31. Burt, R. K., Slavin, S., Burns, W. H., and Marmont, A. M. (2002) Induction of tolerance in autoimmune diseases by hematopoietic stem cell transplantation: getting closer to a cure? *Blood* **99**, 768-784
32. Shapiro, A. M., Lakey, J. R., Ryan, E. A., Korbutt, G. S., Toth, E., Warnock, G. L., Kneteman, N. M., and Rajotte, R. V. (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* **343**, 230-238
33. Tarbell, K. V., Yamazaki, S., and Steinman, R. M. (2006) The interactions of dendritic cells with antigen-specific, regulatory T cells that suppress autoimmunity. *Semin Immunol* **18**, 93-102
34. Woodle, E. S., Xu, D., Zivin, R. A., Auger, J., Charette, J., O'Laughlin, R., Peace, D., Jollife, L. K., Haverty, T., Bluestone, J. A., and Thistlethwaite, J. R., Jr. (1999) Phase I trial of a humanized, Fc receptor nonbinding OKT3 antibody, huOKT3gamma1(Ala-Ala) in the treatment of acute renal allograft rejection. *Transplantation* **68**, 608-616
35. Utset, T. O., Auger, J. A., Peace, D., Zivin, R. A., Xu, D., Jolliffe, L., Alegre, M. L., Bluestone, J. A., and Clark, M. R. (2002) Modified anti-CD3 therapy in psoriatic arthritis: a phase I/II clinical trial. *J Rheumatol* **29**, 1907-1913
36. Orban, T., Bundy, B., Becker, D. J., DiMeglio, L. A., Gitelman, S. E., Goland, R., Gottlieb, P. A., Greenbaum, C. J., Marks, J. B., Monzavi, R., Moran, A., Raskin, P., Rodriguez, H., Russell, W. E., Schatz, D., Wherrett, D., Wilson, D. M., Krischer, J. P., Skyler, J. S., and Type 1 Diabetes TrialNet Abatacept Study, G. (2011) Co-stimulation modulation with abatacept in patients with recent-onset type 1 diabetes: a randomised, double-blind, placebo-controlled trial. *Lancet* **378**, 412-419
37. Ruderman, E. M., and Pope, R. M. (2005) The evolving clinical profile of abatacept (CTLA4-Ig): a novel co-stimulatory modulator for the treatment of rheumatoid arthritis. *Arthritis Res Ther* **7 Suppl 2**, S21-25
38. Wu, Y., Ding, Y., Tanaka, Y., and Zhang, W. (2014) Risk factors contributing to type 2 diabetes and recent advances in the treatment and prevention. *Int J Med Sci* **11**, 1185-1200
39. Samuel, V. T., and Shulman, G. I. (2012) Mechanisms for insulin resistance: common threads and missing links. *Cell* **148**, 852-871
40. Flamment, M., Hajduch, E., Ferre, P., and Foufelle, F. (2012) New insights into ER stress-induced insulin resistance. *Trends Endocrinol Metab* **23**, 381-390
41. Zaccardi, F., Webb, D. R., Yates, T., and Davies, M. J. (2016) Pathophysiology of type 1 and type 2 diabetes mellitus: a 90-year perspective. *Postgrad Med J* **92**, 63-69
42. Roduit, R., Morin, J., Masse, F., Segall, L., Roche, E., Newgard, C. B., Assimacopoulos-Jeannet, F., and Prentki, M. (2000) Glucose down-regulates the expression of the peroxisome proliferator-activated receptor-alpha gene in the pancreatic beta -cell. *J Biol Chem* **275**, 35799-35806
43. Eizirik, D. L., and Coomans de Brachene, A. (2017) Checks and Balances-The Limits of beta-Cell Endurance to ER Stress. *Diabetes* **66**, 1467-1469
44. Uruno, A., Furusawa, Y., Yagishita, Y., Fukutomi, T., Muramatsu, H., Negishi, T., Sugawara, A., Kensler, T. W., and Yamamoto, M. (2013) The Keap1-Nrf2 system prevents onset of diabetes mellitus. *Mol Cell Biol* **33**, 2996-3010

45. Accili, D., Talchai, S. C., Kim-Muller, J. Y., Cinti, F., Ishida, E., Ordelheide, A. M., Kuo, T., Fan, J., and Son, J. (2016) When beta-cells fail: lessons from dedifferentiation. *Diabetes Obes Metab* **18 Suppl 1**, 117-122
46. Holmes, D. (2017) Diabetes: Could broccoli have a role in combating type 2 diabetes mellitus? *Nat Rev Endocrinol* **13**, 437
47. Rorsman, P., and Braun, M. (2013) Regulation of insulin secretion in human pancreatic islets. *Annu Rev Physiol* **75**, 155-179
48. Olokoba, A. B., Obateru, O. A., and Olokoba, L. B. (2012) Type 2 diabetes mellitus: a review of current trends. *Oman Med J* **27**, 269-273
49. Prunier, C., Hocevar, B. A., and Howe, P. H. (2004) Wnt signaling: physiology and pathology. *Growth Factors* **22**, 141-150
50. Lyssenko, V., Lupi, R., Marchetti, P., Del Guerra, S., Orho-Melander, M., Almgren, P., Sjogren, M., Ling, C., Eriksson, K. F., Lethagen, A. L., Mancarella, R., Berglund, G., Tuomi, T., Nilsson, P., Del Prato, S., and Groop, L. (2007) Mechanisms by which common variants in the TCF7L2 gene increase risk of type 2 diabetes. *J Clin Invest* **117**, 2155-2163
51. Rung, J., Cauchi, S., Albrechtsen, A., Shen, L., Rocheleau, G., Cavalcanti-Proenca, C., Bacot, F., Balkau, B., Belisle, A., Borch-Johnsen, K., Charpentier, G., Dina, C., Durand, E., Elliott, P., Hadjadj, S., Jarvelin, M. R., Laitinen, J., Lauritzen, T., Marre, M., Mazur, A., Meyre, D., Montpetit, A., Pisinger, C., Posner, B., Poulsen, P., Pouta, A., Prentki, M., Ribel-Madsen, R., Ruokonen, A., Sandbaek, A., Serre, D., Tichet, J., Vaxillaire, M., Wojtaszewski, J. F., Vaag, A., Hansen, T., Polychronakos, C., Pedersen, O., Froguel, P., and Sladek, R. (2009) Genetic variant near IRS1 is associated with type 2 diabetes, insulin resistance and hyperinsulinemia. *Nat Genet* **41**, 1110-1115
52. Kulkarni, R. N., Winnay, J. N., Daniels, M., Bruning, J. C., Flier, S. N., Hanahan, D., and Kahn, C. R. (1999) Altered function of insulin receptor substrate-1-deficient mouse islets and cultured beta-cell lines. *J Clin Invest* **104**, R69-75
53. Lyssenko, V., Nagorny, C. L., Erdos, M. R., Wierup, N., Jonsson, A., Spegel, P., Bugliani, M., Saxena, R., Fex, M., Pulizzi, N., Isomaa, B., Tuomi, T., Nilsson, P., Kuusisto, J., Tuomilehto, J., Boehnke, M., Altshuler, D., Sundler, F., Eriksson, J. G., Jackson, A. U., Laakso, M., Marchetti, P., Watanabe, R. M., Mulder, H., and Groop, L. (2009) Common variant in MTNR1B associated with increased risk of type 2 diabetes and impaired early insulin secretion. *Nat Genet* **41**, 82-88
54. Kono, T., Ahn, G., Moss, D. R., Gann, L., Zarain-Herzberg, A., Nishiki, Y., Fueger, P. T., Ogihara, T., and Evans-Molina, C. (2012) PPAR-gamma activation restores pancreatic islet SERCA2 levels and prevents beta-cell dysfunction under conditions of hyperglycemic and cytokine stress. *Mol Endocrinol* **26**, 257-271
55. Loos, R. J., and Yeo, G. S. (2014) The bigger picture of FTO: the first GWAS-identified obesity gene. *Nat Rev Endocrinol* **10**, 51-61
56. Holman, R. (2007) Metformin as first choice in oral diabetes treatment: the UKPDS experience. *Journ Annu Diabetol Hotel Dieu*, 13-20
57. Bailey, C. J., and Turner, R. C. (1996) Metformin. *N Engl J Med* **334**, 574-579
58. Kim, Y. D., Park, K. G., Lee, Y. S., Park, Y. Y., Kim, D. K., Nedumaran, B., Jang, W. G., Cho, W. J., Ha, J., Lee, I. K., Lee, C. H., and Choi, H. S. (2008) Metformin inhibits hepatic gluconeogenesis through AMP-activated protein kinase-dependent regulation of the orphan nuclear receptor SHP. *Diabetes* **57**, 306-314
59. Rubin, R. R., Ma, Y., Marrero, D. G., Peyrot, M., Barrett-Connor, E. L., Kahn, S. E., Haffner, S. M., Price, D. W., Knowler, W. C., and Diabetes Prevention Program Research, G. (2008) Elevated depression symptoms, antidepressant

medicine use, and risk of developing diabetes during the diabetes prevention program. *Diabetes Care* **31**, 420-426
60. Madiraju, A. K., Erion, D. M., Rahimi, Y., Zhang, X. M., Braddock, D. T., Albright, R. A., Prigaro, B. J., Wood, J. L., Bhanot, S., MacDonald, M. J., Jurczak, M. J., Camporez, J. P., Lee, H. Y., Cline, G. W., Samuel, V. T., Kibbey, R. G., and Shulman, G. I. (2014) Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature* **510**, 542-546
61. Hundal, R. S., and Inzucchi, S. E. (2003) Metformin: new understandings, new uses. *Drugs* **63**, 1879-1894
62. Erlich, D. R., Slawson, D. C., and Shaughnessy, A. (2013) Diabetes update: new drugs to manage type 2 diabetes. *FP Essent* **408**, 20-24
63. Collier, C. A., Bruce, C. R., Smith, A. C., Lopaschuk, G., and Dyck, D. J. (2006) Metformin counters the insulin-induced suppression of fatty acid oxidation and stimulation of triacylglycerol storage in rodent skeletal muscle. *Am J Physiol Endocrinol Metab* **291**, E182-189
64. Ashcroft, F. M., and Rorsman, P. (2013) K(ATP) channels and islet hormone secretion: new insights and controversies. *Nat Rev Endocrinol* **9**, 660-669
65. Phung, O. J., Schwartzman, E., Allen, R. W., Engel, S. S., and Rajpathak, S. N. (2013) Sulphonylureas and risk of cardiovascular disease: systematic review and meta-analysis. *Diabet Med* **30**, 1160-1171
66. Scheen, A. J. (2005) Drug interactions of clinical importance with antihyperglycaemic agents: an update. *Drug Saf* **28**, 601-631
67. Zander, M., Madsbad, S., Madsen, J. L., and Holst, J. J. (2002) Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and beta-cell function in type 2 diabetes: a parallel-group study. *Lancet* **359**, 824-830
68. Nauck, M., Stockmann, F., Ebert, R., and Creutzfeldt, W. (1986) Reduced incretin effect in type 2 (non-insulin-dependent) diabetes. *Diabetologia* **29**, 46-52
69. Holst, J. J., Vilsboll, T., and Deacon, C. F. (2009) The incretin system and its role in type 2 diabetes mellitus. *Mol Cell Endocrinol* **297**, 127-136
70. Vilsboll, T., Krarup, T., Madsbad, S., and Holst, J. J. (2002) Defective amplification of the late phase insulin response to glucose by GIP in obese Type II diabetic patients. *Diabetologia* **45**, 1111-1119
71. Deacon, C. F., Hughes, T. E., and Holst, J. J. (1998) Dipeptidyl peptidase IV inhibition potentiates the insulinotropic effect of glucagon-like peptide 1 in the anesthetized pig. *Diabetes* **47**, 764-769
72. Deacon, C. F., Knudsen, L. B., Madsen, K., Wiberg, F. C., Jacobsen, O., and Holst, J. J. (1998) Dipeptidyl peptidase IV resistant analogues of glucagon-like peptide-1 which have extended metabolic stability and improved biological activity. *Diabetologia* **41**, 271-278
73. Hansen, L., Deacon, C. F., Orskov, C., and Holst, J. J. (1999) Glucagon-like peptide-1-(7-36)amide is transformed to glucagon-like peptide-1-(9-36)amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine. *Endocrinology* **140**, 5356-5363
74. Deacon, C. F. (2004) Circulation and degradation of GIP and GLP-1. *Horm Metab Res* **36**, 761-765
75. Donath, M. Y., and Burcelin, R. (2013) GLP-1 effects on islets: hormonal, neuronal, or paracrine? *Diabetes Care* **36 Suppl 2**, S145-148
76. Nauck, M. A., Wollschlager, D., Werner, J., Holst, J. J., Orskov, C., Creutzfeldt, W., and Willms, B. (1996) Effects of subcutaneous glucagon-like peptide 1 (GLP-1 [7-36 amide]) in patients with NIDDM. *Diabetologia* **39**, 1546-1553

77. Deacon, C. F., Carr, R. D., and Holst, J. J. (2008) DPP-4 inhibitor therapy: new directions in the treatment of type 2 diabetes. *Front Biosci* **13**, 1780-1794
78. Simonsen, L., Holst, J. J., and Deacon, C. F. (2006) Exendin-4, but not glucagon-like peptide-1, is cleared exclusively by glomerular filtration in anaesthetised pigs. *Diabetologia* **49**, 706-712
79. Edwards, C. M., Stanley, S. A., Davis, R., Brynes, A. E., Frost, G. S., Seal, L. J., Ghatei, M. A., and Bloom, S. R. (2001) Exendin-4 reduces fasting and postprandial glucose and decreases energy intake in healthy volunteers. *Am J Physiol Endocrinol Metab* **281**, E155-161
80. Kashyap, S. R., Gatmaitan, P., Brethauer, S., and Schauer, P. (2010) Bariatric surgery for type 2 diabetes: weighing the impact for obese patients. *Cleve Clin J Med* **77**, 468-476
81. Kim, K. S., Kim, S. K., Sung, K. M., Cho, Y. W., and Park, S. W. (2012) Management of type 2 diabetes mellitus in older adults. *Diabetes Metab J* **36**, 336-344
82. Hevener, A. L., Olefsky, J. M., Reichart, D., Nguyen, M. T., Bandyopadyhay, G., Leung, H. Y., Watt, M. J., Benner, C., Febbraio, M. A., Nguyen, A. K., Folian, B., Subramaniam, S., Gonzalez, F. J., Glass, C. K., and Ricote, M. (2007) Macrophage PPAR gamma is required for normal skeletal muscle and hepatic insulin sensitivity and full antidiabetic effects of thiazolidinediones. *J Clin Invest* **117**, 1658-1669
83. Elayat, A. A., el-Naggar, M. M., and Tahir, M. (1995) An immunocytochemical and morphometric study of the rat pancreatic islets. *J Anat* **186 (Pt 3)**, 629-637
84. Kailey, B., van de Bunt, M., Cheley, S., Johnson, P. R., MacDonald, P. E., Gloyn, A. L., Rorsman, P., and Braun, M. (2012) SSTR2 is the functionally dominant somatostatin receptor in human pancreatic beta- and alpha-cells. *Am J Physiol Endocrinol Metab* **303**, E1107-1116
85. Elliott, A. D., Ustione, A., and Piston, D. W. (2015) Somatostatin and insulin mediate glucose-inhibited glucagon secretion in the pancreatic alpha-cell by lowering cAMP. *Am J Physiol Endocrinol Metab* **308**, E130-143
86. Dezaki, K., Kakei, M., and Yada, T. (2007) Ghrelin uses Galphai2 and activates voltage-dependent K+ channels to attenuate glucose-induced Ca2+ signaling and insulin release in islet beta-cells: novel signal transduction of ghrelin. *Diabetes* **56**, 2319-2327
87. Schwartz, M. W., Woods, S. C., Porte, D., Jr., Seeley, R. J., and Baskin, D. G. (2000) Central nervous system control of food intake. *Nature* **404**, 661-671
88. Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D. J., Honjo, T., Hrabe de Angelis, M., Lendahl, U., and Edlund, H. (1999) Notch signalling controls pancreatic cell differentiation. *Nature* **400**, 877-881
89. Gu, G., Dubauskaite, J., and Melton, D. A. (2002) Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development* **129**, 2447-2457
90. Johansson, K. A., Dursun, U., Jordan, N., Gu, G., Beermann, F., Gradwohl, G., and Grapin-Botton, A. (2007) Temporal control of neurogenin3 activity in pancreas progenitors reveals competence windows for the generation of different endocrine cell types. *Dev Cell* **12**, 457-465
91. Gilon, P., Chae, H. Y., Rutter, G. A., and Ravier, M. A. (2014) Calcium signaling in pancreatic beta-cells in health and in Type 2 diabetes. *Cell Calcium* **56**, 340-361
92. Sekine, N., Cirulli, V., Regazzi, R., Brown, L. J., Gine, E., Tamarit-Rodriguez, J., Girotti, M., Marie, S., MacDonald, M. J., Wollheim, C. B., and et al. (1994) Low

lactate dehydrogenase and high mitochondrial glycerol phosphate dehydrogenase in pancreatic beta-cells. Potential role in nutrient sensing. *J Biol Chem* **269**, 4895-4902
93. Gall, D., Gromada, J., Susa, I., Rorsman, P., Herchuelz, A., and Bokvist, K. (1999) Significance of Na/Ca exchange for Ca2+ buffering and electrical activity in mouse pancreatic beta-cells. *Biophys J* **76**, 2018-2028
94. Gilon, P., and Rorsman, P. (2009) NALCN: a regulated leak channel. *EMBO Rep* **10**, 963-964
95. Henquin, J. C., Nenquin, M., Ravier, M. A., and Szollosi, A. (2009) Shortcomings of current models of glucose-induced insulin secretion. *Diabetes Obes Metab* **11 Suppl 4**, 168-179
96. Arruda, A. P., and Hotamisligil, G. S. (2015) Calcium Homeostasis and Organelle Function in the Pathogenesis of Obesity and Diabetes. *Cell Metab* **22**, 381-397
97. Talchai, C., Xuan, S., Kitamura, T., DePinho, R. A., and Accili, D. (2012) Generation of functional insulin-producing cells in the gut by Foxo1 ablation. *Nat Genet* **44**, 406-412, S401
98. Wang, Z., York, N. W., Nichols, C. G., and Remedi, M. S. (2014) Pancreatic beta cell dedifferentiation in diabetes and redifferentiation following insulin therapy. *Cell Metab* **19**, 872-882
99. English, A. R., Zurek, N., and Voeltz, G. K. (2009) Peripheral ER structure and function. *Curr Opin Cell Biol* **21**, 596-602
100. Schonthal, A. H. (2012) Endoplasmic reticulum stress: its role in disease and novel prospects for therapy. *Scientifica (Cairo)* **2012**, 857516
101. Schroder, M. (2008) Endoplasmic reticulum stress responses. *Cell Mol Life Sci* **65**, 862-894
102. Carvalho, P., Goder, V., and Rapoport, T. A. (2006) Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell* **126**, 361-373
103. Schroder, M., and Kaufman, R. J. (2005) The mammalian unfolded protein response. *Annu Rev Biochem* **74**, 739-789
104. Ron, D., and Walter, P. (2007) Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* **8**, 519-529
105. Malhotra, J. D., and Kaufman, R. J. (2007) The endoplasmic reticulum and the unfolded protein response. *Semin Cell Dev Biol* **18**, 716-731
106. Xu, C., Bailly-Maitre, B., and Reed, J. C. (2005) Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest* **115**, 2656-2664
107. Carrara, M., Prischi, F., and Ali, M. M. (2013) UPR Signal Activation by Luminal Sensor Domains. *Int J Mol Sci* **14**, 6454-6466
108. Walter, P., and Ron, D. (2011) The unfolded protein response: from stress pathway to homeostatic regulation. *Science* **334**, 1081-1086
109. Hollien, J., and Weissman, J. S. (2006) Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science* **313**, 104-107
110. Rao, R. V., and Bredesen, D. E. (2004) Misfolded proteins, endoplasmic reticulum stress and neurodegeneration. *Curr Opin Cell Biol* **16**, 653-662
111. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* **107**, 881-891
112. Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol Cell* **6**, 1099-1108

113. McCullough, K. D., Martindale, J. L., Klotz, L. O., Aw, T. Y., and Holbrook, N. J. (2001) Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Mol Cell Biol* **21**, 1249-1259
114. Puthalakath, H., O'Reilly, L. A., Gunn, P., Lee, L., Kelly, P. N., Huntington, N. D., Hughes, P. D., Michalak, E. M., McKimm-Breschkin, J., Motoyama, N., Gotoh, T., Akira, S., Bouillet, P., and Strasser, A. (2007) ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* **129**, 1337-1349
115. Yamamoto, K., Ichijo, H., and Korsmeyer, S. J. (1999) BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. *Mol Cell Biol* **19**, 8469-8478
116. Bygrave, F. L., and Benedetti, A. (1996) What is the concentration of calcium ions in the endoplasmic reticulum? *Cell Calcium* **19**, 547-551
117. Miyata, H., Silverman, H. S., Sollott, S. J., Lakatta, E. G., Stern, M. D., and Hansford, R. G. (1991) Measurement of mitochondrial free Ca2+ concentration in living single rat cardiac myocytes. *Am J Physiol* **261**, H1123-1134
118. Tarasov, A. I., Griffiths, E. J., and Rutter, G. A. (2012) Regulation of ATP production by mitochondrial Ca(2+). *Cell Calcium* **52**, 28-35
119. Chen, L., Koh, D. S., and Hille, B. (2003) Dynamics of calcium clearance in mouse pancreatic beta-cells. *Diabetes* **52**, 1723-1731
120. Jiang, D., Zhao, L., Clish, C. B., and Clapham, D. E. (2013) Letm1, the mitochondrial Ca2+/H+ antiporter, is essential for normal glucose metabolism and alters brain function in Wolf-Hirschhorn syndrome. *Proc Natl Acad Sci U S A* **110**, E2249-2254
121. Manji, H., Kato, T., Di Prospero, N. A., Ness, S., Beal, M. F., Krams, M., and Chen, G. (2012) Impaired mitochondrial function in psychiatric disorders. *Nat Rev Neurosci* **13**, 293-307
122. Vandecaetsbeek, I., Vangheluwe, P., Raeymaekers, L., Wuytack, F., and Vanoevelen, J. (2011) The Ca2+ pumps of the endoplasmic reticulum and Golgi apparatus. *Cold Spring Harb Perspect Biol* **3**
123. Bandara, S., Malmersjo, S., and Meyer, T. (2013) Regulators of calcium homeostasis identified by inference of kinetic model parameters from live single cells perturbed by siRNA. *Sci Signal* **6**, ra56
124. Periasamy, M., and Kalyanasundaram, A. (2007) SERCA pump isoforms: their role in calcium transport and disease. *Muscle Nerve* **35**, 430-442
125. Chandrasekera, P. C., Kargacin, M. E., Deans, J. P., and Lytton, J. (2009) Determination of apparent calcium affinity for endogenously expressed human sarco(endo)plasmic reticulum calcium-ATPase isoform SERCA3. *Am J Physiol Cell Physiol* **296**, C1105-1114
126. Sakuntabhai, A., Ruiz-Perez, V., Carter, S., Jacobsen, N., Burge, S., Monk, S., Smith, M., Munro, C. S., O'Donovan, M., Craddock, N., Kucherlapati, R., Rees, J. L., Owen, M., Lathrop, G. M., Monaco, A. P., Strachan, T., and Hovnanian, A. (1999) Mutations in ATP2A2, encoding a Ca2+ pump, cause Darier disease. *Nat Genet* **21**, 271-277
127. Foggia, L., and Hovnanian, A. (2004) Calcium pump disorders of the skin. *Am J Med Genet C Semin Med Genet* **131C**, 20-31
128. Ahn, W., Lee, M. G., Kim, K. H., and Muallem, S. (2003) Multiple effects of SERCA2b mutations associated with Darier's disease. *J Biol Chem* **278**, 20795-20801

129. Vangheluwe, P., Sipido, K. R., Raeymaekers, L., and Wuytack, F. (2006) New perspectives on the role of SERCA2's Ca2+ affinity in cardiac function. *Biochim Biophys Acta* **1763**, 1216-1228
130. Haghighi, K., Kolokathis, F., Gramolini, A. O., Waggoner, J. R., Pater, L., Lynch, R. A., Fan, G. C., Tsiapras, D., Parekh, R. R., Dorn, G. W., 2nd, MacLennan, D. H., Kremastinos, D. T., and Kranias, E. G. (2006) A mutation in the human phospholamban gene, deleting arginine 14, results in lethal, hereditary cardiomyopathy. *Proc Natl Acad Sci U S A* **103**, 1388-1393
131. Miyamoto, M. I., del Monte, F., Schmidt, U., DiSalvo, T. S., Kang, Z. B., Matsui, T., Guerrero, J. L., Gwathmey, J. K., Rosenzweig, A., and Hajjar, R. J. (2000) Adenoviral gene transfer of SERCA2a improves left-ventricular function in aortic-banded rats in transition to heart failure. *Proc Natl Acad Sci U S A* **97**, 793-798
132. del Monte, F., Williams, E., Lebeche, D., Schmidt, U., Rosenzweig, A., Gwathmey, J. K., Lewandowski, E. D., and Hajjar, R. J. (2001) Improvement in survival and cardiac metabolism after gene transfer of sarcoplasmic reticulum Ca(2+)-ATPase in a rat model of heart failure. *Circulation* **104**, 1424-1429
133. Hoshijima, M., Ikeda, Y., Iwanaga, Y., Minamisawa, S., Date, M. O., Gu, Y., Iwatate, M., Li, M., Wang, L., Wilson, J. M., Wang, Y., Ross, J., Jr., and Chien, K. R. (2002) Chronic suppression of heart-failure progression by a pseudophosphorylated mutant of phospholamban via in vivo cardiac rAAV gene delivery. *Nat Med* **8**, 864-871
134. Minamisawa, S., Hoshijima, M., Chu, G., Ward, C. A., Frank, K., Gu, Y., Martone, M. E., Wang, Y., Ross, J., Jr., Kranias, E. G., Giles, W. R., and Chien, K. R. (1999) Chronic phospholamban-sarcoplasmic reticulum calcium ATPase interaction is the critical calcium cycling defect in dilated cardiomyopathy. *Cell* **99**, 313-322
135. MacLennan, D. H., and Kranias, E. G. (2003) Phospholamban: a crucial regulator of cardiac contractility. *Nat Rev Mol Cell Biol* **4**, 566-577
136. Lytton, J., Westlin, M., Burk, S. E., Shull, G. E., and MacLennan, D. H. (1992) Functional comparisons between isoforms of the sarcoplasmic or endoplasmic reticulum family of calcium pumps. *J Biol Chem* **267**, 14483-14489
137. Cardozo, A. K., Ortis, F., Storling, J., Feng, Y. M., Rasschaert, J., Tonnesen, M., Van Eylen, F., Mandrup-Poulsen, T., Herchuelz, A., and Eizirik, D. L. (2005) Cytokines downregulate the sarcoendoplasmic reticulum pump Ca2+ ATPase 2b and deplete endoplasmic reticulum Ca2+, leading to induction of endoplasmic reticulum stress in pancreatic beta-cells. *Diabetes* **54**, 452-461
138. Mekahli, D., Bultynck, G., Parys, J. B., De Smedt, H., and Missiaen, L. (2011) Endoplasmic-reticulum calcium depletion and disease. *Cold Spring Harb Perspect Biol* **3**
139. Tong, X., Kono, T., Anderson-Baucum, E. K., Yamamoto, W., Gilon, P., Lebeche, D., Day, R. N., Shull, G. E., and Evans-Molina, C. (2016) SERCA2 Deficiency Impairs Pancreatic beta-Cell Function in Response to Diet-Induced Obesity. *Diabetes* **65**, 3039-3052
140. Zhou, C., Slaughter, B. D., Unruh, J. R., Guo, F., Yu, Z., Mickey, K., Narkar, A., Ross, R. T., McClain, M., and Li, R. (2014) Organelle-based aggregation and retention of damaged proteins in asymmetrically dividing cells. *Cell* **159**, 530-542
141. Barker, C. J., and Berggren, P. O. (2013) New horizons in cellular regulation by inositol polyphosphates: insights from the pancreatic beta-cell. *Pharmacol Rev* **65**, 641-669
142. Braun, M., Ramracheya, R., Amisten, S., Bengtsson, M., Moritoh, Y., Zhang, Q., Johnson, P. R., and Rorsman, P. (2009) Somatostatin release, electrical activity,

membrane currents and exocytosis in human pancreatic delta cells. *Diabetologia* **52**, 1566-1578
143. Zhang, Q., Bengtsson, M., Partridge, C., Salehi, A., Braun, M., Cox, R., Eliasson, L., Johnson, P. R., Renstrom, E., Schneider, T., Berggren, P. O., Gopel, S., Ashcroft, F. M., and Rorsman, P. (2007) R-type Ca(2+)-channel-evoked CICR regulates glucose-induced somatostatin secretion. *Nat Cell Biol* **9**, 453-460
144. Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T., and et al. (1989) Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature* **339**, 439-445
145. Nakai, J., Imagawa, T., Hakamat, Y., Shigekawa, M., Takeshima, H., and Numa, S. (1990) Primary structure and functional expression from cDNA of the cardiac ryanodine receptor/calcium release channel. *FEBS Lett* **271**, 169-177
146. Hakamata, Y., Nakai, J., Takeshima, H., and Imoto, K. (1992) Primary structure and distribution of a novel ryanodine receptor/calcium release channel from rabbit brain. *FEBS Lett* **312**, 229-235
147. Lanner, J. T., Georgiou, D. K., Joshi, A. D., and Hamilton, S. L. (2010) Ryanodine receptors: structure, expression, molecular details, and function in calcium release. *Cold Spring Harb Perspect Biol* **2**, a003996
148. O'Brien, J., Meissner, G., and Block, B. A. (1993) The fastest contracting muscles of nonmammalian vertebrates express only one isoform of the ryanodine receptor. *Biophys J* **65**, 2418-2427
149. Hamada, T., Sakube, Y., Ahnn, J., Kim, D. H., and Kagawa, H. (2002) Molecular dissection, tissue localization and Ca2+ binding of the ryanodine receptor of Caenorhabditis elegans. *J Mol Biol* **324**, 123-135
150. Vazquez-Martinez, O., Canedo-Merino, R., Diaz-Munoz, M., and Riesgo-Escovar, J. R. (2003) Biochemical characterization, distribution and phylogenetic analysis of Drosophila melanogaster ryanodine and IP3 receptors, and thapsigargin-sensitive Ca2+ ATPase. *J Cell Sci* **116**, 2483-2494
151. Mickelson, J. R., and Louis, C. F. (1996) Malignant hyperthermia: excitation-contraction coupling, Ca2+ release channel, and cell Ca2+ regulation defects. *Physiol Rev* **76**, 537-592
152. Capacchione, J. F., Sambuughin, N., Bina, S., Mulligan, L. P., Lawson, T. D., and Muldoon, S. M. (2010) Exertional rhabdomyolysis and malignant hyperthermia in a patient with ryanodine receptor type 1 gene, L-type calcium channel alpha-1 subunit gene, and calsequestrin-1 gene polymorphisms. *Anesthesiology* **112**, 239-244
153. Dirksen, R. T., and Avila, G. (2002) Altered ryanodine receptor function in central core disease: leaky or uncoupled Ca(2+) release channels? *Trends Cardiovasc Med* **12**, 189-197
154. Jungbluth, H. (2007) Multi-minicore Disease. *Orphanet J Rare Dis* **2**, 31
155. Marks, A. R., Priori, S., Memmi, M., Kontula, K., and Laitinen, P. J. (2002) Involvement of the cardiac ryanodine receptor/calcium release channel in catecholaminergic polymorphic ventricular tachycardia. *J Cell Physiol* **190**, 1-6
156. Jayaraman, T., Brillantes, A. M., Timerman, A. P., Fleischer, S., Erdjument-Bromage, H., Tempst, P., and Marks, A. R. (1992) FK506 binding protein associated with the calcium release channel (ryanodine receptor). *J Biol Chem* **267**, 9474-9477
157. Brillantes, A. B., Ondrias, K., Scott, A., Kobrinsky, E., Ondriasova, E., Moschella, M. C., Jayaraman, T., Landers, M., Ehrlich, B. E., and Marks, A. R. (1994)

Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. *Cell* **77**, 513-523
158. Timerman, A. P., Wiederrecht, G., Marcy, A., and Fleischer, S. (1995) Characterization of an exchange reaction between soluble FKBP-12 and the FKBP.ryanodine receptor complex. Modulation by FKBP mutants deficient in peptidyl-prolyl isomerase activity. *J Biol Chem* **270**, 2451-2459
159. Ahern, G. P., Junankar, P. R., and Dulhunty, A. F. (1994) Single channel activity of the ryanodine receptor calcium release channel is modulated by FK-506. *FEBS Lett* **352**, 369-374
160. Gaburjakova, M., Gaburjakova, J., Reiken, S., Huang, F., Marx, S. O., Rosemblit, N., and Marks, A. R. (2001) FKBP12 binding modulates ryanodine receptor channel gating. *J Biol Chem* **276**, 16931-16935
161. Ahern, G. P., Junankar, P. R., and Dulhunty, A. F. (1997) Subconductance states in single-channel activity of skeletal muscle ryanodine receptors after removal of FKBP12. *Biophys J* **72**, 146-162
162. Marx, S. O., Ondrias, K., and Marks, A. R. (1998) Coupled gating between individual skeletal muscle Ca2+ release channels (ryanodine receptors). *Science* **281**, 818-821
163. Tang, W., Ingalls, C. P., Durham, W. J., Snider, J., Reid, M. B., Wu, G., Matzuk, M. M., and Hamilton, S. L. (2004) Altered excitation-contraction coupling with skeletal muscle specific FKBP12 deficiency. *FASEB J* **18**, 1597-1599
164. Shou, W., Aghdasi, B., Armstrong, D. L., Guo, Q., Bao, S., Charng, M. J., Mathews, L. M., Schneider, M. D., Hamilton, S. L., and Matzuk, M. M. (1998) Cardiac defects and altered ryanodine receptor function in mice lacking FKBP12. *Nature* **391**, 489-492
165. Wehrens, X. H., Lehnart, S. E., Huang, F., Vest, J. A., Reiken, S. R., Mohler, P. J., Sun, J., Guatimosim, S., Song, L. S., Rosemblit, N., D'Armiento, J. M., Napolitano, C., Memmi, M., Priori, S. G., Lederer, W. J., and Marks, A. R. (2003) FKBP12.6 deficiency and defective calcium release channel (ryanodine receptor) function linked to exercise-induced sudden cardiac death. *Cell* **113**, 829-840
166. George, C. H., Higgs, G. V., and Lai, F. A. (2003) Ryanodine receptor mutations associated with stress-induced ventricular tachycardia mediate increased calcium release in stimulated cardiomyocytes. *Circ Res* **93**, 531-540
167. Valdivia, H. H., Kaplan, J. H., Ellis-Davies, G. C., and Lederer, W. J. (1995) Rapid adaptation of cardiac ryanodine receptors: modulation by Mg2+ and phosphorylation. *Science* **267**, 1997-2000
168. Marx, S. O., Reiken, S., Hisamatsu, Y., Jayaraman, T., Burkhoff, D., Rosemblit, N., and Marks, A. R. (2000) PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* **101**, 365-376
169. Dalla Volta, S., Battaglia, G., and Zerbini, E. (1961) "Auricularization' of right ventricular pressure curve. *Am Heart J* **61**, 25-33
170. Tiso, N., Stephan, D. A., Nava, A., Bagattin, A., Devaney, J. M., Stanchi, F., Larderet, G., Brahmbhatt, B., Brown, K., Bauce, B., Muriago, M., Basso, C., Thiene, G., Danieli, G. A., and Rampazzo, A. (2001) Identification of mutations in the cardiac ryanodine receptor gene in families affected with arrhythmogenic right ventricular cardiomyopathy type 2 (ARVD2). *Hum Mol Genet* **10**, 189-194
171. Supnet, C., Noonan, C., Richard, K., Bradley, J., and Mayne, M. (2010) Up-regulation of the type 3 ryanodine receptor is neuroprotective in the TgCRND8 mouse model of Alzheimer's disease. *J Neurochem* **112**, 356-365

172. Lamb, G. D. (2000) Excitation-contraction coupling in skeletal muscle: comparisons with cardiac muscle. *Clin Exp Pharmacol Physiol* **27**, 216-224
173. Nakai, J., Sekiguchi, N., Rando, T. A., Allen, P. D., and Beam, K. G. (1998) Two regions of the ryanodine receptor involved in coupling with L-type Ca2+ channels. *J Biol Chem* **273**, 13403-13406
174. Proenza, C., O'Brien, J., Nakai, J., Mukherjee, S., Allen, P. D., and Beam, K. G. (2002) Identification of a region of RyR1 that participates in allosteric coupling with the alpha(1S) (Ca(V)1.1) II-III loop. *J Biol Chem* **277**, 6530-6535
175. Armstrong, C. M., Bezanilla, F. M., and Horowicz, P. (1972) Twitches in the presence of ethylene glycol bis(-aminoethyl ether)-N,N'-tetracetic acid. *Biochim Biophys Acta* **267**, 605-608
176. Dulhunty, A. F., and Gage, P. W. (1988) Effects of extracellular calcium concentration and dihydropyridines on contraction in mammalian skeletal muscle. *J Physiol* **399**, 63-80
177. Bers, D. M., and Stiffel, V. M. (1993) Ratio of ryanodine to dihydropyridine receptors in cardiac and skeletal muscle and implications for E-C coupling. *Am J Physiol* **264**, C1587-1593
178. Fabiato, A. (1983) Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *Am J Physiol* **245**, C1-14
179. Meissner, G., Rios, E., Tripathy, A., and Pasek, D. A. (1997) Regulation of skeletal muscle Ca2+ release channel (ryanodine receptor) by Ca2+ and monovalent cations and anions. *J Biol Chem* **272**, 1628-1638
180. Palade, P., Mitchell, R. D., and Fleischer, S. (1983) Spontaneous calcium release from sarcoplasmic reticulum. General description and effects of calcium. *J Biol Chem* **258**, 8098-8107
181. Kass, R. S., and Tsien, R. W. (1982) Fluctuations in membrane current driven by intracellular calcium in cardiac Purkinje fibers. *Biophys J* **38**, 259-269
182. Yan, Z., Bai, X. C., Yan, C., Wu, J., Li, Z., Xie, T., Peng, W., Yin, C. C., Li, X., Scheres, S. H., Shi, Y., and Yan, N. (2015) Structure of the rabbit ryanodine receptor RyR1 at near-atomic resolution. *Nature* **517**, 50-55
183. Jiang, D., Xiao, B., Yang, D., Wang, R., Choi, P., Zhang, L., Cheng, H., and Chen, S. R. (2004) RyR2 mutations linked to ventricular tachycardia and sudden death reduce the threshold for store-overload-induced Ca2+ release (SOICR). *Proc Natl Acad Sci U S A* **101**, 13062-13067
184. Moore, C. P., Rodney, G., Zhang, J. Z., Santacruz-Toloza, L., Strasburg, G., and Hamilton, S. L. (1999) Apocalmodulin and Ca2+ calmodulin bind to the same region on the skeletal muscle Ca2+ release channel. *Biochemistry* **38**, 8532-8537
185. Tang, W., Sencer, S., and Hamilton, S. L. (2002) Calmodulin modulation of proteins involved in excitation-contraction coupling. *Front Biosci* **7**, d1583-1589
186. Ohrtman, J., Ritter, B., Polster, A., Beam, K. G., and Papadopoulos, S. (2008) Sequence differences in the IQ motifs of CaV1.1 and CaV1.2 strongly impact calmodulin binding and calcium-dependent inactivation. *J Biol Chem* **283**, 29301-29311
187. Halling, D. B., Georgiou, D. K., Black, D. J., Yang, G., Fallon, J. L., Quiocho, F. A., Pedersen, S. E., and Hamilton, S. L. (2009) Determinants in CaV1 channels that regulate the Ca2+ sensitivity of bound calmodulin. *J Biol Chem* **284**, 20041-20051
188. Balshaw, D. M., Xu, L., Yamaguchi, N., Pasek, D. A., and Meissner, G. (2001) Calmodulin binding and inhibition of cardiac muscle calcium release channel (ryanodine receptor). *J Biol Chem* **276**, 20144-20153

189. Xu, X., Yano, M., Uchinoumi, H., Hino, A., Suetomi, T., Ono, M., Tateishi, H., Oda, T., Okuda, S., Doi, M., Kobayashi, S., Yamamoto, T., Ikeda, Y., Ikemoto, N., and Matsuzaki, M. (2010) Defective calmodulin binding to the cardiac ryanodine receptor plays a key role in CPVT-associated channel dysfunction. *Biochem Biophys Res Commun* **394**, 660-666
190. Rodriguez, P., Bhogal, M. S., and Colyer, J. (2003) Stoichiometric phosphorylation of cardiac ryanodine receptor on serine 2809 by calmodulin-dependent kinase II and protein kinase A. *J Biol Chem* **278**, 38593-38600
191. Wehrens, X. H., Lehnart, S. E., Reiken, S. R., and Marks, A. R. (2004) Ca2+/calmodulin-dependent protein kinase II phosphorylation regulates the cardiac ryanodine receptor. *Circ Res* **94**, e61-70
192. Xiao, B., Zhong, G., Obayashi, M., Yang, D., Chen, K., Walsh, M. P., Shimoni, Y., Cheng, H., Ter Keurs, H., and Chen, S. R. (2006) Ser-2030, but not Ser-2808, is the major phosphorylation site in cardiac ryanodine receptors responding to protein kinase A activation upon beta-adrenergic stimulation in normal and failing hearts. *Biochem J* **396**, 7-16
193. Huke, S., and Bers, D. M. (2007) Temporal dissociation of frequency-dependent acceleration of relaxation and protein phosphorylation by CaMKII. *J Mol Cell Cardiol* **42**, 590-599
194. Bers, D. M. (2002) Cardiac excitation-contraction coupling. *Nature* **415**, 198-205
195. Li, L., Desantiago, J., Chu, G., Kranias, E. G., and Bers, D. M. (2000) Phosphorylation of phospholamban and troponin I in beta-adrenergic-induced acceleration of cardiac relaxation. *Am J Physiol Heart Circ Physiol* **278**, H769-779
196. Ai, X., Curran, J. W., Shannon, T. R., Bers, D. M., and Pogwizd, S. M. (2005) Ca2+/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca2+ leak in heart failure. *Circ Res* **97**, 1314-1322
197. Stoyanovsky, D., Murphy, T., Anno, P. R., Kim, Y. M., and Salama, G. (1997) Nitric oxide activates skeletal and cardiac ryanodine receptors. *Cell Calcium* **21**, 19-29
198. Eager, K. R., and Dulhunty, A. F. (1998) Activation of the cardiac ryanodine receptor by sulfhydryl oxidation is modified by Mg2+ and ATP. *J Membr Biol* **163**, 9-18
199. Boraso, A., and Williams, A. J. (1994) Modification of the gating of the cardiac sarcoplasmic reticulum Ca(2+)-release channel by H2O2 and dithiothreitol. *Am J Physiol* **267**, H1010-1016
200. Xu, L., Eu, J. P., Meissner, G., and Stamler, J. S. (1998) Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science* **279**, 234-237
201. Ferdinandy, P., and Schulz, R. (2003) Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. *Br J Pharmacol* **138**, 532-543
202. Porter Moore, C., Zhang, J. Z., and Hamilton, S. L. (1999) A role for cysteine 3635 of RYR1 in redox modulation and calmodulin binding. *J Biol Chem* **274**, 36831-36834
203. Eu, J. P., Hare, J. M., Hess, D. T., Skaf, M., Sun, J., Cardenas-Navina, I., Sun, Q. A., Dewhirst, M., Meissner, G., and Stamler, J. S. (2003) Concerted regulation of skeletal muscle contractility by oxygen tension and endogenous nitric oxide. *Proc Natl Acad Sci U S A* **100**, 15229-15234

204. Bellinger, A. M., Reiken, S., Carlson, C., Mongillo, M., Liu, X., Rothman, L., Matecki, S., Lacampagne, A., and Marks, A. R. (2009) Hypernitrosylated ryanodine receptor calcium release channels are leaky in dystrophic muscle. *Nat Med* **15**, 325-330
205. Sun, J., Yamaguchi, N., Xu, L., Eu, J. P., Stamler, J. S., and Meissner, G. (2008) Regulation of the cardiac muscle ryanodine receptor by O(2) tension and S-nitrosoglutathione. *Biochemistry* **47**, 13985-13990
206. Meissner, G. (1984) Adenine nucleotide stimulation of Ca2+-induced Ca2+ release in sarcoplasmic reticulum. *J Biol Chem* **259**, 2365-2374
207. Laver, D. R., O'Neill, E. R., and Lamb, G. D. (2004) Luminal Ca2+-regulated Mg2+ inhibition of skeletal RyRs reconstituted as isolated channels or coupled clusters. *J Gen Physiol* **124**, 741-758
208. Laver, D. R., Lenz, G. K., and Lamb, G. D. (2001) Regulation of the calcium release channel from rabbit skeletal muscle by the nucleotides ATP, AMP, IMP and adenosine. *J Physiol* **537**, 763-778
209. Yang, J. M., and Huang, W. C. (2004) Sonographic findings in acute urinary retention secondary to retroverted gravid uterus: pathophysiology and preventive measures. *Ultrasound Obstet Gynecol* **23**, 490-495
210. Santulli, G., Pagano, G., Sardu, C., Xie, W., Reiken, S., D'Ascia, S. L., Cannone, M., Marziliano, N., Trimarco, B., Guise, T. A., Lacampagne, A., and Marks, A. R. (2015) Calcium release channel RyR2 regulates insulin release and glucose homeostasis. *J Clin Invest* **125**, 4316
211. Luciani, D. S., Gwiazda, K. S., Yang, T. L., Kalynyak, T. B., Bychkivska, Y., Frey, M. H., Jeffrey, K. D., Sampaio, A. V., Underhill, T. M., and Johnson, J. D. (2009) Roles of IP3R and RyR Ca2+ channels in endoplasmic reticulum stress and beta-cell death. *Diabetes* **58**, 422-432
212. Lu, S., Kanekura, K., Hara, T., Mahadevan, J., Spears, L. D., Oslowski, C. M., Martinez, R., Yamazaki-Inoue, M., Toyoda, M., Neilson, A., Blanner, P., Brown, C. M., Semenkovich, C. F., Marshall, B. A., Hershey, T., Umezawa, A., Greer, P. A., and Urano, F. (2014) A calcium-dependent protease as a potential therapeutic target for Wolfram syndrome. *Proc Natl Acad Sci U S A* **111**, E5292-5301
213. Taylor, C. W., Genazzani, A. A., and Morris, S. A. (1999) Expression of inositol trisphosphate receptors. *Cell Calcium* **26**, 237-251
214. Foskett, J. K., White, C., Cheung, K. H., and Mak, D. O. (2007) Inositol trisphosphate receptor Ca2+ release channels. *Physiol Rev* **87**, 593-658
215. Alzayady, K. J., Sebe-Pedros, A., Chandrasekhar, R., Wang, L., Ruiz-Trillo, I., and Yule, D. I. (2015) Tracing the Evolutionary History of Inositol, 1, 4, 5-Trisphosphate Receptor: Insights from Analyses of Capsaspora owczarzaki Ca2+ Release Channel Orthologs. *Mol Biol Evol* **32**, 2236-2253
216. Berridge, M. J., and Irvine, R. F. (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**, 315-321
217. Pinton, P., Pozzan, T., and Rizzuto, R. (1998) The Golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca2+ store, with functional properties distinct from those of the endoplasmic reticulum. *EMBO J* **17**, 5298-5308
218. Gerasimenko, O. V., Gerasimenko, J. V., Belan, P. V., and Petersen, O. H. (1996) Inositol trisphosphate and cyclic ADP-ribose-mediated release of Ca2+ from single isolated pancreatic zymogen granules. *Cell* **84**, 473-480
219. Gerasimenko, O. V., Gerasimenko, J. V., Tepikin, A. V., and Petersen, O. H. (1995) ATP-dependent accumulation and inositol trisphosphate- or cyclic ADP-ribose-mediated release of Ca2+ from the nuclear envelope. *Cell* **80**, 439-444

220. Essen, L. O., Perisic, O., Katan, M., Wu, Y., Roberts, M. F., and Williams, R. L. (1997) Structural mapping of the catalytic mechanism for a mammalian phosphoinositide-specific phospholipase C. *Biochemistry* **36**, 1704-1718
221. Bosanac, I., Alattia, J. R., Mal, T. K., Chan, J., Talarico, S., Tong, F. K., Tong, K. I., Yoshikawa, F., Furuichi, T., Iwai, M., Michikawa, T., Mikoshiba, K., and Ikura, M. (2002) Structure of the inositol 1,4,5-trisphosphate receptor binding core in complex with its ligand. *Nature* **420**, 696-700
222. Ross, W. N. (2012) Understanding calcium waves and sparks in central neurons. *Nat Rev Neurosci* **13**, 157-168
223. Lechleiter, J., Girard, S., Peralta, E., and Clapham, D. (1991) Spiral calcium wave propagation and annihilation in Xenopus laevis oocytes. *Science* **252**, 123-126
224. Bootman, M. D., Berridge, M. J., and Taylor, C. W. (1992) All-or-nothing Ca2+ mobilization from the intracellular stores of single histamine-stimulated HeLa cells. *J Physiol* **450**, 163-178
225. Demuro, A., and Parker, I. (2008) Multi-dimensional resolution of elementary Ca2+ signals by simultaneous multi-focal imaging. *Cell Calcium* **43**, 367-374
226. Finch, E. A., Turner, T. J., and Goldin, S. M. (1991) Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. *Science* **252**, 443-446
227. Marchant, J. S., and Taylor, C. W. (1997) Cooperative activation of IP3 receptors by sequential binding of IP3 and Ca2+ safeguards against spontaneous activity. *Curr Biol* **7**, 510-518
228. Iino, M. (1987) Calcium dependent inositol trisphosphate-induced calcium release in the guinea-pig taenia caeci. *Biochem Biophys Res Commun* **142**, 47-52
229. Thrower, E. C., Lea, E. J., and Dawson, A. P. (1998) The effects of free [Ca2+] on the cytosolic face of the inositol (1,4,5)-trisphosphate receptor at the single channel level. *Biochem J* **330 (Pt 1)**, 559-564
230. Michikawa, T., Hirota, J., Kawano, S., Hiraoka, M., Yamada, M., Furuichi, T., and Mikoshiba, K. (1999) Calmodulin mediates calcium-dependent inactivation of the cerebellar type 1 inositol 1,4,5-trisphosphate receptor. *Neuron* **23**, 799-808
231. Taylor, C. W., da Fonseca, P. C., and Morris, E. P. (2004) IP(3) receptors: the search for structure. *Trends Biochem Sci* **29**, 210-219
232. Taylor, C. W., and Tovey, S. C. (2010) IP(3) receptors: toward understanding their activation. *Cold Spring Harb Perspect Biol* **2**, a004010
233. Tu, H., Wang, Z., Nosyreva, E., De Smedt, H., and Bezprozvanny, I. (2005) Functional characterization of mammalian inositol 1,4,5-trisphosphate receptor isoforms. *Biophys J* **88**, 1046-1055
234. Baljinnyam, E., De Lorenzo, M. S., Xie, L. H., Iwatsubo, M., Chen, S., Goydos, J. S., Nowycky, M. C., and Iwatsubo, K. (2010) Exchange protein directly activated by cyclic AMP increases melanoma cell migration by a Ca2+-dependent mechanism. *Cancer Res* **70**, 5607-5617
235. Wagner, L. E., 2nd, Joseph, S. K., and Yule, D. I. (2008) Regulation of single inositol 1,4,5-trisphosphate receptor channel activity by protein kinase A phosphorylation. *J Physiol* **586**, 3577-3596
236. Taylor, C. W. (2016) Regulation of IP3 receptors by cyclic AMP. *Cell Calcium*
237. Sienaert, I., Missiaen, L., De Smedt, H., Parys, J. B., Sipma, H., and Casteels, R. (1997) Molecular and functional evidence for multiple Ca2+-binding domains in the type 1 inositol 1,4,5-trisphosphate receptor. *J Biol Chem* **272**, 25899-25906

238. Miyakawa, T., Mizushima, A., Hirose, K., Yamazawa, T., Bezprozvanny, I., Kurosaki, T., and Iino, M. (2001) Ca(2+)-sensor region of IP(3) receptor controls intracellular Ca(2+) signaling. *EMBO J* **20**, 1674-1680
239. Tu, H., Nosyreva, E., Miyakawa, T., Wang, Z., Mizushima, A., Iino, M., and Bezprozvanny, I. (2003) Functional and biochemical analysis of the type 1 inositol (1,4,5)-trisphosphate receptor calcium sensor. *Biophys J* **85**, 290-299
240. Missiaen, L., Taylor, C. W., and Berridge, M. J. (1992) Luminal Ca2+ promoting spontaneous Ca2+ release from inositol trisphosphate-sensitive stores in rat hepatocytes. *J Physiol* **455**, 623-640
241. Nunn, D. L., and Taylor, C. W. (1992) Luminal Ca2+ increases the sensitivity of Ca2+ stores to inositol 1,4,5-trisphosphate. *Mol Pharmacol* **41**, 115-119
242. Sienaert, I., De Smedt, H., Parys, J. B., Missiaen, L., Vanlingen, S., Sipma, H., and Casteels, R. (1996) Characterization of a cytosolic and a luminal Ca2+ binding site in the type I inositol 1,4,5-trisphosphate receptor. *J Biol Chem* **271**, 27005-27012
243. Harzheim, D., Talasila, A., Movassagh, M., Foo, R. S., Figg, N., Bootman, M. D., and Roderick, H. L. (2010) Elevated InsP3R expression underlies enhanced calcium fluxes and spontaneous extra-systolic calcium release events in hypertrophic cardiac myocytes. *Channels (Austin)* **4**, 67-71
244. Harzheim, D., Movassagh, M., Foo, R. S., Ritter, O., Tashfeen, A., Conway, S. J., Bootman, M. D., and Roderick, H. L. (2009) Increased InsP3Rs in the junctional sarcoplasmic reticulum augment Ca2+ transients and arrhythmias associated with cardiac hypertrophy. *Proc Natl Acad Sci U S A* **106**, 11406-11411
245. Signore, S., Sorrentino, A., Ferreira-Martins, J., Kannappan, R., Shafaie, M., Del Ben, F., Isobe, K., Arranto, C., Wybieralska, E., Webster, A., Sanada, F., Ogorek, B., Zheng, H., Liu, X., Del Monte, F., D'Alessandro, D. A., Wunimenghe, O., Michler, R. E., Hosoda, T., Goichberg, P., Leri, A., Kajstura, J., Anversa, P., and Rota, M. (2013) Inositol 1, 4, 5-trisphosphate receptors and human left ventricular myocytes. *Circulation* **128**, 1286-1297
246. Tang, T. S., Guo, C., Wang, H., Chen, X., and Bezprozvanny, I. (2009) Neuroprotective effects of inositol 1,4,5-trisphosphate receptor C-terminal fragment in a Huntington's disease mouse model. *J Neurosci* **29**, 1257-1266
247. Ito, E., Oka, K., Etcheberrigaray, R., Nelson, T. J., McPhie, D. L., Tofel-Grehl, B., Gibson, G. E., and Alkon, D. L. (1994) Internal Ca2+ mobilization is altered in fibroblasts from patients with Alzheimer disease. *Proc Natl Acad Sci U S A* **91**, 534-538
248. Hara, K., Shiga, A., Nozaki, H., Mitsui, J., Takahashi, Y., Ishiguro, H., Yomono, H., Kurisaki, H., Goto, J., Ikeuchi, T., Tsuji, S., Nishizawa, M., and Onodera, O. (2008) Total deletion and a missense mutation of ITPR1 in Japanese SCA15 families. *Neurology* **71**, 547-551
249. Ganesamoorthy, D., Bruno, D. L., Schoumans, J., Storey, E., Delatycki, M. B., Zhu, D., Wei, M. K., Nicholson, G. A., McKinlay Gardner, R. J., and Slater, H. R. (2009) Development of a multiplex ligation-dependent probe amplification assay for diagnosis and estimation of the frequency of spinocerebellar ataxia type 15. *Clin Chem* **55**, 1415-1418
250. Santos, R. M., Rosario, L. M., Nadal, A., Garcia-Sancho, J., Soria, B., and Valdeolmillos, M. (1991) Widespread synchronous [Ca2+]i oscillations due to bursting electrical activity in single pancreatic islets. *Pflugers Arch* **418**, 417-422

251. Rorsman, P., Eliasson, L., Kanno, T., Zhang, Q., and Gopel, S. (2011) Electrophysiology of pancreatic beta-cells in intact mouse islets of Langerhans. *Prog Biophys Mol Biol* **107**, 224-235
252. Busche, M. A., Chen, X., Henning, H. A., Reichwald, J., Staufenbiel, M., Sakmann, B., and Konnerth, A. (2012) Critical role of soluble amyloid-beta for early hippocampal hyperactivity in a mouse model of Alzheimer's disease. *Proc Natl Acad Sci U S A* **109**, 8740-8745
253. Busche, M. A., Grienberger, C., Keskin, A. D., Song, B., Neumann, U., Staufenbiel, M., Forstl, H., and Konnerth, A. (2015) Decreased amyloid-beta and increased neuronal hyperactivity by immunotherapy in Alzheimer's models. *Nat Neurosci* **18**, 1725-1727
254. Basso, M. A., Powers, A. S., and Evinger, C. (1996) An explanation for reflex blink hyperexcitability in Parkinson's disease. I. Superior colliculus. *J Neurosci* **16**, 7308-7317
255. Melo, T., Bigini, P., Sonnewald, U., Balosso, S., Cagnotto, A., Barbera, S., Uboldi, S., Vezzani, A., and Mennini, T. (2010) Neuronal hyperexcitability and seizures are associated with changes in glial-neuronal interactions in the hippocampus of a mouse model of epilepsy with mental retardation. *J Neurochem* **115**, 1445-1454
256. Gwak, Y. S., and Hulsebosch, C. E. (2011) Neuronal hyperexcitability: a substrate for central neuropathic pain after spinal cord injury. *Curr Pain Headache Rep* **15**, 215-222
257. Dong, X. X., Wang, Y., and Qin, Z. H. (2009) Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. *Acta Pharmacol Sin* **30**, 379-387
258. Lee, B. K., Lee, D. H., Park, S., Park, S. L., Yoon, J. S., Lee, M. G., Lee, S., Yi, K. Y., Yoo, S. E., Lee, K. H., Kim, Y. S., Lee, S. H., Baik, E. J., Moon, C. H., and Jung, Y. S. (2009) Effects of KR-33028, a novel Na+/H+ exchanger-1 inhibitor, on glutamate-induced neuronal cell death and ischemia-induced cerebral infarct. *Brain Res* **1248**, 22-30
259. Stafstrom, C. E. (2003) Hyperglycemia Lowers Seizure Threshold. *Epilepsy Curr* **3**, 148-149
260. Chen, W., Wang, R., Chen, B., Zhong, X., Kong, H., Bai, Y., Zhou, Q., Xie, C., Zhang, J., Guo, A., Tian, X., Jones, P. P., O'Mara, M. L., Liu, Y., Mi, T., Zhang, L., Bolstad, J., Semeniuk, L., Cheng, H., Zhang, J., Chen, J., Tieleman, D. P., Gillis, A. M., Duff, H. J., Fill, M., Song, L. S., and Chen, S. R. (2014) The ryanodine receptor store-sensing gate controls Ca2+ waves and Ca2+-triggered arrhythmias. *Nat Med* **20**, 184-192
261. Nuss, H. B., Marban, E., and Johns, D. C. (1999) Overexpression of a human potassium channel suppresses cardiac hyperexcitability in rabbit ventricular myocytes. *J Clin Invest* **103**, 889-896
262. Matthews, D. R., Cull, C. A., Stratton, I. M., Holman, R. R., and Turner, R. C. (1998) UKPDS 26: Sulphonylurea failure in non-insulin-dependent diabetic patients over six years. UK Prospective Diabetes Study (UKPDS) Group. *Diabet Med* **15**, 297-303
263. Remedi, M. S., Rocheleau, J. V., Tong, A., Patton, B. L., McDaniel, M. L., Piston, D. W., Koster, J. C., and Nichols, C. G. (2006) Hyperinsulinism in mice with heterozygous loss of K(ATP) channels. *Diabetologia* **49**, 2368-2378
264. Lupi, R., and Del Prato, S. (2008) Beta-cell apoptosis in type 2 diabetes: quantitative and functional consequences. *Diabetes Metab* **34 Suppl 2**, S56-64

265. Miki, T., Nagashima, K., Tashiro, F., Kotake, K., Yoshitomi, H., Tamamoto, A., Gonoi, T., Iwanaga, T., Miyazaki, J., and Seino, S. (1998) Defective insulin secretion and enhanced insulin action in KATP channel-deficient mice. *Proc Natl Acad Sci U S A* **95**, 10402-10406
266. Seghers, V., Nakazaki, M., DeMayo, F., Aguilar-Bryan, L., and Bryan, J. (2000) Sur1 knockout mice. A model for K(ATP) channel-independent regulation of insulin secretion. *J Biol Chem* **275**, 9270-9277
267. Maedler, K., Carr, R. D., Bosco, D., Zuellig, R. A., Berney, T., and Donath, M. Y. (2005) Sulfonylurea induced beta-cell apoptosis in cultured human islets. *J Clin Endocrinol Metab* **90**, 501-506
268. Bensellam, M., Laybutt, D. R., and Jonas, J. C. (2012) The molecular mechanisms of pancreatic beta-cell glucotoxicity: recent findings and future research directions. *Mol Cell Endocrinol* **364**, 1-27
269. Chang-Chen, K. J., Mullur, R., and Bernal-Mizrachi, E. (2008) Beta-cell failure as a complication of diabetes. *Rev Endocr Metab Disord* **9**, 329-343
270. Weir, G. C., Laybutt, D. R., Kaneto, H., Bonner-Weir, S., and Sharma, A. (2001) Beta-cell adaptation and decompensation during the progression of diabetes. *Diabetes* **50 Suppl 1**, S154-159
271. Bjorklund, A., and Grill, V. (1993) B-cell insensitivity in vitro: reversal by diazoxide entails more than one event in stimulus-secretion coupling. *Endocrinology* **132**, 1319-1328
272. Ma, Z., Portwood, N., Brodin, D., Grill, V., and Bjorklund, A. (2007) Effects of diazoxide on gene expression in rat pancreatic islets are largely linked to elevated glucose and potentially serve to enhance beta-cell sensitivity. *Diabetes* **56**, 1095-1106
273. Ritzel, R. A., Hansen, J. B., Veldhuis, J. D., and Butler, P. C. (2004) Induction of beta-cell rest by a Kir6.2/SUR1-selective K(ATP)-channel opener preserves beta-cell insulin stores and insulin secretion in human islets cultured at high (11 mM) glucose. *J Clin Endocrinol Metab* **89**, 795-805
274. Palladino, A. A., Bennett, M. J., and Stanley, C. A. (2008) Hyperinsulinism in infancy and childhood: when an insulin level is not always enough. *Clin Chem* **54**, 256-263
275. Smith, A. J., Taneja, T. K., Mankouri, J., and Sivaprasadarao, A. (2007) Molecular cell biology of KATP channels: implications for neonatal diabetes. *Expert Rev Mol Med* **9**, 1-17
276. Gilon, P., and Henquin, J. C. (1992) Influence of membrane potential changes on cytoplasmic Ca2+ concentration in an electrically excitable cell, the insulin-secreting pancreatic B-cell. *J Biol Chem* **267**, 20713-20720
277. Charpantier, E., Cancela, J., and Meda, P. (2007) Beta cells preferentially exchange cationic molecules via connexin 36 gap junction channels. *Diabetologia* **50**, 2332-2341
278. Benninger, R. K., Head, W. S., Zhang, M., Satin, L. S., and Piston, D. W. (2011) Gap junctions and other mechanisms of cell-cell communication regulate basal insulin secretion in the pancreatic islet. *J Physiol* **589**, 5453-5466
279. Bergsten, P. (2000) Pathophysiology of impaired pulsatile insulin release. *Diabetes Metab Res Rev* **16**, 179-191
280. Matthews, D. R., Naylor, B. A., Jones, R. G., Ward, G. M., and Turner, R. C. (1983) Pulsatile insulin has greater hypoglycemic effect than continuous delivery. *Diabetes* **32**, 617-621
281. Ward, G. M. (1987) The insulin receptor concept and its relation to the treatment of diabetes. *Drugs* **33**, 156-170

282. Brehm, M. A., Bortell, R., Diiorio, P., Leif, J., Laning, J., Cuthbert, A., Yang, C., Herlihy, M., Burzenski, L., Gott, B., Foreman, O., Powers, A. C., Greiner, D. L., and Shultz, L. D. (2010) Human immune system development and rejection of human islet allografts in spontaneously diabetic NOD-Rag1null IL2rgammanull Ins2Akita mice. *Diabetes* **59**, 2265-2270
283. Toque, H. A., Nunes, K. P., Yao, L., Xu, Z., Kondrikov, D., Su, Y., Webb, R. C., Caldwell, R. B., and Caldwell, R. W. (2013) Akita spontaneously type 1 diabetic mice exhibit elevated vascular arginase and impaired vascular endothelial and nitrergic function. *PLoS One* **8**, e72277
284. Oyadomari, S., Koizumi, A., Takeda, K., Gotoh, T., Akira, S., Araki, E., and Mori, M. (2002) Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J Clin Invest* **109**, 525-532
285. Tovey, S. C., and Taylor, C. W. (2013) High-throughput functional assays of IP3-evoked Ca2+ release. *Cold Spring Harb Protoc* **2013**, 930-937
286. Fulda, S., Gorman, A. M., Hori, O., and Samali, A. (2010) Cellular stress responses: cell survival and cell death. *Int J Cell Biol* **2010**, 214074
287. Tang, Y., Tian, X., Wang, R., Fill, M., and Chen, S. R. (2012) Abnormal termination of Ca2+ release is a common defect of RyR2 mutations associated with cardiomyopathies. *Circ Res* **110**, 968-977
288. Hotamisligil, G. S. (2010) Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell* **140**, 900-917
289. Marciniak, S. J., and Ron, D. (2006) Endoplasmic reticulum stress signaling in disease. *Physiol Rev* **86**, 1133-1149
290. Papa, F. R. (2012) Endoplasmic reticulum stress, pancreatic beta-cell degeneration, and diabetes. *Cold Spring Harb Perspect Med* **2**, a007666
291. Mitchell, K. J., Pinton, P., Varadi, A., Tacchetti, C., Ainscow, E. K., Pozzan, T., Rizzuto, R., and Rutter, G. A. (2001) Dense core secretory vesicles revealed as a dynamic Ca(2+) store in neuroendocrine cells with a vesicle-associated membrane protein aequorin chimaera. *J Cell Biol* **155**, 41-51
292. Ferreiro, E., Resende, R., Costa, R., Oliveira, C. R., and Pereira, C. M. (2006) An endoplasmic-reticulum-specific apoptotic pathway is involved in prion and amyloid-beta peptides neurotoxicity. *Neurobiol Dis* **23**, 669-678
293. Dixit, S. S., Wang, T., Manzano, E. J., Yoo, S., Lee, J., Chiang, D. Y., Ryan, N., Respress, J. L., Yechoor, V. K., and Wehrens, X. H. (2013) Effects of CaMKII-mediated phosphorylation of ryanodine receptor type 2 on islet calcium handling, insulin secretion, and glucose tolerance. *PLoS One* **8**, e58655
294. Marmugi, A., Parnis, J., Chen, X., Carmichael, L., Hardy, J., Mannan, N., Marchetti, P., Piemonti, L., Bosco, D., Johnson, P., Shapiro, J. A., Cruciani-Guglielmacci, C., Magnan, C., Ibberson, M., Thorens, B., Valdivia, H. H., Rutter, G. A., and Leclerc, I. (2016) Sorcin Links Pancreatic beta-Cell Lipotoxicity to ER Ca2+ Stores. *Diabetes* **65**, 1009-1021
295. Belal, C., Ameli, N. J., El Kommos, A., Bezalel, S., Al'Khafaji, A. M., Mughal, M. R., Mattson, M. P., Kyriazis, G. A., Tyrberg, B., and Chan, S. L. (2012) The homocysteine-inducible endoplasmic reticulum (ER) stress protein Herp counteracts mutant alpha-synuclein-induced ER stress via the homeostatic regulation of ER-resident calcium release channel proteins. *Hum Mol Genet* **21**, 963-977
296. Zalk, R., Lehnart, S. E., and Marks, A. R. (2007) Modulation of the ryanodine receptor and intracellular calcium. *Annu Rev Biochem* **76**, 367-385
297. Clapham, D. E. (2007) Calcium signaling. *Cell* **131**, 1047-1058

298. Wehrens, X. H., Lehnart, S. E., Reiken, S., Vest, J. A., Wronska, A., and Marks, A. R. (2006) Ryanodine receptor/calcium release channel PKA phosphorylation: a critical mediator of heart failure progression. *Proc Natl Acad Sci U S A* **103**, 511-518
299. Andersson, D. C., Meli, A. C., Reiken, S., Betzenhauser, M. J., Umanskaya, A., Shiomi, T., D'Armiento, J., and Marks, A. R. (2012) Leaky ryanodine receptors in beta-sarcoglycan deficient mice: a potential common defect in muscular dystrophy. *Skelet Muscle* **2**, 9
300. Shan, J., Kushnir, A., Betzenhauser, M. J., Reiken, S., Li, J., Lehnart, S. E., Lindegger, N., Mongillo, M., Mohler, P. J., and Marks, A. R. (2010) Phosphorylation of the ryanodine receptor mediates the cardiac fight or flight response in mice. *J Clin Invest* **120**, 4388-4398
301. Dyachok, O., and Gylfe, E. (2004) Ca(2+)-induced Ca(2+) release via inositol 1,4,5-trisphosphate receptors is amplified by protein kinase A and triggers exocytosis in pancreatic beta-cells. *J Biol Chem* **279**, 45455-45461
302. Vanderheyden, V., Devogelaere, B., Missiaen, L., De Smedt, H., Bultynck, G., and Parys, J. B. (2009) Regulation of inositol 1,4,5-trisphosphate-induced Ca2+ release by reversible phosphorylation and dephosphorylation. *Biochim Biophys Acta* **1793**, 959-970
303. Brissova, M., Fowler, M. J., Nicholson, W. E., Chu, A., Hirshberg, B., Harlan, D. M., and Powers, A. C. (2005) Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. *J Histochem Cytochem* **53**, 1087-1097
304. Steinbusch, L., Labouebe, G., and Thorens, B. (2015) Brain glucose sensing in homeostatic and hedonic regulation. *Trends Endocrinol Metab* **26**, 455-466
305. O'Brien, B. A., Huang, Y., Geng, X., Dutz, J. P., and Finegood, D. T. (2002) Phagocytosis of apoptotic cells by macrophages from NOD mice is reduced. *Diabetes* **51**, 2481-2488
306. Marselli, L., Thorne, J., Ahn, Y. B., Omer, A., Sgroi, D. C., Libermann, T., Otu, H. H., Sharma, A., Bonner-Weir, S., and Weir, G. C. (2008) Gene expression of purified beta-cell tissue obtained from human pancreas with laser capture microdissection. *J Clin Endocrinol Metab* **93**, 1046-1053
307. Suzuki, J., Kanemaru, K., Ishii, K., Ohkura, M., Okubo, Y., and Iino, M. (2014) Imaging intraorganellar Ca2+ at subcellular resolution using CEPIA. *Nat Commun* **5**, 4153
308. Okumoto, S., Jones, A., and Frommer, W. B. (2012) Quantitative imaging with fluorescent biosensors. *Annu Rev Plant Biol* **63**, 663-706
309. Kamocka, M. M., Mu, J., Liu, X., Chen, N., Zollman, A., Sturonas-Brown, B., Dunn, K., Xu, Z., Chen, D. Z., Alber, M. S., and Rosen, E. D. (2010) Two-photon intravital imaging of thrombus development. *J Biomed Opt* **15**, 016020
310. Karatas, H., Erdener, S. E., Gursoy-Ozdemir, Y., Gurer, G., Soylemezoglu, F., Dunn, A. K., and Dalkara, T. (2011) Thrombotic distal middle cerebral artery occlusion produced by topical FeCl(3) application: a novel model suitable for intravital microscopy and thrombolysis studies. *J Cereb Blood Flow Metab* **31**, 1452-1460
311. Stull, N. D., Breite, A., McCarthy, R., Tersey, S. A., and Mirmira, R. G. (2012) Mouse islet of Langerhans isolation using a combination of purified collagenase and neutral protease. *J Vis Exp*
312. Evans-Molina, C., Robbins, R. D., Kono, T., Tersey, S. A., Vestermark, G. L., Nunemaker, C. S., Garmey, J. C., Deering, T. G., Keller, S. R., Maier, B., and Mirmira, R. G. (2009) Peroxisome proliferator-activated receptor gamma

activation restores islet function in diabetic mice through reduction of endoplasmic reticulum stress and maintenance of euchromatin structure. *Mol Cell Biol* **29**, 2053-2067

313. Ogihara, T., Chuang, J. C., Vestermark, G. L., Garmey, J. C., Ketchum, R. J., Huang, X., Brayman, K. L., Thorner, M. O., Repa, J. J., Mirmira, R. G., and Evans-Molina, C. (2010) Liver X receptor agonists augment human islet function through activation of anaplerotic pathways and glycerolipid/free fatty acid cycling. *J Biol Chem* **285**, 5392-5404
314. Johnson, J. S., Kono, T., Tong, X., Yamamoto, W. R., Zarain-Herzberg, A., Merrins, M. J., Satin, L. S., Gilon, P., and Evans-Molina, C. (2014) Pancreatic and duodenal homeobox protein 1 (Pdx-1) maintains endoplasmic reticulum calcium levels through transcriptional regulation of sarco-endoplasmic reticulum calcium ATPase 2b (SERCA2b) in the islet beta cell. *J Biol Chem* **289**, 32798-32810
315. Tong, X., Kono, T., and Evans-Molina, C. (2015) Nitric oxide stress and activation of AMP-activated protein kinase impair beta-cell sarcoendoplasmic reticulum calcium ATPase 2b activity and protein stability. *Cell Death Dis* **6**, e1790
316. Hohmeier, H. E., Mulder, H., Chen, G., Henkel-Rieger, R., Prentki, M., and Newgard, C. B. (2000) Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes* **49**, 424-430

CURRICULUM VITAE

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EDUCATION

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WORK EXPERIENCES

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May 2013 – August 2017, Graduate Research Assistant

- Using Ca^{2+} imaging and biochemical assays, studying roles of ER Ca^{2+} releasing channels during the progression to diabetes.
- Establishing techniques to do *in vivo* Ca^{2+} imaging on mouse pancreatic β cell.

Indiana University Bloomington, Kyung-Tai Min lab / Department of Biology

Sept 2007 – June 2009, Junior Technician

- Supported the projects to study (1) synaptic dysfuntion in Down syndrome using *Drosophila*, (2) mitochondrial dynamics in neurons.
- Maintained over 500 transgenic *Drosophila* lines.

LANGUAGES

MATLAB, Python.

PUBLICATIONS

Yamamoto, WR., Bone, RN., and Evans-Molina, C. ER Calcium Transporters Are the Key Determinants for the Loss of Calcium Oscillation in Pancreatic β-Cell under Diabetic Stress. In preparation

Tong, X., Kono, T., Anderson-Baucum, EK., Yamamoto, W., Gilon, P., Lebeche, D., Day, RN., Shull, GE., and Evans-Molina, C. (2016) SERCA2 Deficiency Impairs Pancreatic β-Cell Function in Response to Diet-Induced Obesity. *Diabetes* **65**, 3039-3052

Johnson, JS., Kono, T., Tong, X., Yamamoto, WR., Zarain-Herzberg, A., Merrins, MJ., Satin, LS., Gilon, P., and Evans-Molina, C. (2014) Pancreatic and duodenal homeobox protein 1 (Pdx-1) maintains endoplasmic reticulum calcium levels through transcriptional regulation of sarco-endoplasmic reticulum calcium ATPase 2b (SERCA2b) in the islet β cell. *J Biological Chemistry* **289,** 32798-32810

Kono, TM., Sims, EK., Moss, DR., Yamamoto, W., Ahn, G., Diamond, J., Tong, X., Day, KH., Territo, PR., Hanenberg, H., Traktuev, DO., March, KL., and Evans-Molina, C. (2014) Systemic administration of human adipose derived stromal/stem cells protects against streptozotocin-induced hyperglycemia. *Stem Cells* **32,** 1831-1842

REPRESENTATIVE CONFERENCE PRESENTATIONS

Yamamoto, W., Kono, T., Tong, X., and Evans-Molina, C. Stress Induced β cell-ER Calcium Depletion is Mediated Via Calcium Leak From the Ryanodine Receptor. *The Midwest Islet Club*, Indianapolis, IN. May 18, 2016

Yamamoto, W., Kono, T., Tong, X., and Evans-Molina, C. ER Stress Induced Ryanodine Receptor Dysfunction in the Pancreatic Beta Cell. *The Advances & Breakthroughs in Calcium Signaling*, Honolulu, HI. April 8, 2016

Yamamoto, W., Kono, T., Johnson, J., Tong, X., Day, R., and Evans-Molina, C. SERCA2b Overexpression Improves Beta Cell Survival Under Inflammatory and ER Stress Conditions. *The Midwest Islet Club*, Birmingham, AL. May 22, 2014

ACTIVITY

Member of Society for Neuroscience (2012- 2013, 2015 - present).

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