Glucose-Dependent Insulin Secretion in Pancreatic β-Cell Islets from Male Rats Requires Ca\(^{2+}\) Release via ROS-Stimulated Ryanodine Receptors

Paola Llanos\(^{1,2}\)*, Ariel Contreras-Ferrat\(^{1,2}\), Genaro Barrientos\(^{3}\), Marco Valencia\(^{2}\), David Mears\(^{2,4,5}\), Cecilia Hidalgo\(^{2,3,5}\)*

1 Institute for Research in Dental Sciences, Facultad de Odontología, Universidad de Chile, Santiago, Chile, 2 Center of Molecular Studies of the Cell, Facultad de Medicina, Universidad de Chile, Santiago, Chile, 3 Physiology and Biophysics Program, Facultad de Medicina, Universidad de Chile, Santiago, Chile, 4 Human Genetics Program, Institute of Biomedical Sciences, Facultad de Medicina, Universidad de Chile, Santiago, Chile, 5 Biomedical Neuroscience Institute, Facultad de Medicina, Universidad de Chile, Santiago, Chile

* pllanos@med.uchile.cl (PL); chidalgo@med.uchile.cl (CH)

Abstract

Glucose-stimulated insulin secretion (GSIS) from pancreatic β-cells requires an increase in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)])). Glucose uptake into β-cells promotes Ca\(^{2+}\) influx and reactive oxygen species (ROS) generation. In other cell types, Ca\(^{2+}\) and ROS jointly induce Ca\(^{2+}\) release mediated by ryanodine receptor (RyR) channels. Therefore, we explored here if RyR-mediated Ca\(^{2+}\) release contributes to GSIS in β-cell islets isolated from male rats. Stimulatory glucose increased islet insulin secretion, and promoted ROS generation in islets and dissociated β-cells. Conventional PCR assays and immunostaining confirmed that β-cells express RyR2, the cardiac RyR isoform. Extended incubation of β-cell islets with inhibitory ryanodine suppressed GSIS; so did the antioxidant N-acetyl cysteine (NAC), which also decreased insulin secretion induced by glucose plus caffeine. Inhibitory ryanodine or NAC did not affect insulin secretion induced by glucose plus carbachol, which engages inositol 1,4,5-trisphosphate receptors. Incubation of islets with H\(_2\)O\(_2\) in basal glucose increased insulin secretion 2-fold. Inhibitory ryanodine significantly decreased H\(_2\)O\(_2\)-stimulated insulin secretion and prevented the 4.5-fold increase of cytoplasmic [Ca\(^{2+}\)] produced by incubation of dissociated β-cells with H\(_2\)O\(_2\). Addition of stimulatory glucose or H\(_2\)O\(_2\) (in basal glucose) to β-cells disaggregated from islets increased RyR2 S-glutathionylation to similar levels, measured by a proximity ligation assay; in contrast, NAC significantly reduced the RyR2 S-glutathionylation increase produced by stimulatory glucose. We propose that RyR2-mediated Ca\(^{2+}\) release, induced by the concomitant increases in [Ca\(^{2+}\)] and ROS produced by stimulatory glucose, is an essential step in GSIS.
Introduction

In the electrically excitable pancreatic β-cells, an increase in intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) is the primary trigger for glucose-stimulated insulin secretion (GSIS) [1]. Current models propose that GSIS entails a sequence of events initiated by glucose uptake into β-cells via a low affinity glucose transporter (GLUT-2). The ensuing accelerated metabolism of intracellular glucose increases the cytoplasmic ATP/ADP ratio [2, 3], which sequentially causes plasma membrane depolarization through closure of ATP-sensitive K⁺ channels and Ca²⁺ influx through voltage-dependent Ca²⁺ channels; the resulting increase in [Ca²⁺]ᵢ promotes insulin secretion [4].

Previous studies have reported that Ca²⁺ release from intracellular stores contributes to the [Ca²⁺]ᵢ rise induced by glucose in β-cells [5]. Both, the inositol 1,4,5-trisphosphate (InsP₃) receptor [6] and the ryanodine receptor (RyR) [7] channels mediate Ca²⁺ release from sarco/endoplasmic reticulum (SR/ER); these two channel types are present in pancreatic β-cells [8, 9]. The role of InsP₃ receptor-mediated Ca²⁺ release in muscarinic receptor-stimulated insulin secretion is well-established [10]. In addition, β-cells undergo Ca²⁺-induced Ca²⁺ release (CICR) in response to Ca²⁺ entry through plasma membrane L-type Ca²⁺channels [11–13]. Yet, the contribution of CICR and the specific role of RyR-mediated CICR in GSIS remain undefined.

Mammalian cells express three RyR isoforms (RyR1, RyR2, RyR3) that display ~70% identity and are encoded by three different genes [7]. Physiological ions and endogenous molecules (Ca²⁺, ATP, Mg²⁺ and cyclic ADP-ribose), pharmacological agents, phosphorylation and oxidation reactions modulate RyR channel activity [7]. Studies addressing the participation of RyR-mediated Ca²⁺ release in GSIS have not provided conclusive evidence. Membrane fractions isolated from INS-1 and RINmF5 β-cell lines [14] or from MIN6 β-cells [15] contain all three RyR isoforms; the RyR2 isoform has the highest expression level, although it is expressed at much lower density than in primary hippocampal neurons [14] or muscle cells [15]. Of note, RyR agonists elicit Ca²⁺ release from microsomes isolated from islets [16], or from ER isolated from β-cells [16, 17]. By mediating CICR via PKA-independent signaling mechanisms, in INS-1 rat insulinoma cells RyR channels may contribute to the potentiation of GSIS produced by the hormone glucagon-like peptide 1 (GLP-1) [18]. Other reports have suggested RyR involvement in the [Ca²⁺]ᵢ increase produced by glucose or agonists in pancreatic β-cells [14, 16, 17, 19, 20]. Moreover, treatment of the mouse insulinoma cell line MIN6 with inhibitory ryanodine (µM range) decreases GSIS [15]. In contrast, other studies have reported that incubation with inhibitory ryanodine does not prevent insulin secretion in human islets [21] or in the INS-1 rat insulinoma cell line [22]. These conflicting results justify further studies into the role of RyR-mediated Ca²⁺ release on GSIS.

In addition to increasing [Ca²⁺]ᵢ, glucose stimulates by different cellular pathways the generation of reactive oxygen species (ROS) in β-cells [23]; increased cellular ROS levels regulate physiological [24] and pathophysiological processes [23]. In MIN6 cells, elevated glucose levels and sulfonylureas, which stimulate depolarization by inhibition of ATP-sensitive K⁺ channels, seem to enhance ROS production through NADPH oxidase (NOX) activation [25]. Most studies describing the effects of ROS in β-cells have focused on their deleterious actions when present in excess [26]. Yet, ROS act as intracellular signals for insulin secretion when present at physiological levels [24]. Glucose oxidation under physiological conditions results in hydrogen peroxide (H₂O₂) and hydroxyl radical generation [27]. Of note, treatment of rat islets kept at basal glucose concentrations with hydrogen peroxide or alloxan, a molecule which acutely increases intracellular H₂O₂ levels, causes a rapid elevation of [Ca²⁺]ᵢ, and produces a transient increase in insulin release [28, 29].
In other cell types, ROS stimulate RyR-mediated CICR [30]. Given the proposed role of ROS as physiological signals in GSIS [24, 31], plus the redox-sensitivity of RyR-mediated CICR, we hypothesized that glucose, by inducing an initial [Ca^{2+}] increase due to Ca^{2+} entry and increasing cellular ROS levels, promotes RyR-mediated CICR via RyR redox modifications; the resulting amplification of Ca^{2+} entry signals would promote GSIS. Our results support this hypothesis, since a stimulatory glucose concentration generated ROS that increased RyR S-glutathionylation, while RyR inhibition or the antioxidant N-acetyl cysteine (NAC) significantly decreased or abolished GSIS. The main findings of this work were previously presented in abstract form (Biological Research 2009, 42 (Supplement A), R-115).

Materials and Methods

Reagents

All reagents used were of analytical grade. Caffeine, NAC, polylysine, RPMI 1640 culture medium and carbamylcholine chloride (carbachol, CCh) were from Sigma-Aldrich Chemical (St Louis, MO). Fura-2 acetoxyethyl ester (fura-2-AM), Fluo-4 acetoxyethyl ester (Fluo-4-AM), 5-(6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate acetyl ester (CM-H2DCFDA), Dispase-EDTA, Dulbecco modified Eagle’s medium, BODIPY-FL-X Ryanodine (BODIPY-Rya) and Calcium Calibration Kit 1 with Magnesium were from Invitrogen (Eugene, OR). Ryanodine was from Alexis Biochemical (Farmingdale, NY), and H2O2 from Merck (Whitehouse Station, NJ). The Duolink II red starter kit was from Olink-Bioscience (Uppsala, Sweden).

Antibodies

Anti-insulin antibodies were from Dako (Carpinteria, CA), anti-RyR2 from Affinity BioReagents (Golden, CO) or Millipore Corp. (Billerica, MA) and anti-calnexin from Sigma (St Louis, MO). The secondary antibodies used were anti-guinea pig FITC from Jackson Immuno Research (West Grove, PA). Alexa Fluor 635 anti-mouse IgG, and Alexa Fluor 635 anti-rabbit IgG, were both from Invitrogen (Eugene, OR). Antibodies against S-glutathionylated protein adducts were from Virogen Corp. (Watertown, MA).

Animals

Male Sprague-Dawley rats weighing 250–300 g (45–60 days old) were obtained from the Central Animal Facility of the Faculty of Medicine, Universidad de Chile. The animals were kept at 23°C under a 12 h light–dark cycle, with free access to food and water. The Bioethics Committee for Animal Research, Faculty of Medicine, Universidad de Chile, approved all experimental protocols used in this work.

Rat Pancreatic Islet Isolation

The pancreas extracted from male rats was digested with collagenase to isolate the islets of Langerhans as previously described [32]. Islets were picked by hand under a dissecting microscope, rinsed three times in Hanks solution and cultured overnight in an incubator under 95% O2/5% CO2. The RPMI 1640 culture medium was supplemented with 5 mM glucose, 10% fetal bovine serum (FBS), 10 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin. Cell viability was evaluated as described in detail elsewhere [33].

Isolation of Pancreatic β-Cells

For studies on single cells, islets were dispersed into cell suspensions by digestion with dispase-EDTA. The digested suspension was subsequently trituted by passage through a fire-polished
Pasteur pipette. Cells suspended in RPMI 1640 containing 10% FBS and 5 mM glucose were plated onto glass coverslips coated with 40 μg/ml polylysine to facilitate cell adherence.

MIN6 Cell Culture
The mouse insulinoma MIN6 pancreatic β-cell line [34], kindly provided by Dr. Paolo Meda (Geneva, Switzerland), was maintained at 37°C in Dulbecco’s modified Eagle’s medium containing 15% FBS, 100 mU/ml penicillin, 100 mg/ml streptomycin, 11 mM glucose, in an atmosphere of 95% O₂/5% CO₂.

Insulin Secretion
All determinations of insulin secretion were performed in pancreatic islets under static incubation. Briefly, 15 islets of 150–200 μm diameter kept in 24-well plates were pre-incubated for 1 h at 37°C in Krebs–Ringer bicarbonate (KRB) buffer containing (in mM): 120 NaCl, 5 KCl, 1 MgCl₂, 2.5 CaCl₂, 25 NaHCO₃ (equilibrated with 5% CO₂–95% O₂, pH 7.4), 0.5% bovine serum albumin, 2.8 mM glucose. The pre-incubation medium was then replaced with KRB buffer supplemented with different glucose concentrations (basal: 2.8 mM; stimulatory: 16.7 or 27.7 mM) as well as other test agents. After incubation for 1 h at 37°C, the supernatant was collected and stored at -80°C for later analysis of insulin content by ELISA (Mercodia Rat Insulin ELISA, Sweden).

Evaluation of ROS Production in β-Cells and Pancreatic Islets
The commercial probe CM-H₂DCFDA was used to evaluate intracellular ROS generation. Cells or islets were placed on glass coverslips and cultured overnight in RPMI 1640 containing 10% FBS and 5 mM glucose. The coverslips were then washed with Hank’s buffer solution (HBSS; in mM: 125 NaCl, 5 KCl, 0.3 Na₂HPO₄, 0.4 KH₂PO₄, 5.5 glucose, 10 HEPES-Na; pH 7.4), and incubated for 1 h at 37°C with KRB buffer containing 2.8 mM glucose, 16.7 mM glucose or 2.8 mM glucose plus 100 μM H₂O₂. Cells were loaded next with 10 μM CM-H₂DCFDA and after 60 min digital fluorescence images were obtained in a confocal microscope (Pascal 5, Zeiss, Germany), using an excitation wavelength of 488 nm and a 515 nm long pass emission filter.

[Ca²⁺]i Measurements
Isolated β-cells were maintained on glass coverslips overnight prior to each experiment. Cells were loaded with the Ca²⁺-sensitive dye fura-2 AM (2 μM with 0.02% Pluronic acid in HBSS) by incubation for 45 min at 37°C. To test the effects of H₂O₂, cells were incubated for 1 h with 100 μM H₂O₂ and then loaded with fura-2 AM for 30 min. All fluorescence determinations were performed at room temperature. Dual wavelength excitation microspectrofluorimetry was performed ratiometrically at 1-s intervals using a digital video imaging system (Ionwizard 4.4; IonOptix Corp., Milton, MA, USA). Calibration of raw fluorescence values was performed using fura-2 pentapotassium salt dissolved in calibration buffer solutions (Calcium Calibration Kit 1 with Magnesium). Solutions containing H₂O₂ were prepared each time just prior to use.

To evaluate ER Ca²⁺ content, we inhibited the SERCA pump by adding thapsigargin in Ca²⁺-free solution, and monitored with Fluo-4 (Kₐ = 345 nM) the cytoplasmic Ca²⁺ signals arising from the ensuing net Ca²⁺ efflux from the ER. To this purpose, isolated β-cells were pre-incubated for 30 min at 37°C with 5 μM Fluo-4-AM (with 0.02% Pluronic acid in HBSS). After washing isolated β-cells for 10 min in modified HBBS solution to allow complete dye de-esterification, cultures were transferred to Ca²⁺-free medium just prior to fluorescence recording.
Fluorescence images of cytoplasmic Ca^{2+} signals were obtained at 1-s intervals with an inverted confocal microscope (Carl Zeiss, Axiovert 200, LSM 5 Pascal, Oberkochen, Germany, Plan Apochromatic 63x Oil DIC objective, optical slice 1000 μm, excitation 488 nm, argon laser beam). Image data were acquired from different regions of optical interest (ROI) defined with the same area and located in the cell bodies, excluding the nucleus; frame scans were averaged using the equipment data acquisition program. All experiments were done at room temperature (20–22°C).

### Binding of BODIPY FL-X Ryanodine

Binding of BODIPY FL-X ryanodine to pancreatic islets was evaluated by confocal microscopy. The islets were loaded with 50 μM BODIPY FL-X ryanodine for 1 or 12 h at 37°C and then washed with KRB three times and maintained in this solution. Digital images of BODIPY FL-X fluorescence were acquired in a confocal microscope (Pascal 5, Zeiss, Germany) using an excitation wavelength of 488 nm and a 515 nm long-pass emission filter.

### Immunofluorescence Staining

Pancreatic β-cells or MIN6 cells grown on coverslips were fixed in phosphate-buffered saline (PBS; in mM: 137 NaCl, 2.7 KCl, 8 Na_{2}HPO_{4}, 1.46 KH_{2}PO_{4}; pH 7.4) containing 3% formaldehyde at room temperature for 15 min. Cells were treated next with 0.25% Triton X-100 in PBS for an additional 15 min, and incubated with anti-insulin, anti-RyR2 or anti-calnexin antibodies. Anti-guinea pig FITC, Alexa Fluor 635 anti-mouse IgG or Alexa Fluor 635 anti-rabbit IgG were used as secondary antibodies. Nuclei were stained with Hoechst as described elsewhere [35]. The cross sections of pancreatic tissue were 5 μm thick.

### In situ Proximity Ligation Assay (PLA)

To detect RyR2 S-glutathionylation in situ, we used a proximity ligation assay (Duolink II red starter kit) according to the manufacturer instructions, plus primary antibodies against RyR2 (Millipore Corp.) and S-glutathionylated protein adducts. Briefly, β-cells disaggregated from islets and incubated 24 h in RPMI 1640 culture medium containing 10% FBS and 5 mM glucose, were incubated overnight at 4°C in a humid chamber with the above primary antibodies. Cells were incubated next for 1 h at 37°C with Duolink, plus and minus secondary antibodies; these secondary antibodies contain oligonucleotides that in Duolink Ligation Solution form a closed circle when in close proximity (optimal resolution, 30–40 nm). Circle formation was detected by subsequent addition of polymerase to amplify the closed circles, which were detected next with the complementary oligonucleotides, fluorescently labeled, provided in the Duolink kit. Fluorescence images were acquired in a confocal microscope as described above. After incubation with the PLA probes, β-cells were identified by immunofluorescence with insulin antibodies.

**Statistical analysis**—Data are expressed as Mean ± SEM. One-way ANOVA followed by Tukey’s multiple comparison test was used to compare groups. A p-value ≤ 0.05 was considered significant.

### Results

**Pancreatic Islet β-cells Express the RyR2 Isoform**

Previous reports indicate that β-cell lines express the three mammalian RyR isoforms [14, 15], plus a newly described RyR isoform [36]. By immunohistochemical analysis, we detected the presence of the cardiac RyR2 isoform in rat endocrine pancreas. In cross sections of pancreatic...
tissue, RyR2 fluorescent label was present in islets (endocrine pancreas) and pancreatic acini (exocrine pancreas) (Fig 1A). Within the islets, the RyR2 signal co-localized with insulin, a specific marker of pancreatic β-cells. In disaggregated islets, immunostaining for RyR2 was apparent in both insulin-positive and insulin-negative cells (Fig 1B). The RyR2 signal in β-cells, which have a highly developed ER typical of secretory cells, was strongest in the cell periphery near the plasma membrane. By immunocytochemical analysis, we also detected RyR2 in the mouse pancreatic β-cell line MIN6 and in pancreatic β-cells dissociated from islets. In both cell types, the RyR2 signal co-localized with calnexin (S1 Fig), a well known ER marker [37]. Immunoblot analysis of MIN6 cell homogenates revealed a distinct band corresponding to RyR2 (S2 Fig). In contrast, we did not detect a band corresponding to RyR2 in immunoblots of islet homogenates. Presumably, RyR2 density in whole islet homogenates is too low for detection by this technique; this feature would explain why there are no reports in the literature describing the presence of RyR2 in islets by immunoblot analysis. Taken together, these results confirm that pancreatic β-cells express the RyR2 protein isoform, which seems to be the predominant RyR isoform present in β-cells [9, 14]. We did not examine the presence of other RyR isoforms. Additionally, semi-quantitative RT-PCR analysis showed that rat pancreatic islets expressed RyR2 mRNA (S2 Fig), confirming previous findings [16, 17, 38].

Equilibration of a Fluorescent Ryanodine Analog in Pancreatic β-Cell Islets

Ryanodine is a plant alkaloid that acts as a RyR channel agonist at nM concentrations but is a potent and highly selective channel inhibitor at μM concentrations. Because of these distinctive actions and its high degree of specificity (to date no other cellular targets have been reported), ryanodine is widely considered the “gold standard” to test RyR channel function and is often
used to functionally identify RyR channels [7]. Ryanodine is membrane permeable, so within
cells it targets ER-resident RyR channels where it binds preferentially to RyR channels in the
open state. Hence, effective inhibition of RyR channels present in complex systems, such as the
pancreatic β-cell islets, is likely to require both high concentrations of ryanodine and long incu-
bation times to ensure access of inhibitory ryanodine concentrations to all cells within the islet.
To test if incubation time affected the distribution of ryanodine, rat islets were incubated for 1
h or 12 h with BODIPY-ryanodine, a permeable and fluorescent ryanodine analog. BODIPY-
ryanodine showed a relatively homogeneous distribution throughout the islet after prolonged
incubation (12 h; S3B Fig); in contrast, after 1 h of incubation the fluorescent probe was found
only in cells present at the periphery of the islet (S3A Fig). Accordingly, we tested below the in-
hibitory effects of ryanodine on GSIS after incubating islets for 12 h with this plant alkaloid. As
detailed below, this long incubation period with inhibitory ryanodine did not prevent insulin
secretion in response to carbachol plus stimulatory glucose concentration.

**Glucose-Stimulated Insulin Secretion Requires Functional RyR**

Stimulatory glucose (16.7 mM) increased insulin secretion rate (μg/l h⁻¹) from an average basal
value of 4.7 ± 0.7 to a value of 12.6 ± 2.1 (Fig 2A, left panel). Incubation with inhibitory ryan-
odine for 12 h decreased GSIS rate to 5.6 ± 1.6 (μg/l h⁻¹), a value not significantly different to the
average basal level determined in the absence of ryanodine. After 12 h incubation with ryano-
dine, the average insulin secretion rate in basal glucose (2.8 mM) was 1.7 ± 1.0 (μg/l h⁻¹) (Fig
2A, left panel), not significantly different from the average basal value. In agreement with the
lack of penetration of BODIPY-ryanodine into the islet after 1 h, pre-incubation with inhibito-
ry ryanodine for 1 h did not affect insulin secretion from islets incubated with basal (2.8 mM)
or stimulatory (16.7 mM) glucose compared to controls (Fig 2A, right panel).

To test if islets remained functional and with the ER loaded with Ca²⁺ after prolonged incu-
bation (12 h) with 200 μM ryanodine, we treated islets with 30 μM carbachol to stimulate insu-
lin secretion. Previous reports have established that carbachol, a pharmacological agonist of
muscarinic receptors, stimulates insulin secretion from pancreatic β-cells in a strictly glucose-
dependent manner, through a pathway that engages Ca²⁺ release mediated by InsP3 receptors
[39, 40]. As expected, carbachol did not stimulate insulin secretion when added at basal glucose
concentration, but at stimulatory glucose concentration it significantly increased insulin secre-
tion, from 13.7 ± 1.6 to 38.9 ± 16.7 (μg/l h⁻¹) (Fig 2B). Joint addition of glucose and carbachol
to islets pre-incubated 12 h with inhibitory ryanodine produced insulin secretion rates of
37.5 ± 6.9 (μg/l h⁻¹). These values are not significantly different to those produced by carbachol
plus glucose in the absence of ryanodine, indicating that inhibitory ryanodine did not affect
carbachol-mediated pathways. In addition, by using thapsigargin to inhibit the SERCA pump
in Ca²⁺-free solution, and thus promote net Ca²⁺ efflux from the ER, we tested directly if pro-
longed incubation with inhibitory ryanodine promoted ER depletion. Both control and ryano-
dine-treated isolated β-cells exhibited similar Ca²⁺ signals in response to thapsigargin addition
(S4 Fig), strongly suggesting that ryanodine-treated β-cells had similar ER Ca²⁺ contents as
control cells, even after overnight incubation with 200 μM ryanodine. Moreover, ryanodine-
treated islets displayed similar ROS levels as controls (S4 Fig), indicating that RyR inhibition
did not modify basal ROS production.

**Glucose Stimulates ROS Production in Isolated Islets and Single
Pancreatic β-Cells**

In islets and single β-cells loaded with the ROS-sensitive probe CM-H₂DCF, stimulatory glu-
cose (16.7 mM) increased probe fluorescence 1.3 fold and 2.5-fold, respectively, relative to the
Fig 2. Overnight incubation of pancreatic islets with 200 μM ryanodine inhibits insulin secretion stimulated by glucose but not by glucose plus carbachol. Insulin secretion was determined in groups of 15 islets after incubation for 1 h at 37°C in basal (2.8 mM) or stimulatory glucose (16.7 mM). (A, left) Rya ON: islets were pre-incubated with 200 μM ryanodine for 12 h before determination of insulin secretion after 1 h incubation in ryanodine-free solutions. (A, right) Rya 1 h: islets were pre-incubated with 100 μM ryanodine for 1 h before determination of insulin secretion after 1 h incubation in ryanodine-free solutions; G: glucose. (B) CCh: 30 μM carbachol was added during the 1 h incubation period used to measure insulin secretion. All data represent Mean ± SEM; N = 3 experiments (each condition in triplicate). Statistical significance was determined with one-way ANOVA followed by Tukey multiple comparison test. *: p < 0.05; **: p < 0.01; ***p < 0.001.

doi:10.1371/journal.pone.0129238.g002
basal condition (Fig 3). These results confirm previous reports that glucose increases ROS generation in islets and β-cells [24]. Incubation with H₂O₂ for 1 h of islets or β-cells maintained in basal glucose concentration (2.8 mM) also increased probe fluorescence, 1.4 fold in islets and 2.8-fold in cells relative to the basal condition, indicating that H₂O₂ addition in basal glucose produces a comparable increase in probe fluorescence as that produced by stimulatory glucose.

N-Acetyl Cysteine Suppresses GSIS and Inhibits Insulin Secretion Stimulated by Glucose and Caffeine

Pre-incubation with the antioxidant NAC for 1 h did not affect basal insulin secretion but fully inhibited GSIS, which decreased from 14.6 ± 2.1 to 5.5 ± 1 (μg/l h⁻¹) (Fig 4A). Addition of 2.5 mM caffeine, which at this concentration acts primarily as a pharmacological RyR agonist [22], did not stimulate insulin secretion when measured at basal glucose levels (Fig 4B). As reported earlier [41], caffeine markedly stimulated insulin secretion, from 14.0 ± 1.3 to 90.6 ± 15.0 (μg/l h⁻¹) when tested at a stimulatory glucose concentration, whereas NAC significantly decreased insulin secretion jointly stimulated by glucose and caffeine (Fig 4B). In contrast, incubation with NAC did not affect insulin secretion jointly stimulated by carbachol plus 16.7 mM glucose (Fig 4C) or by 27.7 mM glucose (S5 Fig).

Exogenous H₂O₂ Has a Dual Effect on Insulin Secretion

Pre-incubation of pancreatic islets for 1 h with H₂O₂ added as an exogenous ROS source had a dual effect on insulin secretion. Under conditions of low glucose (2.8 mM), addition of H₂O₂ stimulated insulin secretion to a value of 11.7 ± 1.7 (μg/l h⁻¹); this value is nearly 2-fold higher than the basal value of 6.1 ± 0.9 (μg/l h⁻¹) (Fig 5A). Pre-incubation with 100 μM H₂O₂ for 1 h of islets kept in low glucose produced a modest decrease (13%) in cell viability. Under conditions of stimulatory glucose (16.7 mM) concentrations of H₂O₂ ≥ 100 μM significantly decreased insulin secretion (Fig 5B); these results are in agreement with a previous report showing that 200 μM H₂O₂ significantly decreased GSIS in islets [29]. Concentrations of H₂O₂ < 100 μM were ineffective either at basal or stimulatory glucose concentrations.

Insulin Secretion Induced by H₂O₂ at Basal Glucose Concentration Requires Functional RyR Channels

To test RyR participation in the enhancement of insulin secretion induced by H₂O₂ in basal glucose concentration, we incubated islets with inhibitory ryanodine for 12 h prior to H₂O₂ addition. In these conditions, addition of H₂O₂ in basal glucose did not stimulate insulin secretion (Fig 6). In contrast, as illustrated in Fig 6, H₂O₂ stimulated insulin secretion > 2-fold in islets kept in basal glucose and not treated with ryanodine, while islets incubated for 12 h with inhibitory ryanodine had comparable levels of insulin secretion (2.4 ± 0.2 μg/l h⁻¹) as islets kept in basal glucose (3.4 ± 0.7 μg/l h⁻¹).

RyR-Mediated Ca²⁺ Release Underlies the [Ca²⁺]i Increase Produced by H₂O₂

Addition of H₂O₂ stimulates RyR-mediated CICR in other cell types [30]. The results illustrated in Fig 6 led us to hypothesize that addition of H₂O₂ activates RyR-mediated Ca²⁺ release in pancreatic β-cells; the resulting increase in [Ca²⁺]i would cause the increase in insulin secretion induced by H₂O₂. To test this hypothesis, we measured [Ca²⁺]i with the fluorescent probe fura-2 (Fig 7A). Incubation for 1 h of disaggregated β-cells with H₂O₂ increased [Ca²⁺]i, from a basal level of 99.7 ± 21 nM to 455.2 ± 69.6 nM. Cells pre-incubated with inhibitory ryanodine for
12 h displayed an average value of $[\text{Ca}^{2+}]_i = 142.6 \pm 21.5$ nM, which did not change after addition of H$_2$O$_2$ (Fig 7A). As illustrated in Fig 7B, H$_2$O$_2$ addition to control cells increased $[\text{Ca}^{2+}]_i$ rapidly (within 10 s) to a value of 324 ± 5.4 nM (mean value, first minute after H$_2$O$_2$ addition, N = 3). This increase occurred as a consequence of RyR-mediated Ca$^{2+}$ release since overnight incubation with inhibitory ryanodine prevented the fast $[\text{Ca}^{2+}]_i$ increase produced by H$_2$O$_2$ (Fig 7C). Yet, these same cells did respond to subsequent addition of 90 mM KCl with a marked increase in $[\text{Ca}^{2+}]_i$ (Fig 7C). The observations that disaggregated β-cells incubated overnight with inhibitory ryanodine maintained $[\text{Ca}^{2+}]_i$, at resting levels, and responded to
KCl, show that Ca\(^{2+}\) homeostasis and depolarization-induced Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels remained largely unaffected by this treatment.

Glucose-Dependent ROS Production Increases S-glutathionylation of RyR Cysteine Residues

Previous studies have established that the RyR1 and RyR2 mammalian isoforms present reactive cysteines that readily undergo redox modifications, such as S-glutathionylation, which enhance RyR-mediated CICR [30]. To evaluate if glucose modified RyR2 S-glutathionylation levels, we used a novel proximity ligation assay (PLA) that generates a fluorescence signal if the targets lie within an optimal distance of 30–40 nm. In this particular case, the two targets were the RyR2 protein and S-glutathionylated protein adducts. Isolated β-cells stimulated for 1 h with 16.7 mM glucose displayed a significant increase in fluorescent dot density (Fig 8A), which increased from a basal value (in arbitrary units) of 37 ± 5 in 2.8 mM glucose, to 129 ± 14 in 16.7 mM glucose (Fig 8B). Incubation of cells with H\(_2\)O\(_2\) for 1 h induced a similar stimulation of fluorescence intensity (Fig 8A, third row), yielding a fluorescent dot density of 136 ± 15 (Fig 8B). Lastly, β-cells pre-incubated with NAC for 1 h and subsequently stimulated with glucose (16.7 mM) for 1 h displayed a significant reduction of fluorescent dot density (Fig 8A, fourth row), with values of 73 ± 14, dots per cell (Fig 8B). Images of these cells taken at different confocal planes are illustrated in S6 Fig. These results strongly suggest that glucose-induced ROS generation promotes S-glutathionylation of RyR2 cysteine residues, which decreases in cells pre-incubated with NAC.

Discussion

Previous reports have shown that pancreatic islets and β-cell lines express functional RyR channels [9] that give rise to nuclear Ca\(^{2+}\) signals [42]. To explore the presence of RyR channels in β-cells, previous studies have employed pharmacological tools [11], endogenous RyR ligands [16, 19, 43], detection of RyR mRNA levels [16, 17, 38] and protein [14, 15, 21, 44], or quantitative determinations of ryanodine binding using fluorescently-labeled ryanodine [38]. Here, we confirmed that β-cells dissociated from pancreatic rat islets and MIN6 pancreatic β-cells express the RyR2 isoform. In contrast to previous studies describing RyR2 localization in insulin secretory vesicles [45] and/or endosomes [21], we found RyR2 co-localized with the ER marker calnexin in both cell types.

A Role of RyR Channels in GSIS

In spite of the fact that β-cells express functional RyR channels, there is no consensus that RyR-mediated Ca\(^{2+}\) release plays a significant role in GSIS [9]. Previous studies have shown that RyR-mediated Ca\(^{2+}\) release determines cell viability in pancreatic islets [46] and mediates insulin secretion in INS-1E cells [22]. Additionally, RyR-mediated Ca\(^{2+}\) release mediates the activation of TRP-type channels, leading to subsequent depolarization of the plasma...
membrane [47]; RyR channels also mediate CICR in MIN6 pancreatic β-cells [12] and RyR-mediated Ca^{2+} release contributes to mitochondrial ATP synthesis via GLP-1 [48]. In agreement with previous studies [14, 22], we found that caffeine, which at the low concentrations used in this work acts primarily as a RyR agonist [22], increased GSIS but did not stimulate insulin secretion when added at basal glucose levels. Presumably, activation of RyR-mediated Ca^{2+} release by caffeine does not occur at the resting [Ca^{2+}]_{i} and ROS levels present in cells.
maintained in basal glucose. Furthermore, the antioxidant agent NAC markedly decreased insulin secretion jointly stimulated by glucose and caffeine, suggesting that caffeine requires glucose-induced ROS generation to effectively trigger RyR-mediated CICR and stimulate GSIS.

To examine more directly the role of RyR-mediated Ca\(^{2+}\) release on GSIS in pancreatic β-cell islets, we inhibited RyR function with inhibitory concentrations of ryanodine, an agent which to date has no other reported cellular targets. We observed complete GSIS suppression in islets incubated with inhibitory ryanodine for 12 h. This condition did not produce extensive cellular damage, since cholinergic stimulation with CCh of glucose-induced insulin secretion, a process that includes membrane depolarization, InsP\(_3\) generation, InsP\(_3\) receptor-mediated Ca\(^{2+}\) release and the ensuing fusion of insulin-containing vesicles [39], was not affected. In addition, we show that β-cells retained their ER Ca\(^{2+}\) content after prolonged incubation with inhibitory ryanodine, in agreement with a recent report in primary hippocampal neurons [49].

In contrast to the results observed after overnight incubation with ryanodine, we found that exposure of islets for 1 h to inhibitory ryanodine did not affect GSIS. These results are similar to other findings reported in the literature, which provided support for the lack of RyR involvement in GSIS. For example, in isolated human islets, incubation for 1 h with different concentrations of ryanodine (inhibitory and stimulatory) stimulates insulin secretion [21], while 1 h exposure of INS-1 cells to inhibitory ryanodine does not inhibit insulin secretion [22]. Our findings indicate that the exposure time to inhibitory ryanodine is critical to assess the functional roles of RyR in pancreatic islets, and may provide a methodological explanation for the discrepant findings reported in the literature. Based on the slow diffusion of the fluorescent ryanodine analog BODIPY-Ryanodine into the islets, we propose that ryanodine requires a long time to reach inhibitory concentrations in all cells within the islets, which are composed of a highly compact cluster of 1,000–5,000 cells.

**Fig 6. RyR inhibition prevents H\(_2\)O\(_2\)-dependent insulin secretion.** Islets were pre-incubated for 1 h at 37°C in Krebs bicarbonate buffer supplemented with 2.8 mM glucose. Groups of 15 islets were then incubated for 1 h at 37°C in the presence or absence of 100 μM H\(_2\)O\(_2\) in basal glucose (2.8 mM) to measure insulin secretion. Rya ON: islets were pre-incubated with 200 μM ryanodine for 12 h before the 1 h incubation period used to measure insulin secretion. Values represent Mean ± SEM; N = 3. Statistical significance was determined with one-way ANOVA followed by Tukey multiple comparison test. *: p < 0.05.

doi:10.1371/journal.pone.0129238.g006
RyR-Mediated GSIS Requires ROS

While ROS are damaging to cells when present in excess, controlled ROS generation plays a central role in cell signaling [50, 51]. Previous reports indicate that β-cells express antioxidant enzymes at relatively low levels [52, 53], a trait which may make β-cells particularly susceptible to oxidative damage. In fact, oxidative stress may be an important factor in the development of β-cell failure during the progression of type-2 diabetes, since excessive ROS production is deleterious for β-cell function [23, 54], and increased ROS production may underlie the cellular

Fig 7. Incubation with exogenous H2O2 increases [Ca2+]i in pancreatic β-cells via activation of RyR-mediated Ca2+ release. (A) Records of [Ca2+]i vs time obtained from rat pancreatic β-cells pre-incubated for 1 h with 2 μM fura-2-AM in Hanks basal solution (2.8 mM glucose). Control: cells were kept in basal Hanks solution. H2O2: cells were pre-incubated for 1 h with 100 μM H2O2 in basal Hanks solution. H2O2 + Rya ON: cells were pre-incubated with 200 μM ryanodine (Rya) for 12 h and were then incubated for 1 h with 100 μM H2O2 (in ryanodine-free solution) prior to recording in basal Hanks solution (H2O2 free). Rya ON: cells were pre-incubated with 200 μM ryanodine for 12 h. At right, quantification of these results, given as Mean ± SEM, N = 3–7. Statistical significance was determined with one-way ANOVA followed by Tukey multiple comparison test. ***: p < 0.001. (B) Average record (N = 3) of Ca2+ signals elicited by 100 μM H2O2 in the absence of ryanodine. (C) Average record (N = 3) of Ca2+ signals registered in cells pre-incubated with 200 μM ryanodine for 12 h (Rya ON); 100 μM H2O2 or 90 mM KCl were added in succession, as indicated by the arrows.

doi:10.1371/journal.pone.0129238.g007
Fig 8. Stimulatory glucose concentrations and H2O2 promote RyR2 S-glutathionylation; NAC inhibits this response. (A) Representative image of β-cells disaggregated from islets showing RyR2 S-glutathionylation with the PLA assay (red fluorescence) and insulin immunostaining (in green). H2O2: 100 μM; NAC: 10 mM. Calibration bars = 20 μm. (B) Quantification of the effects illustrated in A (Mean ± SEM, N = 3). Statistical significance was determined with one-way ANOVA followed by Tukey multiple comparison test. ***: p < 0.001.

doi:10.1371/journal.pone.0129238.g008
damage produced by both lipo- and gluco-toxicity [23, 55]. Nonetheless, other studies [24, 31] support a role for physiological ROS concentrations as second messengers in insulin secretion. An increase in extracellular glucose concentration enhances ROS generation in pancreatic β-cells [56], as confirmed here, while other studies indicate that GSIS requires mitochondrial ROS production [31]. The low antioxidant enzyme levels of β-cells are likely to make them especially sensitive to ROS-mediated signaling under physiological conditions. Our results, showing that incubation of islets with the antioxidant agent NAC prevented GSIS and markedly decreased insulin secretion jointly stimulated by glucose and caffeine, support and extend these previous findings. NAC has been widely used as an effective antioxidant agent in vivo and in vitro [57]. Results similar to ours have been described in INS-1 cells, where the exogenous application of NAC inhibits insulin secretion stimulated by glucose [24]. We found that NAC did not modify carbachol-stimulated insulin secretion, suggesting that NAC does not prevent alternative cellular mechanisms underlying insulin secretion. Hence, we propose ROS production is a requisite step for GSIS but not for insulin secretion jointly stimulated by glucose and carbachol.

Previous studies in other cell types indicate that RyR channels are highly susceptible to changes in cellular redox state, making RyR a potential cellular redox sensor protein that does not respond to activation by Ca\(^{2+}\) when key cysteine residues are in the reduced state [30]. We found that a reduced cellular environment is not conducive to GSIS. Additionally, we observed a direct correlation between GSIS inhibition by NAC and the marked decrease in RyR2 S-glutathionylation levels produced by NAC. Consequently, we suggest that GSIS inhibition by NAC is due to reduction of RyR2 cysteine residues, a redox modification that prevents activation of RyR channels by Ca\(^{2+}\) in muscle and neurons [55], and that hinders RyR-mediated CICR in other excitable cell types [30]. Supporting our proposal, a recent study in patients with rare RyR2 mutations that produce leaky RyR2 channels (that display intracellular Ca\(^{2+}\) leak via oxidized/nitrosylated RyR2 channels), concluded that RyR2 plays a crucial role in the regulation of insulin secretion and glucose homeostasis [58].

**Effects of H\(_2\)O\(_2\) on Insulin Secretion**

Exogenous H\(_2\)O\(_2\) and diethyl maleate, which increases intracellular H\(_2\)O\(_2\) levels, stimulate insulin secretion, whereas high concentrations of exogenous antioxidants inhibit GSIS [24]. Our hypothesis predicts that H\(_2\)O\(_2\)-induced insulin secretion at basal glucose concentration involves RyR oxidation, which causes increased RyR-mediated Ca\(^{2+}\) release. Our results corroborate this prediction, because both RyR inhibition and NAC prevented insulin secretion induced by H\(_2\)O\(_2\). Since at basal glucose concentrations H\(_2\)O\(_2\) enhanced RyR2 S-glutathionylation, we propose that this oxidative change contributes to promote RyR-mediated Ca\(^{2+}\) release, thereby increasing [Ca\(^{2+}\)], to the levels required for insulin secretion. This proposed mechanism is supported by the present results, showing RyR-dependent [Ca\(^{2+}\)], increase after addition of H\(_2\)O\(_2\), as discussed below, and by results obtained in other cell types, where addition of exogenous H\(_2\)O\(_2\) promotes RyR redox modifications and specifically stimulates RyR-mediated Ca\(^{2+}\) release [30, 59].

Additionally, we found that 100 μM H\(_2\)O\(_2\) disrupted GSIS, confirming previous reports in rat islets [29] and mouse pancreatic β-cells [60]. Chronically high glucose concentrations increase superoxide production and proton leak in mitochondria, reducing ATP levels and causing impaired GSIS in islets from rodents [54]. Hence, we propose that addition of 100 μM H\(_2\)O\(_2\) in stimulatory glucose produces an abnormal ROS increase and causes oxidative damage,
which the weak antioxidant capacity of β-cells presumably fails to neutralize [53], resulting in inhibition of GSIS.

**Effects of H2O2 on [Ca2+]i**

Thimerosal, an oxidizing agent that effectively enhances the activity of skeletal RyR1 and cardiac RyR2 channels [61], releases Ca2+ from InsP3-insensitive ER Ca2+ pools in RINm5F insulinoma cells and from β-cells isolated from ob/ob mice [62]. Our results show that addition of exogenous H2O2 to dissociated β-cells maintained in basal glucose increased [Ca2+]i, which reached values close to 400 nM after H2O2 addition. These levels are within the range of the [Ca2+]i increases elicited by depolarization of human β-cells [63], or elicited by increased glucose levels in cell lines and pancreatic β-cells [9]. This result strengthens our proposal that the increased insulin secretion promoted by H2O2 at basal glucose concentration is due to an
increase in [Ca\textsuperscript{2+}]\textsubscript{i}, and extends previous reports showing that H\textsubscript{2}O\textsubscript{2} increases [Ca\textsuperscript{2+}]\textsubscript{i} to similar levels in islets and β-cell lines through a process that implicates Ca\textsuperscript{2+} release from the ER [29, 64]. A requirement for Ca\textsuperscript{2+} entry has been suggested as well, since removal of extracellular Ca\textsuperscript{2+} suppresses insulin secretion in INS-1 cells in response to H\textsubscript{2}O\textsubscript{2} [24]. Addition of H\textsubscript{2}O\textsubscript{2} to rat islets in basal glucose increases [Ca\textsuperscript{2+}]\textsubscript{i}, in a dose-dependent manner; this increase is partially sensitive to blockers of L-type channels and is abolished by thapsigargin [65].

In summary, there is consensus that at basal glucose concentration H\textsubscript{2}O\textsubscript{2} increases [Ca\textsuperscript{2+}]\textsubscript{i} to levels that promote exocytosis of insulin-containing granules, albeit the source of Ca\textsuperscript{2+} remained undefined. Our findings suggest that H\textsubscript{2}O\textsubscript{2}-induced RyR-mediated Ca\textsuperscript{2+} release is a major contributor to the increase in [Ca\textsuperscript{2+}]\textsubscript{i}, since H\textsubscript{2}O\textsubscript{2} did not increase [Ca\textsuperscript{2+}]\textsubscript{i} in cells pre-incubated overnight with inhibitory ryanodine. The present results provide the first evidence that RyR channels are involved in the [Ca\textsuperscript{2+}]\textsubscript{i} increase induced by H\textsubscript{2}O\textsubscript{2} in β-cells.

Conclusions

According to the model proposed in this study (Fig 9), the increased ROS generation produced by cellular glucose metabolism makes possible the activation of RyR channels by the local and moderate [Ca\textsuperscript{2+}]\textsubscript{i} increase produced by Ca\textsuperscript{2+} entry from the extracellular medium in response to glucose-induced β-cell depolarization. Although not directly tested here, the glucose-induced increase in ATP concentration may also contribute to enhance RyR channel activation by Ca\textsuperscript{2+}, as reported in single RyR channels from neuronal cells [66]. The resulting RyR-mediated CICR would provide the [Ca\textsuperscript{2+}]\textsubscript{i} increase required for insulin secretion. Our hypothesis, presenting GSIS as the combined result of glucose-induced Ca\textsuperscript{2+} entry and glucose-induced ROS generation leading to enhanced RyR-mediated CICR, adds a new concept to the physiology of the pancreatic β-cell. Our results may also explain why prolonged glucose elevations, which promote oxidative stress [67], adversely affect the function of pancreatic β-cells, since excessive activation of RyR-mediated CICR by ROS may promote cellular damage leading to cell death.

Supporting Information

S1 Fig. RyR2 and calnexin immunostaining in MIN6 and pancreatic β-cells. (A) MIN6 cells. Immunostaining directed against RyR2 (green) and the ER marker calnexin (red). The right hand panel illustrates the combined red and green fluorescence plus the blue (Hoechst) nuclear staining. (B) Images were collected from a single pancreatic β-cell. Immunostaining directed against RyR2 (green) and the ER marker calnexin (red). The image at right shows the superposition of green and red fluorescence. Bars indicate 20 μm. (TIF)

S2 Fig. Expression of RyR2 mRNA in rat pancreatic islets and of RyR2 protein in MIN6 cells. (A) RyR2 mRNA was determined by conventional PCR, using the following primer sequences, which are specific for the RyR2 isoform: RyR2sense: 5'-CTACTCAGGATGAGGTCGGA-3'; RyR2antisense: 5'-CTCTCTCCAGATCAGAAGCCA-3'. Lane ST: standard; lanes 1, 2, 5 and 6: RNA extracted from rat primary hippocampal neurons. Lanes 3 and 4: RNA extracted from rat pancreatic islets. Lanes 5 and 9: negative controls. The amplified fragment for RyR2 corresponds to 157 bp. (B) RyR2 protein levels in primary hippocampal neurons and MIN6 cells were assayed by Western blot analysis as described in the text. (TIF)

S3 Fig. Distribution of BODIPY-ryanodine. Images were acquired after incubation of pancreatic islets with this probe for 1 h (A) or 12 h (B); both images were obtained by confocal
microscopy with identical acquisition parameters, allowing qualitative comparisons. The images at left correspond to fluorescence and at right to transmitted light. Calibration bars: 50 μm.

(TIF)

S4 Fig. Ryanodine-treated isolated β-cells displayed similar thapsigargin-elicited Ca²⁺ signals and ROS levels as control cells. (A). Time course of Fluo-4 fluorescence recorded from isolated β-cells before and after addition of thapsigargin to cultures loaded with Fluo-4 AM and transferred to Ca²⁺-free solution just before starting the record. Fluorescence values are expressed as (F/F₀), where F₀ represents the basal fluorescence recorded before addition of thapsigargin. Addition of 5 μM thapsigargin (Tg, arrow) elicited similar Ca²⁺ signals in controls (upper panel) as in isolated β-cells pre-incubated with 200 μM ryanodine for 1 h (middle panel) or overnight (bottom panel). (B) Quantification of the areas under the curve. (C) Quantification of maximum fluorescence intensity. In A to C, values represent Mean ± SEM, (N = 3–6 cells from 2 rats). Statistical significance was determined with one-way ANOVA followed by Tukey’s multiple comparison test. ns: no significant differences. (D). Representative fluorescence images (upper) of islets loaded with 10 μM CM-H₂DCFDA, collected by confocal microscopy; at bottom, light-contrast images. (E) Quantification of H₂DCFDA fluorescence intensity determined in control islets, in islets pre-incubated with 200 μM ryanodine for 1 h or overnight, or treated with 0.5 mM H₂O₂ for 1 h. N = 4–10 islets. ***: p < 0.001, determined by statistical analysis with One-way ANOVA, followed by Tukey’s post-hoc test.

(TIF)

S5 Fig. N-acetyl cysteine (NAC) does not prevent insulin secretion induced by carbachol. The effects of NAC were tested in either basal (2.8 mM) or stimulatory (27.7 mM) glucose (G) concentrations. Values represent Mean ± SEM, N = 3. Statistical significance was determined with one-way ANOVA followed by Tukey’s Multiple Comparison Test. *: p < 0.05; ***: p <0.001; ns: no significant differences.

(TIF)

S6 Fig. Determination of RyR2 S-glutathionylation with the PLA assay. The figure displays representative confocal images acquired in disaggregated β-cells from islets, showing PLA labeling (red), insulin immunostaining (green) and the merged images. From left to right, images were taken at different depths, from the bottom to the top of cells incubated in basal glucose (2.8 mM), stimulatory glucose (16.7 mM), basal glucose (2.8 mM) plus H₂O₂ (100 μM) or stimululatory glucose (16.7 mM) plus NAC (10 mM).

(JPG)

Acknowledgments

We are grateful to A. Garcia and Dr. J. Hidalgo for their excellent advice and help with confocal microscope determinations. We thank specially Dr I. Atwater for many insightful discussions on β-cell function and Dr T. Adasme for her kind help in semiquantitative RT-PCR experiments.

Author Contributions

Conceived and designed the experiments: PL ACF GB DM CH. Performed the experiments: PL MV ACF. Analyzed the data: PL MV ACF GB. Contributed reagents/materials/analysis tools: PL DM CH. Wrote the paper: PL CH.
References

1. Henquin JC. Triggering and amplifying pathways of regulation of insulin secretion by glucose. Diabetes. 2000; 49(11):1751–60. PMID:11079440.

2. Cook DL, Hales CN. Intracellular ATP directly blocks K+ channels in pancreatic B-cells. Nature. 1984; 311(5983):271–3. PMID:6909390.

3. Kohler M, Norgren S, Berggren PO, Fredholm BB, Larsson O, Rhodes CJ, et al. Changes in cytoplasmic ATP concentration parallels changes in ATP-regulated K+-channel activity in insulin-secreting cells. FEBS letters. 1998; 441(1):97–102. PMID:9877173.

4. Mears D. Regulation of insulin secretion in islets of Langerhans by Ca(2+)channels. The Journal of membrane biology. 2004; 200(2):57–66. PMID:15520904.

5. Tengholm A, Hellman B, Gylfe E. The endoplasmic reticulum is a glucose-modulated high-affinity sink for Ca2+ in mouse pancreatic beta-cells. The Journal of physiology. 2001; 530(Pt 3):533–40. PMID:11158282.

6. Patterson RL, Boehning D, Snyder SH. Inositol 1,4,5-trisphosphate receptors as signal integrators. Annual review of biochemistry. 2004; 73:437–65. PMID:15189149.

7. Fill M, Copello JA. Ryanodine receptor calcium release channels. Physiological reviews. 2002; 82(4):893–922. PMID:12270947.

8. Blondel O, Moody MM, Depaoli AM, Sharp AH, Ross CA, Swift H, et al. Localization of inositol trisphosphate receptor subtype 3 to insulin and somatostatin secretory granules and regulation of expression in islets and insulinoma cells. Proceedings of the National Academy of Sciences of the United States of America. 1994; 91(16):7777–81. PMID:7914371.

9. Islam MS. The ryanodine receptor calcium channel of beta-cells: molecular regulation and physiological significance. Diabetes. 2002; 51(5):329–39. PMID:11978625.

10. Wolfle CB, Biden TJ. Second messenger function of inositol 1,4,5-trisphosphate. Early changes in inositol phosphates, cytosolic Ca2+, and insulin release in carbamylcholine-stimulated RINm5F cells. The Journal of biological chemistry. 1986; 261(18):8314–9. PMID:3522567.

11. Islam MS, Rorsman P, Berggren PO. Ca(2+)-induced Ca2+ release in insulin-secreting cells. FEBS letters. 1992; 296(3):287–91. PMID:1537406.

12. Graves TK, Hinkle PM. Ca(2+)-induced Ca(2+) release in the pancreatic beta-cell: direct evidence of endoplasmic reticulum Ca(2+) release. Endocrinology. 2003; 144(8):3565–74. PMID:12865339.

13. Beauvois MC, Arredouani A, Jonas JC, Rolland JF, Schuit F, Henquin JC, et al. Atypical Ca2+-induced Ca2+ release from a sarco-endoplasmic reticulum Ca2+-ATPase 3-dependent Ca2+ pool in mouse pancreatic beta-cells. The Journal of physiology. 2004; 559(Pt 1):141–56. PMID:15218077.

14. Gamberucci A, Fulceri R, Pralong W, Banhegyi G, Marcolongo P, Watkins SL, et al. Caffeine releases a glucose-primed endoplasmic reticulum Ca2+ pool in the insulin secreting cell line INS-1. FEBS letters. 1999; 466(2–3):309–12. PMID:10100864.

15. Mitchell KJ, Lai FA, Rutter GA. Ryanodine receptor type I and nicotinic acid adenine dinucleotide phosphate receptors mediate Ca2+ release from insulin-containing vesicles in living pancreatic beta-cells (MIN6). The Journal of biological chemistry. 2003; 278(13):11057–64. PMID:12538591.

16. Takasawa S, Akiyama T, Nata K, Kuroki M, Tohgo A, Noguchi N, et al. Cyclic ADP-ribose and inositol 1,4,5-trisphosphate as alternate second messengers for intracellular Ca2+ mobilization in normal and diabetic beta-cells. The Journal of biological chemistry. 1998; 273(5):2497–500. PMID:9446548.

17. Islam MS, Leibiger I, Leibiger B, Rossi D, Sorrentino V, Ekstrom TJ, et al. In situ activation of the type 2 ryanodine receptor in pancreatic beta cells requires cAMP-dependent phosphorylation. Proceedings of the National Academy of Sciences of the United States of America. 1998; 95(11):6145–50. PMID:9600932.

18. Kang G, Chepurny OG, Holz GG. cAMP-regulated guanine nucleotide exchange factor II (Epac2) mediates Ca2+-induced Ca2+ release in INS-1 pancreatic beta-cells. The Journal of physiology. 2001; 536(Pt 2):375–85. PMID:11600673.

19. Woolcott QQ, Gustafsson AJ, Dzabic M, Pierro C, Tedeschi P, Sandgren J, et al. Arachidonic acid is a physiological activator of the ryanodine receptor in pancreatic beta-cells. Cell calcium. 2006; 39(6):529–37. PMID:16620964.

20. Choi KJ, Cho DS, Kim JY, Kim BJ, Lee KM, Kim SH, et al. Ca-induced Ca Release from Internal Stores in INS-1 Rat Insulinoma Cells. Korean J Physiol Pharmacol. 15(1):53–9. PMID:21461241. doi:10.4196/kjpp.2011.15.1.53

21. Johnson JD, Kuang S, Misler S, Polonsky KS. Ryanodine receptors in human pancreatic beta cells: localization and effects on insulin secretion. Faseb J. 2004; 18(7):878–80. PMID:15033925.
22. Brustan JD, Lemmens R, Shi CL, Persson-Sjogren S, Westerblad H, Ahmed M, et al. Ryanodine receptors of pancreatic beta-cells mediate a distinct context-dependent signal for insulin secretion. Faseb J. 2003; 17(2):301–3. PMID: 12478592.

23. Robertson RP. Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. The Journal of biological chemistry. 2004; 279(41):42351–4. PMID: 15258147.

24. Pi J, Bai Y, Zhang Q, Wong V, Floering LM, Daniel K, et al. Reactive oxygen species as a signal in glucose-stimulated insulin secretion. Diabetes. 2007; 56(7):1783–91. PMID: 17400930.

25. Tsubouchi H, Inoguchi T, Inou M, Kakimoto M, Sonta T, Sonoda N, et al. Sulfonylurea as well as elevated glucose levels stimulate reactive oxygen species production in the pancreatic beta-cell line, MIN6-a role of NAD(P)H oxidase in beta-cells. Biochemical and biophysical research communications. 2005; 326(1):60–5. PMID: 15567152.

26. Sakai K, Matsumoto K, Nishikawa T, Suefuji M, Nakamaru K, Hirashima Y, et al. Mitochondrial reactive oxygen species reduce insulin secretion by pancreatic beta-cells. Biochemical and biophysical research communications. 2003; 300(1):216–22. PMID: 12480546.

27. Wolff SP, Dean RT. Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes. The Biochemical journal. 1987; 245(1):243–50. PMID: 3117042.

28. Janjic D, Maechler P, Sekine N, Bartley C, Annen AS, Wolheim CB. Free radical modulation of insulin release in INS-1 cells exposed to alloxan. Biochemical pharmacology. 1999; 57(6):639–48. PMID: 10037448.

29. Maechler P, Jornot L, Wolheim CB. Hydrogen peroxide alters mitochondrial activation and insulin secretion in pancreatic beta-cells. The Journal of biological chemistry. 1999; 274(39):27905–13. PMID: 10488138.

30. Hidalgo C, Donoso P. Crosstalk between calcium and redox signaling: from molecular mechanisms to health implications. Antioxidants & redox signaling. 2008; 10(7):1275–312. PMID: 18377233.

31. Leloup C, Tourrel-Cuzin C, Magnan C, Karaca M, Castel J, Carneiro L, et al. Mitochondrial reactive oxygen species are obligatory signals for glucose-induced insulin secretion. Diabetes. 2009; 58(3):673–81. PMID: 19073765. doi: 10.2337/db07-1056

32. Mears D, Leighton X, Atwater I, Rojas E. Tetracaine stimulates insulin secretion from the pancreatic beta-cell by release of intracellular calcium. Cell calcium. 1999; 25(1):59–68. PMID: 10191960.

33. Paula-Lima AC, Adasme T, SanMartin C, Sebollela A, Hetz C, Carrasco MA, et al. Amyloid beta-peptide oligomers stimulate RyR-mediated Ca2+ release inducing mitochondrial fragmentation in hippocampal neurons and prevent RyR-mediated dendritic spine remodeling produced by BDNF. Antioxidants & redox signaling. 14(7):1209–23. PMID: 20712397.

34. Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, et al. Establishment of a pancreatic beta-cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. Endocrinology. 1990; 127(1):126–32. Epub 1990/07/01. doi: 10.1210/endo-127-1-126 PMID: 2163307.

35. Adasme T, Haeger P, Paula-Lima AC, Espinoza I, Casas-Alarcon MM, Carrasco MA, et al. Involvement of ryanodine receptors in neurotrophin-induced hippocampal synaptic plasticity and spatial memory formation. Proceedings of the National Academy of Sciences of the United States of America. 108(7):3029–34. PMID: 21282625. doi: 10.1073/pnas.1013580108

36. Takasawa S, Kuroki M, Naka K, Noguchi N, Ikeda T, Yamauchi A, et al. A novel ryanodine receptor expressed in pancreatic islets by alternative splicing from type 2 ryanodine receptor gene. Biochemical and biophysical research communications. 2003; 300(1):216–22. PMID: 12480546.

37. Malaisse WJ, Malaisse-Lagae F, Mayhew D. A possible role for the adenylcyclase system in insulin secretion. The Journal of clinical investigation. 1967; 46(11):1724–34. PMID: 4294054.
55. Bindokas VP, Kuznetsov A, Sreenan S, Polonsky KS, Roe MW, Philipson LH. Visualizing superoxide
46. Johnson JD, Han Z, Otani K, Ye H, Zhang Y, Wu H, et al. RyR2 and calpain-10 delineate a novel apo-
Mitchell KJ, Pinton P, Varadi A, Tacchetti C, Ainscow EK, Pozzan T, et al. Dense core secretory vesi-
45. Willmott NJ, Galione A, Smith PA. Nitric oxide induces intracellular Ca2+ mobilization and increases se-
44. Zheng J, Chen Z, Yin W, Miao L, Zhou Z, Ji G. Ryanodine receptors are involved in nuclear calcium os-
43. Gustafsson AJ, Ingelman-Sundberg H, Dzabic M, Awasum J, Nguyen KH, Ostenson CG, et al. Ryano-
47. 44. Shigeto M, Katsura M, Matsuda M, Ohkuma S, Kaku K. Nateglinide and mitiglinide, but not sulfonyl-
Santulli G, Pagano G, Sardu C, Xie W, Reiken S, D’Ascia SL, et al. Calcium release channel RyR2 reg-
58. 57. Durham WJ, Aracena-Parks P, Long C, Rossi AE, Goonasekera SA, Boncompagni S, et al. RyR1 S-
57. Krauss S, Zhang CY, Scorrano L, Dalgaard LT, St-Pierre J, Grey ST, et al. Superoxide-mediated activa-
56. 55. Grankvist K, Marklund SL, Taljedal IB. CuZn-superoxide dismutase, Mn-superoxide dismutase, cata-
lase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. The Biochemical journal. 1981; 199(2):393–8. PMID: 7041886.
54. Robertson RP, Harmon J, Tran PO, Poitout V. Beta-cell glucose toxicity, lipotoxicity, and chronic oxida-
tive stress in type 2 diabetes. Diabetes. 2004; 53 Suppl 1:S119–24. PMID: 14749276.
53. 52. Tiedge M, Lortz S, Drinkgern J, Lenzen S. Relation between antioxidant enzyme gene expression and
81. PMID: 802. S0006-291X(15)00095-9 [pii] doi:10.1016/j.bbrc.2015.01.065 PMID: 25623539.
50. 49. Rhee SG. Cell signaling. H2O2, a necessary evil for cell signaling. Science (New York, NY. 2006; 312(5782):1882–3. PMID: 16609515.
48. 47. Droge W. Free radicals in the physiological control of cell function. Physiological reviews. 2002; 82 (1):47–95. PMID: 11773609.
46. 45. Tiedge M, Paula-Lima A, Hidalgo C. Inhibitory ryanodine prevents ryanodine receptor-mediated Ca(2+)
44. Tsubo T, da Silva Xavier G, Holz GG, Jouaville LS, Thomas AP, Rutter GA. Glucagon-like peptide-1
43. 42. Tiedge M, Paula-Lima A, Hidalgo C. Inhibitory ryanodine prevents ryanodine receptor-mediated Ca(2+)
42. Tiedge M, Paula-Lima A, Hidalgo C. Inhibitory ryanodine prevents ryanodine receptor-mediated Ca(2+)
41. Krauss S, Zhang CY, Scorrano L, Dalgaard LT, St-Pierre J, Grey ST, et al. Superoxide-mediated activa-
40. 39. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
38. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
37. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
36. 35. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
34. 33. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
32. 31. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
30. 29. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
28. 27. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
26. 25. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
24. 23. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
22. 21. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
20. 19. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
18. 17. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
16. 15. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
14. 13. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
12. 11. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
10. 9. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
8. 7. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
6. 5. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
4. 3. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)
2. 1. Bennett S, Balsamo A, Fischetti VA, Hidalgo C. Ryanodine receptor-mediated Ca(2+)

62. Islam MS, Larsson O, Berggren PO. Cyclic ADP-ribose in beta cells. Science (New York, NY. 1993; 262(5133):584–6. PMID: 8211188.

63. Rojas E, Carroll PB, Ricordi C, Boschero AC, Stojilkovic SS, Atwater I. Control of cytosolic free calcium in cultured human pancreatic beta-cells occurs by external calcium-dependent and independent mechanisms. Endocrinology. 1994; 134(4):1771–81. PMID: 8137742.

64. Nakagaki I, Sasaki S, Hori S, Kondo H. Ca2+ and electrolyte mobilization following agonist application to the pancreatic beta cell line HIT. Pflugers Arch. 2000; 440(6):828–34. PMID: 11041547.

65. Tang J, Zhang JH. Mechanisms of [Ca2+]i elevation by H2O2 in islets of rats. Life sciences. 2000; 68 (4):475–81. PMID: 11205896.

66. Bull R, Finkelstein JP, Humeres A, Behrens MI, Hidalgo C. Effects of ATP, Mg2+, and redox agents on the Ca2+ dependence of RyR channels from rat brain cortex. Am J Physiol Cell Physiol. 2007; 293(1): C162–71. Epub 2007/03/16. doi: 10.1152/ajpcell.00518.2006 PMID: 17360812.

67. Tanaka Y, Tran PO, Harmon J, Robertson RP. A role for glutathione peroxidase in protecting pancreatic beta cells against oxidative stress in a model of glucose toxicity. Proceedings of the National Academy of Sciences of the United States of America. 2002; 99(19):12363–8. PMID: 12218186.