Endocrine (2015) 50:110–122
DOI 10.1007/s12020-015-0568-z

ORIGINAL ARTICLE

Autocrine effect of Zn^{2+} on the glucose-stimulated insulin secretion

Kira G. Slepchenko[1] · Nigel A. Daniels[2] · Aili Guo[2] · Yang V. Li[1]

Received: 16 October 2014 / Accepted: 2 March 2015 / Published online: 15 March 2015

Abstract It is well known that zinc (Zn^{2+}) is required for the process of insulin biosynthesis and the maturation of insulin secretory granules in pancreatic beta (β)-cells, and that changes in Zn^{2+} levels in the pancreas have been found to be associated with diabetes. Glucose-stimulation causes a rapid co-secretion of Zn^{2+} and insulin with similar kinetics. However, we do not know whether Zn^{2+} regulates insulin availability and secretion. Here we investigated the effect of Zn^{2+} on glucose-stimulated insulin secretion (GSIS) in isolated mouse pancreatic islets. Whereas Zn^{2+} alone (control) had no effect on the basal secretion of insulin, it significantly inhibited GSIS. The application of CaEDTA, by removing the secreted Zn^{2+} from the extracellular milieu of the islets, resulted in significantly increased GSIS, suggesting an overall inhibitory role of secreted Zn^{2+} on GSIS. The inhibitory action of Zn^{2+} was mostly mediated through the activities of K_{ATP}/Ca^{2+} channels. Furthermore, during brief paired-pulse glucose-stimulated Zn^{2+} secretion (GSZS), Zn^{2+} secretion following the second pulse was significantly attenuated, probably by the secreted endogenous Zn^{2+} after the first pulse. Such an inhibition on Zn^{2+} secretion following the second pulse was completely reversed by Zn^{2+} chelation, suggesting a negative feedback mechanism, in which the initial glucose-stimulated Zn^{2+} release inhibits subsequent Zn^{2+} secretion, subsequently inhibiting insulin co-secretion as well. Taken together, these data suggest a negative feedback mechanism on GSZS and GSIS by Zn^{2+} secreted from β-cells, and the co-secreted Zn^{2+} may act as an autocrine inhibitory modulator.

Keywords Insulin · Zinc · Beta-cells · Autocrine · Feedback · Islet

Introduction

Zinc (Zn^{2+}) is an essential element crucial for growth and development, and also plays a role in cell signaling for cellular processes like cell division and apoptosis. In the mammalian pancreas, where the total Zn^{2+} content is among the highest in the body, Zn^{2+} is essential for the correct processing, storage, secretion, and action of insulin in beta (β)-cells [1]. Insulin is stored inside secretory vesicles or granules where two Zn^{2+} ions coordinate six insulin monomers to form the hexameric structure on which maturated insulin crystals are based. The relationship between co-stored Zn^{2+} and insulin undoubtedly is, therefore, important to normal β-cell function. A link between Zn^{2+} and diabetes has also been proposed [2–4]. Hyperglycemia in diabetes is usually associated with increased urinary loss of Zn^{2+}, which is responsible for decreases in total body Zn^{2+} [5–7]. Zn^{2+} deficiency combined with over-secretion of insulin can affect the ability of the islet cells to respond appropriately, and might then compound the problem, particularly in type II diabetes (T2D).

Recent studies reveal that Zn^{2+} acts as an intracellular and extracellular signaling molecule, and is involved in intra- and inter-cellular communications [8–12]. Zn^{2+} appears to exhibit profound modulatory effects on a variety of ligand- and voltage-gated mammalian ion channels, including attenuation of current through voltage-gated Ca^{2+} channels

✉ Yang V. Li
Liy1@ohio.edu

[1] Department of Biomedical Sciences, Heritage College of Osteopathic Medicine, Ohio University, Athens, OH 45701, USA

[2] Department of Specialty Medicine, Heritage College of Osteopathic Medicine, Ohio University, Athens, OH 45701, USA

(VGCCs), NMDA and GABA receptor-gated channels [13–16]. Among VGCCs, Zn^{2+} has particularly high affinity for T-type channels [17], which are also expressed in human β-cells [18]. The insulin secretion is initiated by the influx of glucose, a prominent physiological insulin secretagogue, into β-cells, which consequently increases the ATP/ADP ratio [19, 20]. As a result, ATP-regulated K_{ATP} channels are inhibited, leading to membrane depolarization that facilitates Ca^{2+} entry, which triggers insulin secretion [21, 22]. Studies indicate that Zn^{2+} directly inhibits Ca^{2+} channels, or may do so indirectly through its action in K_{ATP} conductance [23] (also see [24, 25] and [26]). There is evidence for a role of the G protein-coupled receptor (GPR)-39 in the regulation of insulin secretion [27]. Several groups have suggested that this Zn^{2+}-sensitive receptor (GPR39) is important for pancreatic function [28]. However, there is no study that has shown the direct action of Zn^{2+} on glucose-stimulated insulin secretion (GSIS) in pancreatic islets.

A better understanding of insulin exocytosis in β-cells is of intense interest because of the possible role of defective insulin secretion or reduced insulin production in T2D [4, 29–33]. Many studies have shown that Zn^{2+} is secreted from pancreatic β-cells in response to elevated glucose concentration [29, 34–38], as Zn^{2+}/insulin complex. Our previous study showed that Zn^{2+} was secreted in the same manner as the secretion of insulin [39]. Once in the extracellular microenvironment, the Zn^{2+}/insulin complex begins the process of rapid dissociation in an almost exact reverse order of its synthesis. After all, insulin must dissociate from Zn^{2+} because the extracellular milieu favors the dissociation and, essentially, the insulin monomer is the biologically active form [1, 38, 40, 41], yielding a substantial amount of free Zn^{2+} in the interstitial space surrounding a β-cell. The present study was to examine the direct effects of secreted or extracellular Zn^{2+} on GSIS in the mouse pancreatic β-cells. We observed a robust glucose-stimulated Zn^{2+} secretion (GSZS) in pancreatic islets. Overall action of the secreted Zn^{2+} appeared to be inhibitory to GSIS. We also investigated the mechanism by which Zn^{2+} inhibited GSIS. Our study suggests that there may be a feedback mechanism regulating insulin secretion, which involves co-secreted Zn^{2+}.

Materials and methods

Mouse pancreatic islet preparation

Pancreatic islets were isolated by collagenase digestion of pancreas from adult female mice C57BL/6. Briefly, 3 ml of ice cold collagenase Liberase TL (Roche Diagnostics) at 1.3 U/ml was injected into common bile duct and pancreas was removed following the injection. Digestion was carried out at 37 °C for 10–12 min. Islets were removed from the collagenase solution by pipetting. Islets were washed three times with RPMI medium with additional 10 % fetal bovine serum (v/v) (complete medium). Following isolation, islets to be tested for GSIS were transferred to an ultra-low attachment Petri dish containing complete medium (see Insulin Collection and Measurements with Islets). Islets intended for visualization and fluorescence experiments were plated onto glass bottom dishes (see Fluorescence Experiments). All islets were incubated for 48 h prior to the experiments, to allow recovery from the isolation.

Cell culture

Clonal HIT-T15 cells (number CRL-1777) were purchased from ATCC (Manassa, VA). HIT-T15 cells were established by transforming Syrian hamster primary culture cells with SV40. The HIT-T15 cell has been widely used in the study of insulin secretion [42]. It secretes insulin in response to glucose and other cell secretagogues. Among insulin-producing cell lines, HIT-T15 cells contain modest numbers of membrane-bound secretory granules and have the ability to release insulin following glucose stimulation, i.e., GSIS, similar to that of isolated pancreatic islets [43–45]. Cells were used between passages 64–78. In the constraint of these passages, cells maintained normal insulin secretion and glucose sensitivity. They were maintained in Ham's F12K medium supplemented with 2.5 % fetal bovine serum and 10 % dialyzed horse serum in 5 % CO_2–95 % humidified air at 37 °C (as suggested by ATCC).

Fluorescence experiments

The experiments with islets were performed similarly to the experiments using cultured cells described previously [46, 47]. Isolated islets were placed into 35 mm glass bottom Petri dishes (P35G-4.5-14-C. MatTek Corp, Ashland, MA) with 2 ml of RPMI culture medium supplemented with 10 % fetal bovine serum in 5 % CO_2–95 % humidified air at 37 °C for about 48 h prior to the experiments to allow attachment to the dish and recovery from the isolation process. Attached islets were washed three times with 1 mL of basal HEPES buffer (in mM) 25 HEPES, 125 NaCl, 3 KCl, 1.28 $CaCl_2$, 1.1 $MgCl_2$, 0.8 glucose, and loaded for 30 min with membrane-permeable fluorescent Zn^{2+} indicator Zinpyr-1 (5 μM) then incubated in 5 % CO_2–95 % humidified air at 37 °C. The experiments with cells were performed as described previously [39, 46]. Briefly, cells were detached with trypsin and plated into 35 mm glass bottom Petri dishes (MatTek Corp, Ashland, MA). Similar to islets, cells were washed three times with 1 mL of basal HEPES buffer before loading with Zinpyr-1.

Zinpyr-1 is a chelating agent, a di-2-picolylamine or DPA-derivatized fluorescein, and has a high affinity for

Zn^{2+} ($K_D \sim 1$ nM) and a relative high k_{off} value (2.3×10^{-3} s^{-1}) [48]. Zinpyr-1 has high quantum yield and essentially no measurable affinity for Ca^{2+} or Mg^{2+}. Furthermore, Zinpyr-1 is membrane permeable and binds Zn^{2+} in a TPEN-like manner, owing to its binding moiety similarity to the membrane-permeable heavy metal chelator *N*,*N*,*N*¢,*N*¢-tetra(2-picolyl)ethylenediamine (TPEN) [49–51]. Images of cells were taken using camera QImaging Retiga 1300i on an inverted microscope MoticAE31 using Olympus U Plan FL 40×, 0.75 NA, or Zeiss LSM 510 confocal microscope (100×/1.4). Image analysis was performed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) or Zen 2011 imaging software (Zeiss, Oberkochen, Germany). Images were collected every 1 or 5 s. Autofluorescence was below the detection limits of the camera, and photobleaching was negligible; neither was subtracted from the data. For glucose stimulation, the stimulating medium consisted of (in mM) 25 HEPES, 125 NaCl, 3 KCl, 1.28 $CaCl_2$, 1.1 $MgCl_2$, 20 glucose, pH 7.4. Final glucose concentration was 10 mM, unless otherwise specified. Care was taken to minimize potential Zn^{2+} contamination that might introduce background fluorescence.

Transfection of HIT-T15 cells with AdIox-mInsCmCherry

The construct was kindly gifted by Dr.Drain [52, 53]. AdIox-mInsCmCherry is red fluorescent mCherry reporter (Ins-C-mCherry). It is fused with the C peptide of proinsulin which localizes in the insulin secretory granules. The reporter construct is inserted into the E1 region of an E1, E3-deleted adenoviral vector [52, 53]. HIT-T15 cells were prepared for transfection similar to fluorescence experiments (see fluorescence experiments above). Cells were incubated in complete medium for 24 h before transfection. Lipofectamine® LTX with PLUS™ reagent (Invitrogen, Carlsbad, CA) was used as directed by the manufacturer. For each 35 mm petri dish we used 0.5 μg purified construct DNA and 7 μl of Lipofectamine. Transfection reagents and DNA were dissolved in Opti-MEM medium (Gibco, Grand Island). The solution was added dropwise to the cells which were in complete medium. Transfected cells were incubated for 48 h, and the medium was not exchanged during this time. We estimated that about 50 % of the cells expressed the Ins-C-mCherry. Transfected cells were loaded with Zinpyr-1 as described in fluorescent experiments above. Cells were visualized with an inverted Zeiss LSM510 confocal microscope, using 100× oil lens. This microscope system has multitracking data collection to reduce signal cross-talk. To visualize green Zn^{2+} fluorescence the 505–530 nm emission and 488 nm excitation filters were used. For red insulin fluorescence 650 nm emission and 543 nm excitation filters were used.

Paired-pulse glucose stimulations

Islets were loaded with Zn^{2+} indicator Zinpyr-1 and manipulated, as described in "Fluorescence experiments" with a modified glucose stimulation method. The test was carried out by directly applying a high concentration but small volume (<0.01 ml) of glucose to β-cells through a modified VC-6 perfusion valve "drop perfusion" device [39]. Perfusion was guided with a micromanipulator under the microscope with the tip placed near the top of the islet of interest, but not touching it, to achieve a final glucose concentration of 10 mM. This method allowed for short intervals between two glucose stimulations.

Insulin collection and measurements with islets

Islets were grown in ultra-low attachment culture dishes (60 × 15 mm Corning, Corning, NY) to keep the islets in suspension under the same conditions as for experiments as described above. Experiments were performed 48 h after islets were isolated. Groups of ten size matched islets were transferred out of the culture medium into labeled eppendorf tubes containing 500 μl of basal HEPES buffer (see "Fluorescence experiments" for the formulation of the buffer) and incubated in 5 % CO_2–95 % humidified air for 30 min. Tubes were then agitated to collect islets at the bottom of the tube so that the liquid above could be aspirated and fresh basal buffer was added (500 μl) to repeat the 30 min incubation. Finally, as much buffer as possible, without drying the islets, was aspirated and 600 μl of treatment solutions was added to each tube. After 60 min the tubes were gently agitated and additional 600 μl of the appropriate buffers were added to each tube (to mix the content of the tube and to dilute the samples). Each experiment was performed in triplicate (three tubes per treatment). Collected samples were kept on ice and were analyzed the same day as soon as sample collection was completed. To analyze collected samples, Alpco insulin (mouse) ultrasensitive ELISA kit (Salem, NH) was used according to manufacturer's instructions. Spectrophotometer uQuant (Bio-tek instruments, Winooski, VT) was used to measure absorbance.

Insulin collection and measurements with clonal HIT-T15 β-cells

Cells grown in a 75-cm flask (70 % confluency) were trypsinized and diluted into one large seed solution (12 mL), then distributed equally into 12-well plates by adding 1 mL of seed into each well [39]. Before seeding, cells were counted in seed solution using a hemocytometer (Bright-Line, Sigma, St. Louis, MO) and dissecting microscope. The average density of cells was 4.5×10^6 cells

per mL. After 48 h of incubation, once cells had attached to the culture wells, they were washed three times with 1 mL of basal HEPES buffer (contains 0.8 mM glucose; see composition in fluorescence experiments section) and incubated in the same buffer for 30 min. Following this, fresh buffer was added and cells were incubated for an additional 30 min at 37 °C. After a total of 1 h incubation in basal buffer, cells were washed three times with 1 mL basal buffer. Appropriate solutions were then added to each well as follows: basal buffer to control wells, or 10 mM glucose in HEPES buffer for experimental wells. Cells were incubated at 37 °C in 5 % CO_2–95 % humidified air. Incubation time began as soon as appropriate buffer was added and 30 µl samples were collected from each well at 60 min after stimulation. The collection and measurement of insulin were similar to islets.

Data analyses

For the time-lapse GSZS experiments, baseline intensity was acquired and expressed as the mean stable fluorescence intensity of a bright granular region over the 5–10 min period preceding the glucose stimulation. Background intensity was determined from cell free regions in images. The glucose-induced Zn^{2+} fluorescence change for each individual cell or granular region was expressed as the percent changes of fluorescence intensity (a percent deduction in the present study) to a maximum intensity recorded in the baseline intensity. The percent deduction in Zn^{2+} fluorescence intensity was calculated per the following equation: $(F_{max} - F)/(F_{max} - F_{min}) \times \%$, where F was the measured fluorescence intensity. F_{max} was the average of the baseline intensity (before glucose stimulation) in the granular region of β-cells, and F_{min} was the background fluorescence. In some plots, the changes of glucose-stimulated Zn^{2+} fluorescence were expressed as the change in fluorescence intensity relative to the baseline of each region. Changes in cell fluorescence intensity were measured through simple two-group comparisons and analyzed by Student's paired t tests or single-factor ANOVA, and $p < 0.05$ was considered significant.

Results

Glucose-stimulated Zn^{2+} secretion (GSZS)

Pancreatic islets loaded with cell membrane-permeable fluorescent Zn^{2+} indicator yielded intense fluorescence in islet cells (Fig. 1a). Since the majority of cells (70–80 %) in pancreatic islets are β-cells that contain the highest amount of Zn^{2+} in their secretory granules [54, 55], cells labeled by the Zn^{2+} indicator are most likely pancreatic

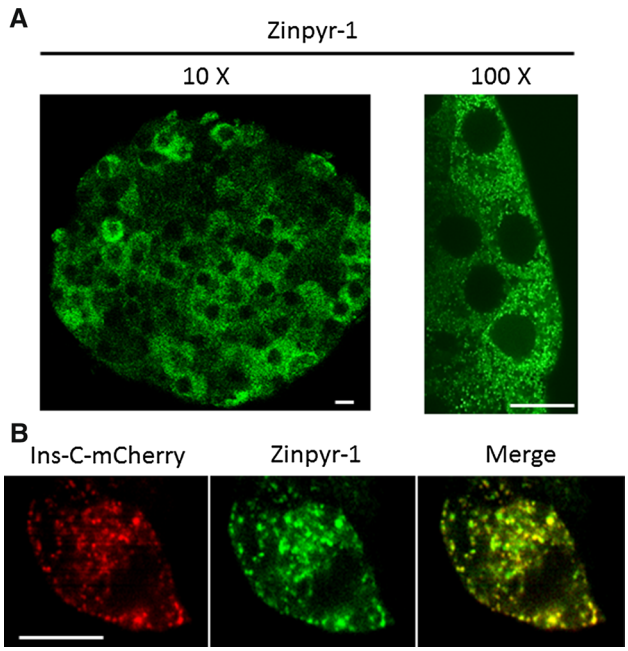

Fig. 1 Confocal images of Fluorescent Zn^{2+} staining and its co-localization with insulin-C-Cherry fusion protein (Ins-C-mCherry). **a** Cells within a pancreatic islet labeled with fluorescent Zn^{2+} indicator Zinpyr-1 (10 µM). The image at *right* is a close-up image (×100) showing the apparent granular *dots* in the cytosol. **b** *Left* A HIT-T15 cell was transfected with Ins-C-Cherry (*red* fluorescence), showing insulin secretory granules. *Middle* The application of fluorescent Zn^{2+} indicator Zinpyr-1 (*green* fluorescence) shows the similar pattern of punctate structures in the same cell. *Right* The *yellow* puncta of the merged insulin-mCherry fusion protein and *green* Zn^{2+} staining show the obvious colocalization of insulin secretory granules and punctuate Zn^{2+} staining. All images were captured at ×100 (except for the image on *left* in **a**). *Scale bars* are 10 µm in each of the images

β-cells. In individual β-cells, Zn^{2+} staining appeared to be distributed primarily in the cytosolic space between the eccentrically located nucleus (not labeled) and the plasma membrane at the opposite side of the cells, consistent with Zn^{2+} accumulation in secretory granules as previously reported [54–56] and also corresponding to the location of the insulin secretory granules. We further demonstrated that the granule-like Zn^{2+} staining was localized to insulin secretory granules by co-visualizing green Zn^{2+} fluorescence together with the red fluorescent "Ins-C-mCherry" reporter of live cell insulin granules [52, 53] in the HIT-T15 β-cell line. Our results showed the similar pattern of punctuate structures of Ins-C-mCherry as reported previously [52, 53]. Application of green fluorescent Zn^{2+} indicator Zinpyr-1 onto cells expressing the red fluorescent Ins-C-mCherry resulted in colocalization of the green and red fluorescent reporters at the same cytoplasmic punctate structures as indicated by yellow puncta in the merged image (Fig. 1b). The results suggest that the fluorescent Zn^{2+} indicator is indeed localized to the insulin secretory

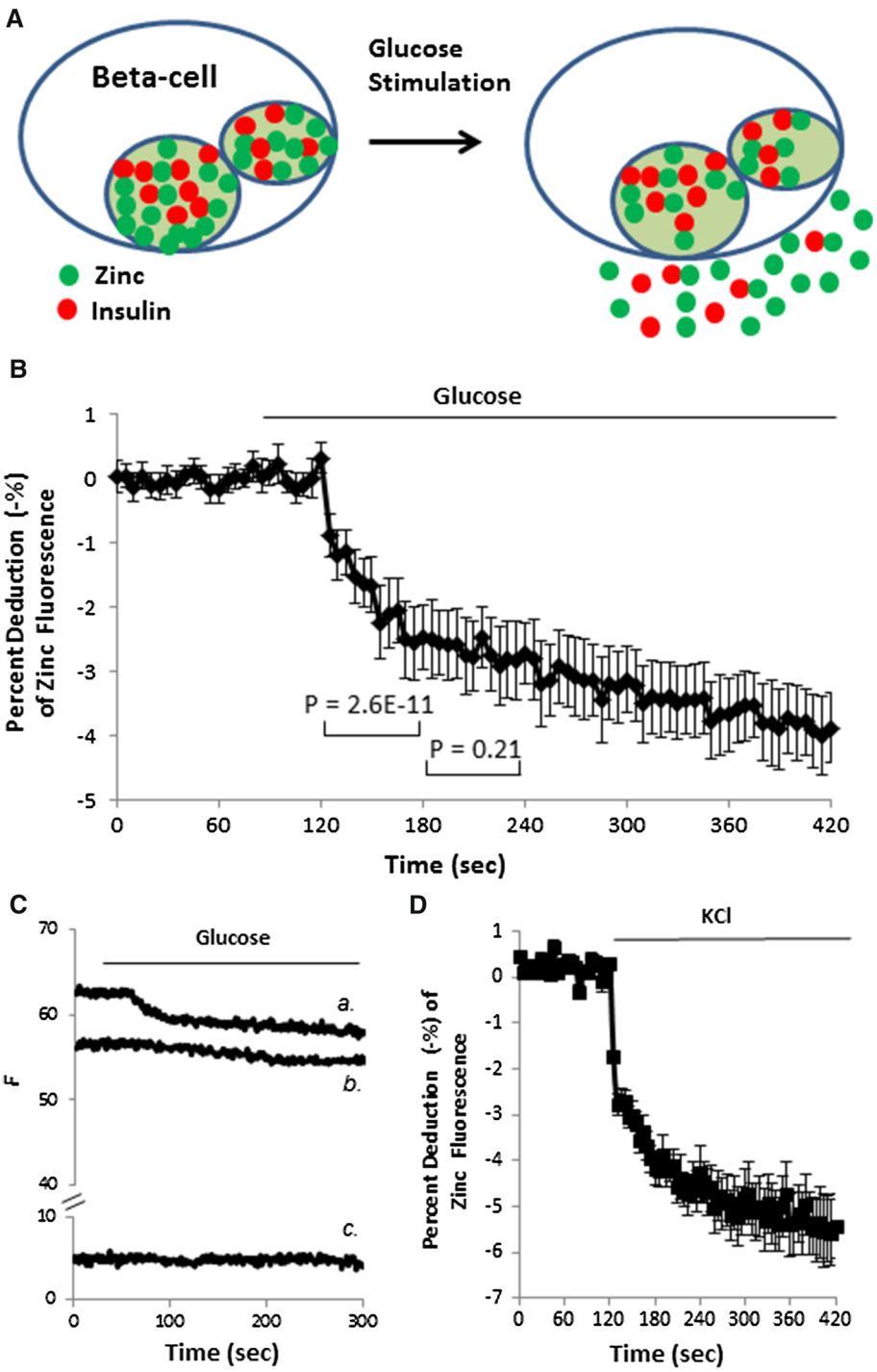

Fig. 2 Measurements of Zn^{2+} secretion in the Zinpyr-1-labeled β-cells of pancreatic islets. **a** Schematic model of a Zinpyr-1-labeled pancreatic β-cell showing the response to glucose stimulation. Zinpyr-1 bound to Zn^{2+} yields bright *green* fluorescence, which decreases upon glucose-stimulated Zn^{2+} secretion (GSZS) due to Zn^{2+} and insulin being released from the β-cells. **b** Percent reduction of Zn^{2+} fluorescence upon glucose stimulation. A rapid decrease ($p = 2.6E{-}11$ between the baseline and the sample at the end of the 1st min) in fluorescence was followed by a slow decline ($p = 0.21$ between samples at the beginning and the end of the 2nd min). Data represent mean ± SD of 51 cells in 14 islets. **c** Representative traces of real-time fluorescence measurements in a pancreatic β-cell. The upper traces (**a**, **b**) show the effect of glucose (10 mM) stimulation on the Zn^{2+} fluorescence at the cell edge (**a**) and at the region adjacent to nucleus (**b**). The lower trace (**c**) is the background fluorescence measured in a cell free region from the same test. *F*, Fluorescence Intensity. **d** Percent reduction of Zn^{2+} fluorescence by K^+ stimulation (30 mM KCl). Data represent mean ± SD of 14 cells in 3 islets

granules, which supports that Zn^{2+} and insulin are co-stored in the granules.

To study the secretion of Zn^{2+} from islets, we also loaded the islets with Zinpyr-1, which is a membrane-permeable Zn^{2+} indicator. Once loaded into a β-cell, Zinpyr-1 will stay in the Zn^{2+} containing granules. When Zn^{2+} is secreted from Zinpyr-1 loaded β-cells, the fluorescence in the granular region will be reduced. The model shown in Fig. 2a summarizes the protocol for using Zinpyr-1-labeled granular Zn^{2+} for measuring GSZS. When glucose triggers exocytosis and insulin-Zn^{2+} complexes are secreted, a reduction of Zn^{2+} fluorescence is detected directly in granules or the granular area. In this study, glucose (10 mM) (basal glucose: 0.8 mM) induced a substantial decrease in Zinpyr-1 fluorescence recorded in the granular regions of pancreatic β-cells, indicating exocytosis of granular Zn^{2+} in response

to glucose stimulation (Fig. 2b, c). The fluorescence reduction observed at the edge of cells was usually larger compared to the inner regions, and the region adjacent to the nucleus and the nucleus itself yielded little or no deduction in Zn^{2+} fluorescence during the stimulation (Fig. 2c). In unstimulated cells, the Zinpyr-1 signal remained stable over the experimental period and photobleaching was negligible [39, 46]. When islets were treated with the basal buffer (containing 0.8 mM glucose), no perceptible changes were observed. The background fluorescence (in cell free regions) did not change during the glucose stimulation (Fig. 2c). As shown in Fig. 2b the initial decrease in Zn^{2+} fluorescence in the presence of glucose stimulation was rapid and lasted about one min (rapid phase), followed by a slow decline. The percent reduction in Zn^{2+} fluorescence intensity was calculated per the following equation: $(F_{max} - F)/(F_{max} - F_{min}) \times \%$ (see "Materials and Methods" Section). During the initial rapid phase, glucose stimulation induced a less than 3 % reduction in Zn^{2+} fluorescence (2.5 % per min). After the rapid phase, the rate of fluorescence reduction by glucose stimulation was significantly slower at about 0.5 % per minute. The changes in Zn^{2+} fluorescence occurred generally 10–30 s after glucose stimulation. The application of a high concentration of potassium (30 mM KCl), that is widely used to depolarize the β-cell membrane, induced the immediate reduction of Zn^{2+} fluorescence (Fig. 2d).

Effect of Zn^{2+} on GSIS in islets

To measure the action of Zn^{2+} on GSIS from isolated islets, islets were stimulated with medium containing glucose and Zn^{2+}. Insulin collection and measurement were carried out by ultrasensitive ELISA assay (see "Materials and Methods" Section). Figure 3a shows the effect of Zn^{2+} on GSIS in islets. As anticipated, increases in the extracellular glucose concentration, from 0.8 mM basal concentration to 10 mM, induced the secretion of insulin. The application of a moderate concentration of Zn^{2+} (50 μM) alone, without glucose stimulation, had no perceptible effect on the basal secretion of insulin. However, the application of Zn^{2+} in combination with glucose stimulation, significantly inhibited GSIS, suggesting an inhibitory effect of Zn^{2+} on GSIS. On the other hand, the application of a low concentration of Zn^{2+} (1 μM) enhanced GSIS in islets (Fig. 3b). These results suggest that Zn^{2+} inhibits GSIS at higher concentrations but enhances GSIS at lower concentrations.

Effect of endogenously secreted Zn^{2+} on GSIS

Since Zn^{2+}-insulin hexamers dissociate rapidly (within seconds) after exposure of the granule interior to the extracellular milieu [41], high concentrations of unbound Zn^{2+} are likely to be produced locally in the immediate vicinity of

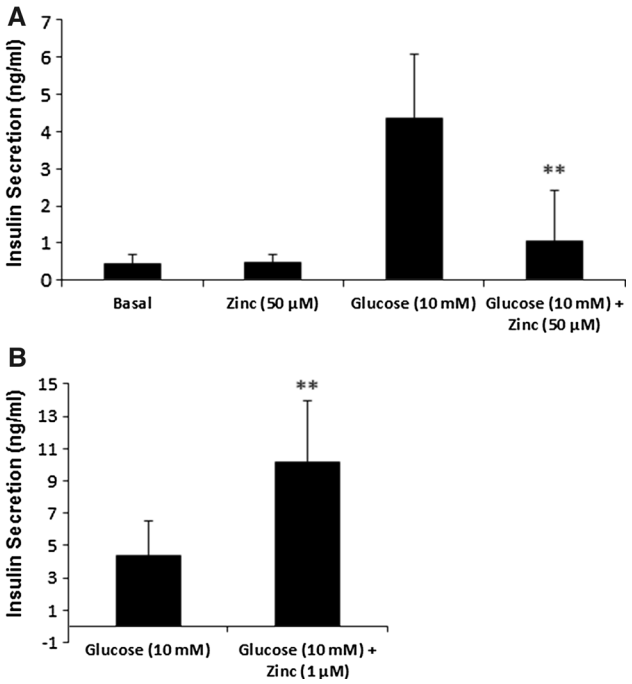

Fig. 3 Effect of Zn^{2+} on GSIS in islets. **a** Inhibitory effect of Zn^{2+} on GSIS. Pancreatic islets (10 islets in each tube) were incubated for 1 h in the presence of basal glucose (0.8 mM), high glucose (10 mM), Zn^{2+} (50 μM) alone, or glucose (10 mM)/Zn^{2+} (50 μM) as indicated. High concentration glucose stimulated insulin secretion and significantly enhanced insulin content in the medium as compared to insulin content in the basal buffer (0.8 mM glucose). Zn^{2+} alone had no effect on insulin secretion. The co-application of Zn^{2+} (50 μM) and glucose (10 mM) significantly decreased insulin secretion or insulin content compared to insulin secretion by 10 mM glucose alone. Data are expressed as mean ± SD of 6–19 independent tests (**$p < 0.01$). **b** Low concentration Zn^{2+} (1 μM) enhanced GSIS. The insulin secretion by the co-application of glucose (10 mM) and Zn^{2+} (1 μM) significantly increased insulin secretion or insulin content in the medium compared to insulin secretion by 10 mM glucose alone. Data are expressed as mean ± SD of 7–9 independent tests (**$p < 0.01$)

a β-cell. The effect of secreted Zn^{2+} on GSIS can be revealed by exposing islets to an extracellular Zn^{2+} chelator, which binds and removes the Zn^{2+} upon secretion. In this experiment, we examined the effect of applying CaEDTA ($K_{D\ zinc} = 10^{-16.4}$ M), a cell-impermeable extracellular Zn^{2+} chelator, on GSIS in pancreatic islets. CaEDTA does not alter Ca^{2+} concentrations because it is already saturated with calcium [56, 57]. Since GSIS is affected by the presence of extracellular Ca^{2+}; it is essential to use a chelator that has no effect on extracellular Ca^{2+} concentration. The application of CaEDTA with glucose (10 mM) enhanced GSIS, suggesting that overall action of Zn^{2+} on GSIS in pancreatic islets is inhibitory (Fig. 4).

Since Zn^{2+} was co-secreted with insulin, the accumulation of Zn^{2+} in the interstitium could act in a feedback loop to regulate further insulin secretion in an autocrine manner. This hypothesis was tested by directly applying glucose to

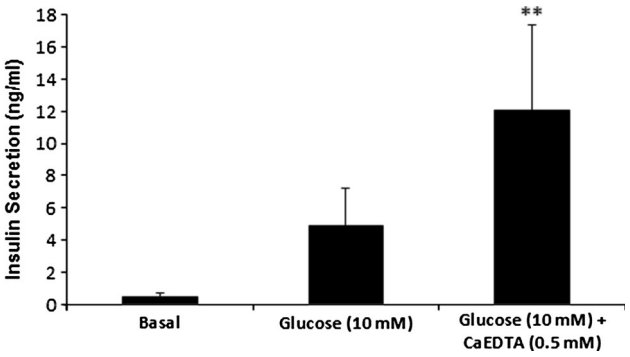

Fig. 4 Effect of Zn^{2+} chelator CaEDTA on GSIS in pancreatic islets. The application of CaEDTA (0.5 mM) enhanced GSIS. Data are expressed as mean ± SD of 8–15 independent tests (**$p < 0.01$)

pancreatic islets through a modified VC-6 perfusion valve device (Warner Instrument). Perfusion was guided with a micromanipulator under the microscope and the tip was placed near the top of the islet of interest but not touching it. An advantage of this treatment was that the islet was only briefly exposed (~2 s) to a stimulative concentration of glucose. As shown in Fig. 5a, b, the β-cell responses to glucose stimulation recovered quickly to the baseline usually within 10 s of stimulation (2 s stimulation), or much longer when the duration of stimulation increased to 5 s. This method allowed for a short interval between two or multiple brief consecutive glucose stimulations, to investigate the autocrine effect of secreted Zn^{2+}, before it diffused away, on the successive Zn^{2+} secretion or GSZS.

In the present study, two identical strengths (concentrations) of glucose stimulation were applied (20 mM) in close succession to study paired-pulse responses. When paired-pulses or two consecutive (in 20 s interval) glucose stimulations were delivered as shown in Fig. 5c, the magnitude of the second Zn^{2+} fluorescence reduction was inhibited. This was paired-pulse inhibition, which was not seen in the presence of CaEDTA (1 mM), a membrane-impermeable or extracellular Zn^{2+} chelator (Fig. 5d, e).

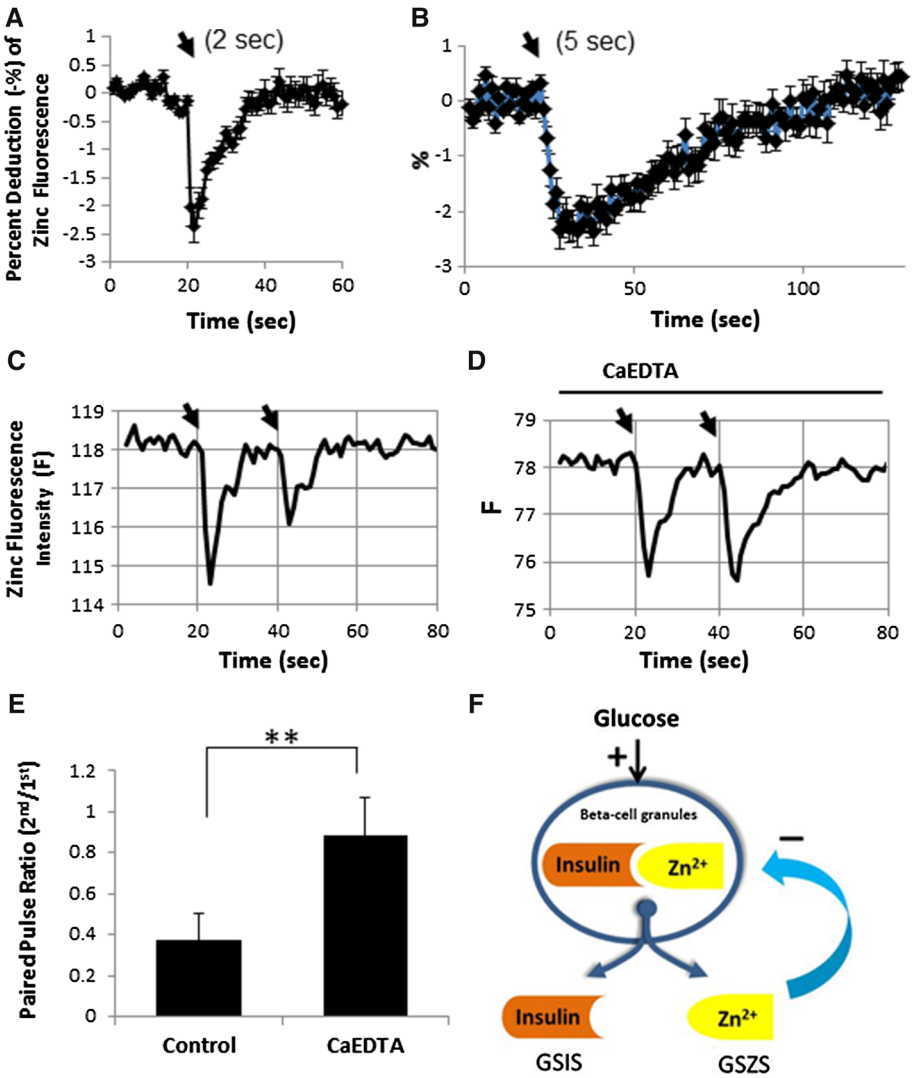

Fig. 5 Paired-pulse inhibition of pancreatic β-cell Zn^{2+} secretion. **a, b** Comparison of the reductions of Zn^{2+} fluorescence or Zn^{2+} secretion induced by short (2 s, in **a**) or longer (5 s, **b**) glucose stimulation (20 mM). **c** Paired-pulse glucose stimulations with a 20 s interval between stimulations, showing that the reduction of Zn^{2+} fluorescence following the 2nd stimulation was inhibited (paired-pulse inhibition). **d** The same experiment was done as in C, with addition of 1 mM CaEDTA (a membrane-impermeable chelator of Zn^{2+}). In the presence of the chelator the second response was similar or larger than the first compared to data in C. **e** The *bar graph* demonstrates the average paired-pulse ratios in which two stimuli were given 20 s apart in the presence and absence of CaEDTA. Data represent mean ± SD of 10 cells in 4 islets ($n = 10$; **$p < 0.01$). **f** Proposed model for an underlying Zn^{2+} feedback loop within the GSIS coupling pathway. An increase in extracellular glucose leads to the secretion of insulin and Zn^{2+} into the extracellular space and inhibits subsequent GSZS and GSIS

Therefore, the removal of secreted Zn^{2+} reversed this inhibition. Taken together, above data suggest that secreted Zn^{2+} could act as an autocrine feedback modulator of Zn^{2+} and insulin secretion.

The mechanism of Zn^{2+} inhibition in GSIS

Studies suggest that GSIS is mediated largely through a sequence of cellular events that lead to the inhibition of K_{ATP} channels and the depolarization of the β-cell membrane. This depolarization opens VGCC that facilitates Ca^{2+} entry from the extracellular environment and triggers the glucose- and voltage-dependent mechanism of the insulin exocytotic pathway. In the present study, we investigated whether the action of Zn^{2+} on GSIS is mediated through K_{ATP} channel activity since this channel is closely associated with GSIS. This experiment was carried out using the insulin releasing β-cell line, HIT-T15 to avoid possible interference by other types of cells in the islets and to ensure data being generated strictly from β-cells. Since the medium in the present study was buffered to the physiological pH at 7.4, it would prevent the formation of Zn^{2+} and insulin crystallization. We previously showed that the application of Zn^{2+} inhibited GSIS in HIT-T15 cells [39]. We interfered with the K_{ATP} channel activity by using tolbutamide, a widely used K_{ATP} blocker and an insulin secretagogue, which keeps K_{ATP} channels closed. When tolbutamide was applied, it stimulated the secretion of insulin (Fig. 6a). If the site of Zn^{2+}'s inhibitory action is on K_{ATP} channels, by opening K_{ATP} channels, Zn^{2+} would be able to reduce the tolbutamide-induced insulin secretion. However, while the moderate concentration of Zn^{2+} (50 μM) showed significant inhibition of GSIS, it has little effect on tolbutamide-stimulated insulin secretion. Only the higher concentration of Zn^{2+} (100 μM) significantly inhibited tolbutamide-stimulated insulin secretion.

To further examine the inhibitory action of Zn^{2+}, we used a K_{ATP} channel activator, diazoxide, which hyperpolarizes the β-cell membrane by opening K_{ATP} channels and thus inhibits the secretion of insulin. As shown in Fig. 6b, the application of diazoxide, as expected, inhibited GSIS. If Zn^{2+} acts on the K_{ATP} channel, then using diazoxide would blind the inhibitory action of Zn^{2+} in GSIS. The application of Zn^{2+} with diazoxide caused significantly greater inhibition of GSIS than that of diazoxide alone (Fig. 6b).

Zn^{2+} may affect electrical activity of β-cells by blocking voltage-dependent calcium channels, reducing the influx of calcium [25, 29]. The activation of L-type Ca^{2+} channels increases Ca^{2+} entry and stimulates insulin release [58, 59]. Ca^{2+} channel antagonists, such as verapamil, inhibit GSIS [60]. In this study, we used verapamil (50 μM) to block L-type Ca^{2+} channels in β-cells. As shown in Fig. 7, verapamil effectively inhibited GSIS. If the inhibitory action of

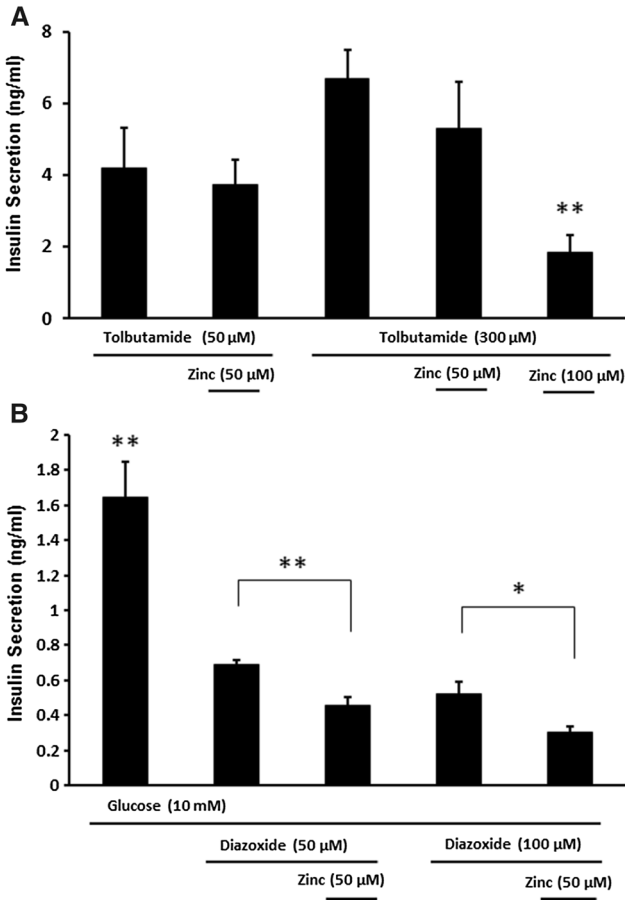

Fig. 6 Effect of Zn^{2+} on K_{ATP} channel-mediated insulin secretion. **a** The inhibition of K_{ATP} channels by tolbutamide (50 or 300 μM) stimulated the secretion of insulin. The application of 50 μM Zn^{2+} produced moderate inhibition of tolbutamide-stimulated insulin secretion. However, the application of 100 μM Zn^{2+} significantly reduced the tolbutamide-stimulated insulin secretion. Data are expressed as mean ± SD of 7 tests ($n = 7$; $^{**}p < 0.01$). **b** The activation of K_{ATP} by diazoxide (50 or 100 μM) inhibited GSIS. The addition of Zn^{2+} in the presence of diazoxide further inhibited GSIS. Data are expressed as mean ± SD of 5–7 tests ($n = 5$–7; $^{*}p < 0.05$, $^{**}p < 0.01$)

Zn^{2+} in GSIS is due to Zn^{2+}'s actions on L-type channels, then using verapamil would blind the inhibitory action of Zn^{2+} in GSIS. However, the application of Zn^{2+} with verapamil caused greater inhibition of GSIS than by the application of verapamil alone. There was significant difference between verapamil alone and verapamil + zinc in GSIS.

Discussion

The present study demonstrated that, in isolated mouse pancreatic islets, endogenous Zn^{2+}, secreted during GSIS, primarily in turn played an inhibitory role on GSIS. The

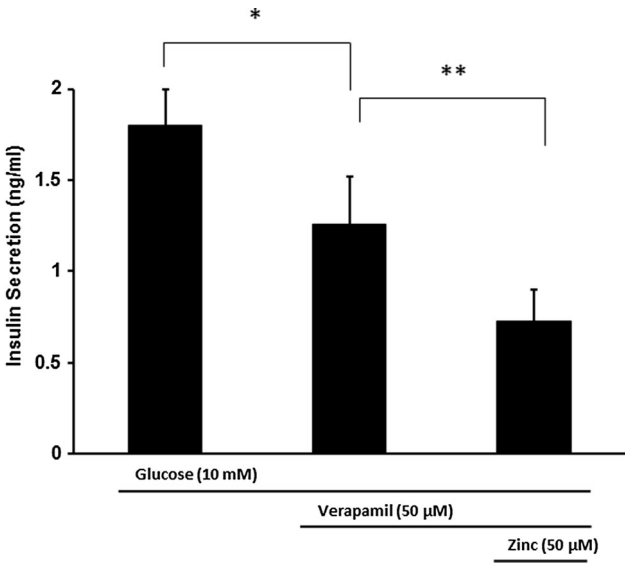

Fig. 7 Effect of Zn^{2+} and L-type channel blocker, verapamil on GSIS. Insulin secretion was measured in the presence of glucose (10 mM), verapamil (50 μM), and Zn^{2+} (50 μM). The application of glucose induced GSIS. The application of verapamil significantly inhibited the GSIS. GSIS was significantly inhibited further when Zn^{2+} was co-applied with verapamil. Data are expressed as mean ± SD of 5 tests ($n = 5$; *$p < 0.05$, **$p < 0.01$)

application of Zn^{2+} chelator, CaEDTA, to remove extracellular Zn^{2+}, revealed that an overall effect of Zn^{2+} on the insulin secretion of pancreatic islets was inhibitory. In the study of GSZS, data supported that Zn^{2+}, co-packaged with insulin, was co-secreted with insulin during the glucose stimulation of islets. When paired-pulse glucose stimulations were applied at a short interval, the second pulse response of Zn^{2+} (to glucose) was significantly blunted; however, this paired-pulse inhibition was also reversed by Zn^{2+} chelation, suggesting a negative feedback mechanism, in which the initial glucose-stimulated Zn^{2+} release inhibits subsequent Zn^{2+} secretion, subsequently inhibiting insulin co-secretion as well. Investigation of the mechanism of Zn^{2+} action on GSIS revealed that similar to GSIS, GSZS was triggered by membrane depolarization and the inhibitory action of Zn^{2+} was mostly mediated by K_{ATP} channels and voltage-dependent calcium channels. However, other mechanisms may also be involved in Zn^{2+} modulation of GSIS. The present findings strongly support the hypothesis that secreted Zn^{2+} during GSIS can regulate β-cell insulin secretion possibly through an autocrine feedback mechanism.

A better understanding of stimulus-secretion coupling and exocytosis in β-cells is of intense interest due to mounting evidence for the importance of defective insulin secretion in T2D [4, 32, 33]. Previous studies using various techniques, including atomic absorption spectroscopy, X-ray microprobe analysis, and fluorescent Zn^{2+} indicators, have demonstrated that the insulin secretory granules of pancreatic β-cells contain large amounts of Zn^{2+} [34, 38, 61–64]. Membrane-impermeable or extracellular fluorescent Zn^{2+} indicator has been used to monitor Zn^{2+} as it is released from the β-cells [35, 36]. In the present study, glucose triggers insulin-Zn^{2+} complex exocytosis and dissociation giving rise to a small but detectable reduction of Zn^{2+} fluorescence in granular areas during GSIS (Fig. 2b), which complements previous studies that showed glucose-stimulated increase of extracellular Zn^{2+} [35, 36]. Because Zn^{2+} is co-stored with insulin, it is expected that the temporal patterns of its secretion could be similar to those of insulin. Furthermore, the temporal relationship between membrane potential, capacitance (a measure of exocytosis), and Zn^{2+} release has been described using the Zn^{2+} sensor ZIMIR [65]. As we know, glucose stimulation of pancreatic β-cells induces the secretion of insulin [30, 45, 66], in which insulin is released by Ca^{2+}-dependent exocytosis of the secretory granules [8, 67]. We have previously shown that, during GSZS, Zn^{2+} secretion was also calcium and glucose concentration dependent [39]. Furthermore, the current study revealed that treatment of pancreatic islets with high K^+, to induce the depolarization of the plasma membrane, led to an increase in Zn^{2+} secretion similar to GSZS (Fig. 2d).

The biphasic pattern of GSZS in isolated pancreatic islets in the current study appears very similar to that in β-cell lines in our previous report [39]. During the initial rapid phase, glucose stimulation induced about 2–10 % reduction in Zn^{2+} fluorescence (2.5 % per min in the islets), reflecting a swift glucose-induced Zn^{2+} release, which consistently peaked at 1 min (Fig. 2b). After the rapid phase, the rate of fluorescence reduction following glucose stimulation was significantly slower at about 0.5–2 % per min (0.5 % per min in the islets). Biphasic insulin secretion is the normal response of β-cells to a rapid and sustained increase in glucose concentration. Impairment of the first phase of GSIS has long and repeatedly been recognized as an early sign of β–cell dysfunction in type 2 diabetic patients [68, 69]. Strikingly, the fast initial drop in Zn^{2+} fluorescence recorded in the present study markedly resembles the first-phase of insulin response. Further studies to explore whether this biphasic pattern of GSZS corresponds to biphasic insulin secretion would provide deeper understanding of the physiological regulation of insulin secretion and any alterations in pathophysiological conditions such as in diabetes mellitus.

The study of Zn^{2+} and insulin interactions has been focused on the requirement of Zn^{2+} for the assembly and stability of insulin. In the present study, we have demonstrated that Zn^{2+}, secreted from pancreatic β-cells, is capable of regulating insulin secretion. Although the application of Zn^{2+} inhibits or enhances GSIS depending on Zn^{2+} concentration in

pancreatic islet β-cells (Fig. 3), removal of secreted Zn^{2+} by Zn^{2+} chelation increased GSIS, suggesting that the overall action of secreted Zn^{2+} on pancreatic islets is inhibitory (Fig. 4). In particular, the paired-pulse glucose stimulation study provided direct evidence that the secretion of Zn^{2+} can inhibit GSZS (Fig. 5). This paired-pulse inhibition of Zn^{2+} secretion may represent a negative feedback phenomenon that involves Zn^{2+} acting on β-cell insulin exocytosis to inhibit its own release and insulin secretion. Because Zn^{2+} is co-secreted with insulin, it is expected that the secreted Zn^{2+} could also affect insulin exocytosis. Such a local inhibition may provide an important negative feedback control against excessive excitatory inputs by the continuous presence of glucose. However, the mechanism underlying the proposed negative feedback regulation is unclear at present.

It has been shown that the electrical excitability of pancreatic β-cells controls the triggering phase of insulin secretion and depends on the coordinated activity of specific ion channels including the L-type calcium channel [21], Na^+ channels [70], and ATP-regulated potassium channels (K_{ATP}) [8, 71]. As described above, Zn^{2+} exhibits profound modulatory effects on a variety of ligand- and voltage-gated mammalian ion channels [14]. Specifically relevant to the present study, Zn^{2+} has been identified to reversibly regulate the L-type calcium channel [72] and K_{ATP} channel [26] (also see [16, 73]). Although very little is known about the regulation of insulin secretion by Zn^{2+}, especially by co-secreted Zn^{2+}, there are previous reports on the inhibitory effect of Zn^{2+} on glucose-induced electrical activity and GSIS from pancreatic β-cells [24, 25].

The concentration of Zn^{2+} in the insulin secretory granules was estimated at about 20 mM [63, 74]. Whereas the low pH environment ($pH < 6$) inside granules of β-cells favors the binding of Zn^{2+} to insulin, the higher pH in the extracellular milieu favors the dissociation of Zn^{2+} from insulin. Since Zn^{2+}-insulin hexamers start dissociating immediately (within seconds) after exposure of the granule interior to the extracellular milieu [38, 41], high concentrations of unbound free Zn^{2+} are likely to be produced locally in the interstitium surrounding the β-cells. In addition, β-cell granules contain Zn^{2+} in 1–1.5-fold excess of that necessary to form Zn^{2+}-insulin hexamers [63]. The latter suggests that Zn^{2+} and insulin secretions may not be in a 1:1 ratio and that glucose stimulation could release more Zn^{2+} than insulin. Several studies have proposed that Zn^{2+} can influence insulin secretion in pancreatic β-cells through a negative feedback loop, involving both K_{ATP} and VGCC conductance. Meanwhile, Zn^{2+}, at μM concentration, activates K_{ATP} channels [23] and inhibits the voltage-gated calcium channels [23, 72]. Zn^{2+} is both an intracellular and extracellular regulator of K_{ATP} channel function [75]. Taken together, it is conceivable that secreted Zn^{2+}, if the concentration is sufficiently high, alters the function of pancreatic β-cells by acting on ion channels, or another mechanism that regulates insulin secretion. This view is further supported by the findings of the present study that Zn^{2+} inhibits insulin secretion or GSIS (Fig. 4 and see [24, 25]). Furthermore, our results show that a moderate concentration (50 μM) of Zn^{2+} exerts profound inhibition of GSIS with no significant effect on tolbutamide-stimulated insulin secretion (Fig. 6a). Comparison of the effects of the K_{ATP} channel blocker, diazoxide and the Ca^{2+} channel blocker, verapamil on GSIS shows that they mediate the majority of the Zn^{2+} inhibition (Fig. 6b, Fig. 7). However, the remaining unidentified inhibition suggests another mechanism may also be involved in Zn^{2+}-mediated regulation of GSIS.

In pancreatic β-cells the transport of Zn^{2+} into secretory granules seems to be mainly accomplished by Zn^{2+} transporter 8 (ZnT8), which is identified primarily in the secretory granules of β-cells [37, 76]. ZnT8 knockout or down-regulation reduced dense core granules and insulin content, although they displayed normal proinsulin biosynthesis [37, 76]. The changes in ZnT8 expression may affect β-cell function. However, there are discrepancies from these studies in terms of glucose tolerance, insulin homeostasis, and the risk for diabetes. To this point, the reasons for these differences remain unclear and require further investigation. For example, in the study by Wijesekara et al. [77], that ZnT8 knockout mice were glucose intolerant and the isolated ZnT8 knockout islets displayed reduced first-phase insulin secretion, which could have been due to reduced cargo or to the reduced or delayed exocytosis of insulin granules. On the other hand, in the study by Lemaire et al. [4] ZnT8 knockout mice showed normal glucose tolerance and insulin homeostasis, whereas KCl stimulation evoked higher, but not significant, insulin secretion from islets of ZnT8 knockout mice. Further, the study by Nicolson et al. [78] showed that ZnT8 knockout mice were glucose intolerant with reduced insulin secretion in vivo. However, in the same line with our findings, they ([78]) did demonstrate that glucose-induced insulin secretion was significantly enhanced in ZnT8 knockout mice islets in vitro.

In summary, this work suggests a critical role for Zn^{2+} in regulation of GSIS in pancreatic islets. Specifically, the phenomenon that released Zn^{2+} exhibits a negative feedback and inhibits further secretion in an autocrine manner is a novel finding. We have shown that β-cell-secreted Zn^{2+} exerts a feedback control on its own release in pancreatic islets. An inhibitory control mechanism of endogenous Zn^{2+} acting locally on insulin secretion of β-cells may be of great importance in the physiology of pancreatic function. This negative feedback mechanism might limit the excess of insulin (or Zn^{2+}) released during prolonged or repetitive stimulations by glucose or that of other insulin secretagogues. This finding may provide a new insight into the physiological modulation of an

inhibitory mechanism on insulin-secreting β-cells to prevent β-cell fatigue and preserve the insulin capacity during prolonged glucose stimulation, subsequently reducing susceptibility to develop diabetes. Therefore, it would be reasonable to expect that further understanding of this inhibitory mechanism would permit a novel therapeutic approach to diabetes mellitus.

Acknowledgments We thank Dr. Calvin James for his advice of preparing pancreatic islets, Dr. Peter Drain for mCherry-tagged insulin fusion protein, and Dr. Tomohiko Sugiyama for helping with propagation and purification of construct DNA kindly gifted to us by Dr. Drain. We also wish to thank the Imaging Facility of the Ohio University Neuroscience Program for their support.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Y.V. Li, Zinc and insulin in pancreatic beta-cells. Endocrine **45**(2), 178–189 (2014). doi:10.1007/s12020-013-0032-x
2. D.A. Scott, A.M. Fisher, The insulin and the zinc content of normal and diabetic pancreas. J. Clin. Investig. **17**(6), 725–728 (1938). doi:10.1172/JCI101000
3. A.B. Chausmer, Zinc, insulin and diabetes. J. Am. Coll. Nutr. **17**(2), 109–115 (1998)
4. K. Lemaire, M.A. Ravier, A. Schraenen, J.W. Creemers, R. Van de Plas, M. Granvik, L. Van Lommel, E. Waelkens, F. Chimienti, G.A. Rutter, P. Gilon, P.A. in't Veld, F.C. Schuit, Insulin crystallization depends on zinc transporter ZnT8 expression, but is not required for normal glucose homeostasis in mice. Proc Natl Acad Sci U S A **106**(35), 14872–14877 (2009). doi:10.1073/pnas.0906587106
5. J. Brandao-Neto, C.A. Silva, T. Shuhama, J.A. Silva, L. Oba, Renal handling of zinc in insulin-dependent diabetes mellitus patients. Biometals **14**(1), 75–80 (2001)
6. J.J. Cunningham, A. Fu, P.L. Mearkle, R.G. Brown, Hyperzincuria in individuals with insulin-dependent diabetes mellitus: concurrent zinc status and the effect of high-dose zinc supplementation. Metabolism **43**(12), 1558–1562 (1994)
7. W.B. Kinlaw, A.S. Levine, J.E. Morley, S.E. Silvis, C.J. McClain, Abnormal zinc metabolism in type II diabetes mellitus. Am. J. Med. **75**(2), 273–277 (1983). doi:10.1016/0002-9343(83)91205-6
8. P. Proks, J.D. Lippiat, Membrane ion channels and diabetes. Curr. Pharm. Des. **12**(4), 485–501 (2006)
9. G.A. Rutter, Think zinc: new roles for zinc in the control of insulin secretion. Islets **2**(1), 49–50 (2011). doi:10.4161/isl.2.1.10259
10. M. Foster, S. Samman, Zinc and redox signaling: perturbations associated with cardiovascular disease and diabetes mellitus. Antioxid. Redox Signal **13**(10), 1549–1573 (2010). doi:10.1089/ars.2010.3111
11. M.D. Bosco, D.M. Mohanasundaram, C.J. Drogemuller, C.J. Lang, P.D. Zalewski, P.T. Coates, Zinc and zinc transporter regulation in pancreatic islets and the potential role of zinc in islet transplantation. Rev Diabet Stud **7**(4), 263–274 (2010). doi:10.1900/RDS.2010.7.263
12. N. Wijesekara, F. Chimienti, M.B. Wheeler, Zinc, a regulator of islet function and glucose homeostasis. Diabetes Obes. Metab. **11**(Suppl 4), 202–214 (2009). doi:10.1111/j.1463-1326.2009.01110.x
13. Y.V. Li, Zinc Overload in Stroke, in *Metal Ion in Stroke*, ed. by Y.V. Li, J.H. Zhang (Springer Science+Business Media, New York, 2012), pp. 167–189
14. Y.V. Li, C.J. Hough, J.M. Sarvey, Do we need zinc to think? Sci. STKE **182**, 19 (2003)
15. C.J. Frederickson, J.Y. Koh, A.I. Bush, The neurobiology of zinc in health and disease. Nat. Rev. Neurosci. **6**(6), 449–462 (2005). doi:10.1038/nrn1671
16. S.L. Sensi, P. Paoletti, A.I. Bush, I. Sekler, Zinc in the physiology and pathology of the CNS. Nat. Rev. Neurosci. **10**(11), 780–791 (2009). doi:10.1038/nrn2734
17. A. Mathie, G.L. Sutton, C.E. Clarke, E.L. Veale, Zinc and copper: pharmacological probes and endogenous modulators of neuronal excitability. Pharmacol. Ther. **111**(3), 567–583 (2006)
18. D.W. Barnett, D.M. Pressel, S. Misler, Voltage-dependent Na+ and Ca2+ currents in human pancreatic islet beta-cells: evidence for roles in the generation of action potentials and insulin secretion. Pflugers Arch. **431**(2), 272–282 (1995)
19. C.B. Newgard, J.D. McGarry, Metabolic coupling factors in pancreatic beta-cell signal transduction. Annu. Rev. Biochem. **64**, 689–719 (1995). doi:10.1146/annurev.bi.64.070195.003353
20. A. Tarasov, J. Dusonchet, F. Ashcroft, Metabolic regulation of the pancreatic beta-cell ATP-sensitive K+ channel: a pas de deux. Diabetes **53**(Suppl 3), S113–S122 (2004)
21. F.M. Ashcroft, P. Rorsman, G. Trube, Single calcium channel activity in mouse pancreatic beta-cells. Ann. N. Y. Acad. Sci. **560**, 410–412 (1989)
22. I. Findlay, F.M. Ashcroft, R.P. Kelly, P. Rorsman, O.H. Petersen, G. Trube, Calcium currents in insulin-secreting beta-cells. Ann. N. Y. Acad. Sci. **560**, 403–409 (1989)
23. A. Bloc, T. Cens, H. Cruz, Y. Dunant, Zinc-induced changes in ionic currents of clonal rat pancreatic -cells: activation of ATP-sensitive K+ channels. J. Physiol. **529**(Pt 3), 723–734 (2000)
24. R. Ferrer, B. Soria, C.M. Dawson, I. Atwater, E. Rojas, Effects of Zn2+ on glucose-induced electrical activity and insulin release from mouse pancreatic islets. Am. J. Physiol. **246**(5 Pt 1), C520–C527 (1984)
25. T. Ghafghazi, M.L. McDaniel, P.E. Lacy, Zinc-induced inhibition of insulin secretion from isolated rat islets of Langerhans. Diabetes **30**(4), 341–345 (1981)
26. V. Bancila, I. Nikonenko, Y. Dunant, A. Bloc, Zinc inhibits glutamate release via activation of pre-synaptic K channels and reduces ischaemic damage in rat hippocampus. J. Neurochem. **90**(5), 1243–1250 (2004). doi:10.1111/j.1471-4159.2004.02587.xJNC2587
27. B. Holst, K.L. Egerod, C. Jin, P.S. Petersen, M.V. Ostergaard, J. Hald, A.M. Sprinkel, J. Storling, T. Mandrup-Poulsen, J.J. Holst, P. Thams, C. Orskov, N. Wierup, F. Sundler, O.D. Madsen, T.W. Schwartz, G protein-coupled receptor 39 deficiency is associated with pancreatic islet dysfunction. Endocrinology **150**(6), 2577–2585 (2009). doi:10.1210/en.2008-1250
28. P. Popovics, A.J. Stewart, GPR39: a Zn(2+)-activated G protein-coupled receptor that regulates pancreatic, gastrointestinal and neuronal functions. Cell Mol. Life Sci. **68**(1), 85–95 (2011). doi:10.1007/s00018-010-0517-1
29. C.A. Aspinwall, S.A. Brooks, R.T. Kennedy, J.R. Lakey, Effects of intravesicular H+ and extracellular H+ and Zn2+ on insulin secretion in pancreatic beta cells. J. Biol. Chem. **272**(50), 31308–31314 (1997)
30. B. Ahren, Type 2 diabetes, insulin secretion and beta-cell mass. Curr. Mol. Med. **5**(3), 275–286 (2005)
31. D.M. Muoio, C.B. Newgard, Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. Nat. Rev. Mol. Cell Biol. **9**(3), 193–205 (2008). doi:10.1038/nrm2327

32. Y. Lin, Z. Sun, Current views on type 2 diabetes. J. Endocrinol. **204**(1), 1–11 (2012). doi:10.1677/JOE-09-0260
33. G. Tian, S. Sandler, E. Gylfe, A. Tengholm, Glucose- and hormone-induced cAMP oscillations in alpha- and beta-cells within intact pancreatic islets. Diabetes **60**(5), 1535–1543 (2011). doi:10.2337/db10-1087
34. P.D. Zalewski, S.H. Millard, I.J. Forbes, O. Kapaniris, A. Slavotinek, W.H. Betts, A.D. Ward, S.F. Lincoln, I. Mahadevan, Video image analysis of labile zinc in viable pancreatic islet cells using a specific fluorescent probe for zinc. J. Histochem. Cytochem. **42**(7), 877–884 (1994)
35. W.J. Qian, K.R. Gee, R.T. Kennedy, Imaging of Zn2 + release from pancreatic beta-cells at the level of single exocytotic events. Anal. Chem. **75**(14), 3468–3475 (2003)
36. W.J. Qian, J.L. Peters, G.M. Dahlgren, K.R. Gee, R.T. Kennedy, Simultaneous monitoring of Zn2+ secretion and intracellular Ca2+ from islets and islet cells by fluorescence microscopy. Biotechniques **37**(6), 922–924 (2004). 926, 928-930 passim
37. F. Chimienti, S. Devergnas, F. Pattou, F. Schuit, R. Garcia-Cuenca, B. Vandewalle, J. Kerr-Conte, L. Van Lommel, D. Grunwald, A. Favier, M. Seve, In vivo expression and functional characterization of the zinc transporter ZnT8 in glucose-induced insulin secretion. J. Cell Sci. **119**(Pt 20), 4199–4206 (2006). doi:10.1242/jcs.03164
38. B. Formby, F. Schmid-Formby, G.M. Grodsky, Relationship between insulin release and 65zinc efflux from rat pancreatic islets maintained in tissue culture. Diabetes **33**(3), 229–234 (1984)
39. K.G. Slepchenko, C.B. James, Y.V. Li, Inhibitory effect of zinc on glucose-stimulated zinc/insulin secretion in an insulin-secreting beta-cell line. Exp. Physiol. **98**(8), 1301–1311 (2013). doi:10.1113/expphysiol.2013.072348
40. D.P. Figlewicz, B. Formby, A.T. Hodgson, F.G. Schmid, G.M. Grodsky, Kinetics of 65zinc uptake and distribution in fractions from cultured rat islets of langerhans. Diabetes **29**(10), 767–773 (1980)
41. G. Gold, G.M. Grodsky, Kinetic aspects of compartmental storage and secretion of insulin and zinc. Experientia **40**(10), 1105–1114 (1984)
42. E.D. Kilpatrick, R.P. Robertson, Differentiation between glucose-induced desensitization of insulin secretion and beta-cell exhaustion in the HIT-T15 cell line. Diabetes **47**(4), 606–611 (1998)
43. R.F. Santerre, R.A. Cook, R.M. Crisel, J.D. Sharp, R.J. Schmidt, D.C. Williams, C.P. Wilson, Insulin synthesis in a clonal cell line of simian virus 40-transformed hamster pancreatic beta cells. Proc. Natl. Acad. Sci. U.S.A. **78**(7), 4339–4343 (1981)
44. M. Skelin, M. Rupnik, A. Cencic, Pancreatic beta cell lines and their applications in diabetes mellitus research. Altex **27**(2), 105–113 (2010)
45. J. Miyazaki, K. Araki, E. Yamato, H. Ikegami, T. Asano, Y. Shibasaki, Y. Oka, K. Yamamura, Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. Endocrinology **127**(1), 126–132 (1990)
46. K.G. Slepchenko, Y.V. Li, Rising intracellular zinc by membrane depolarization and glucose in insulin-secreting clonal HIT-T15 beta cells. Exp. Diabetes Res. **2012**, 190309 (2012). doi:10.1155/2012/190309
47. J.K. Ketterman, Y.V. Li, Presynaptic evidence for zinc release at the mossy fiber synapse of rat hippocampus. J. Neurosci. Res. **86**(2), 422–434 (2008)
48. S.C. Burdette, G.K. Walkup, B. Spingler, R.Y. Tsien, S.J. Lippard, Fluorescent sensors for Zn(2+) based on a fluorescein platform: synthesis, properties and intracellular distribution. J. Am. Chem. Soc. **123**(32), 7831–7841 (2001)
49. G.K. Walkup, S.C. Burdette, S.J. Lippard, R.Y. Tsien, A new cell-permeable fluorescent probe for Zn2+. J. Am. Chem. Soc. **122**(23), 5644–5645 (2000). doi:10.1021/ja000868p
50. G. Anderegg, E. Hubmann, N.G. Podder, F. Wenk, Pyridinderivate als Komplexbildner. XI. Die Thermodynamik der Metallkomplexbildung mit Bis-, Tris- und Tetrakis[(2-pyridyl)methyl]-aminen. Helv. Chim. Acta **60**(1), 123–140 (1977). doi:10.1002/hlca.19770600115
51. P. Arslan, F. Di Virgilio, M. Beltrame, R.Y. Tsien, T. Pozzan, Cytosolic Ca2+ homeostasis in Ehrlich and Yoshida carcinomas. A new, membrane-permeant chelator of heavy metals reveals that these ascites tumor cell lines have normal cytosolic free Ca2+. J. Biol. Chem. **260**(5), 2719–2727 (1985)
52. K.Y. Chu, M.J. Briggs, T. Albrecht, P.F. Drain, J.D. Johnson, Differential regulation and localization of carboxypeptidase D and carboxypeptidase E in human and mouse beta-cells. Islets **3**(4), 155–165 (2011)
53. S. Watkins, X. Geng, L. Li, G. Papworth, P.D. Robbins, P. Drain, Imaging secretory vesicles by fluorescent protein insertion in propeptide rather than mature secreted peptide. Traffic **3**(7), 461–471 (2002)
54. D. Baetens, F. Malaisse-Lagae, A. Perrelet, L. Orci, Endocrine pancreas: three-dimensional reconstruction shows two types of islets of langerhans. Science **206**(4424), 1323–1325 (1979)
55. O. Cabrera, D.M. Berman, N.S. Kenyon, C. Ricordi, P.O. Berggren, A. Caicedo, The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. Proc. Natl. Acad. Sci. U.S.A. **103**(7), 2334–2339 (2006). doi:10.1073/pnas.0510790103
56. D.M. Bers, C.W. Patton, R. Nuccitelli, A practical guide to the preparation of Ca2+ buffers. Methods Cell Biol. **40**, 3–29 (1994)
57. Y. Li, C.J. Hough, C.J. Frederickson, J.M. Sarvey, Induction of mossy fiber –> Ca3 long-term potentiation requires translocation of synaptically released Zn2+. J. Neurosci. **21**(20), 8015–8025 (2001)
58. H.L. Hellmich, C.J. Frederickson, D.S. DeWitt, R. Saban, M.O. Parsley, R. Stephenson, M. Velasco, T. Uchida, M. Shimamura, D.S. Prough, Protective effects of zinc chelation in traumatic brain injury correlate with upregulation of neuroprotective genes in rat brain. Neurosci. Lett. **355**(3), 221–225 (2004)
59. T.J. Kamp, J.W. Hell, Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C. Circ. Res. **87**(12), 1095–1102 (2000)
60. G. Devis, G. Somers, E. Van Obberghen, W.J. Malaisse, Calcium antagonists and islet function. I. Inhibition of insulin release by verapamil. Diabetes **24**(6), 247–251 (1975)
61. S. Falkmer, R. Odselius, B. Blondel, M. Prentki, C.B. Wollheim, Energy dispersive X-ray microanalysis of zinc and calcium in organelles of insulin-producing cells of the mouse, rat, and a fish. Biomed. Biochim. Acta **44**(1), 37–43 (1985)
62. U. Lindh, L. Juntti-Berggren, P.O. Berggren, B. Hellman, Proton microprobe analysis of pancreatic beta-cells. Biomed. Biochim. Acta **44**(1), 55–61 (1985)
63. M.C. Foster, R.D. Leapman, M.X. Li, I. Atwater, Elemental composition of secretory granules in pancreatic islets of Langerhans. Biophys. J. **64**(2), 525–532 (1993). doi:10.1016/S0006-3495(93)81397-3
64. W.J. Qian, C.A. Aspinwall, M.A. Battiste, R.T. Kennedy, Detection of secretion from single pancreatic beta-cells using extracellular fluorogenic reactions and confocal fluorescence microscopy. Anal. Chem. **72**(4), 711–717 (2000)
65. D. Li, S. Chen, E.A. Bellomo, A.I. Tarasov, C. Kaut, G.A. Rutter, W.H. Li, Imaging dynamic insulin release using a fluorescent zinc indicator for monitoring induced exocytotic release (ZIMIR). Proc. Natl. Acad. Sci. U.S.A. **108**(52), 21063–21068 (2011). doi:10.1073/pnas.1109773109
66. L.S. Satin, Localized calcium influx in pancreatic beta-cells: its significance for Ca2+-dependent insulin secretion from the islets of Langerhans. Endocrine **13**(3), 251–262 (2000). doi:10.1385/ENDO:13:3:251
67. S. Jitrapakdee, A. Wutthisathapornchai, J.C. Wallace, M.J. MacDonald, Regulation of insulin secretion: role of

mitochondrial signalling. Diabetologia **53**(6), 1019–1032 (2010). doi:10.1007/s00125-010-1685-0
68. J.C. Henquin, M. Nenquin, P. Stiernet, B. Ahren, In vivo and in vitro glucose-induced biphasic insulin secretion in the mouse: pattern and role of cytoplasmic Ca2+ and amplification signals in beta-cells. Diabetes **55**(2), 441–451 (2006)
69. S. Seino, T. Shibasaki, K. Minami, Dynamics of insulin secretion and the clinical implications for obesity and diabetes. J. Clin. Investig. **121**(6), 2118–2125 (2011). doi:10.1172/JCI45680
70. I.I. Nita, M. Hershfinkel, C. Kantor, G.A. Rutter, E.C. Lewis, I. Sekler, Pancreatic beta-cell Na+ channels control global Ca2+ signaling and oxidative metabolism by inducing Na+ and Ca2+ responses that are propagated into mitochondria. FASEB J. **28**(8), 3301–3312 (2014). doi:10.1096/fj.13-248161
71. L. Aguilar-Bryan, J. Bryan, Molecular biology of adenosine triphosphate-sensitive potassium channels. Endocr. Rev. **20**(2), 101–135 (1999)
72. B. Turan, Zinc-induced changes in ionic currents of cardiomyocytes. Biol. Trace Elem. Res. **94**(1), 49–60 (2003). doi:10.1385/BTER:94:1:49
73. X.P. Chu, J.A. Wemmie, W.Z. Wang, X.M. Zhu, J.A. Saugstad, M.P. Price, R.P. Simon, Z.G. Xiong, Subunit-dependent high-affinity zinc inhibition of acid-sensing ion channels. J. Neurosci. **24**(40), 8678–8689 (2004). doi:10.1523/JNEUROSCI.2844-04.2004
74. M. Hutton, The effects of environmental lead exposure and in vitro zinc on tissue delta-aminolevulinic acid dehydratase in urban pigeons. Comp. Biochem. Physiol. C **74**(2), 441–446 (1983)
75. A.L. Prost, A. Bloc, N. Hussy, R. Derand, M. Vivaudou, Zinc is both an intracellular and extracellular regulator of KATP channel function. J. Physiol. **559**(Pt 1), 157–167 (2004). doi:10.1113/jphysiol.2004.065094
76. F. Chimienti, S. Devergnas, A. Favier, M. Seve, Identification and cloning of a beta-cell-specific zinc transporter, ZnT-8, localized into insulin secretory granules. Diabetes **53**(9), 2330–2337 (2004)
77. N. Wijesekara, F.F. Dai, A.B. Hardy, P.R. Giglou, A. Bhattacharjee, V. Koshkin, F. Chimienti, H.Y. Gaisano, G.A. Rutter, M.B. Wheeler, Beta cell-specific Znt8 deletion in mice causes marked defects in insulin processing, crystallisation and secretion. Diabetologia **53**(8), 1656–1668 (2010). doi:10.1007/s00125-010-1733-9
78. T.J. Nicolson, E.A. Bellomo, N. Wijesekara, M.K. Loder, J.M. Baldwin, A.V. Gyulkhandanyan, V. Koshkin, A.I. Tarasov, R. Carzaniga, K. Kronenberger, T.K. Taneja, G. da Silva Xavier, S. Libert, P. Froguel, R. Scharfmann, V. Stetsyuk, P. Ravassard, H. Parker, F.M. Gribble, F. Reimann, R. Sladek, S.J. Hughes, P.R. Johnson, M. Masseboeuf, R. Burcelin, S.A. Baldwin, M. Liu, R. Lara-Lemus, P. Arvan, F.C. Schuit, M.B. Wheeler, F. Chimienti, G.A. Rutter, Insulin storage and glucose homeostasis in mice null for the granule zinc transporter ZnT8 and studies of the type 2 diabetes-associated variants. Diabetes **58**(9), 2070–2083 (2009). doi:10.2337/db09-0551