The Zn\(^{2+}\)-transporting Pathways in Pancreatic \(\beta\)-Cells

A ROLE FOR THE L-TYPE VOLTAGE-GATED Ca\(^{2+}\) CHANNEL\(^*\)

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In pancreatic \(\beta\)-cells Zn\(^{2+}\) is crucial for insulin biosynthesis and exocytosis. Despite this, little is known about mechanisms of Zn\(^{2+}\) transport into \(\beta\)-cells or the regulation and compartmentalization of Zn\(^{2+}\) within this cell type. Evidence suggests that Zn\(^{2+}\) in part enters neurons and myocytes through specific voltage-gated calcium channels (VGCC). Using a Zn\(^{2+}\)-selective fluorescent dye with high affinity and quantum yield, FluoroZin-3 AM and the plasma membrane potential dye DiBAC\(4(3)\) we applied fluorescent microscopy techniques for analysis of Zn\(^{2+}\)-accumulating pathways in mouse islets, dispersed islet cells, and \(\beta\)-cell lines (MIN6 and \(\beta\)-TC6\(\gamma7\) cells). Because the stimulation of insulin secretion is associated with cell depolarization, Zn\(^{2+}\) (5–10 \(\mu\)M) uptake was analyzed under basal (1 mM glucose) and stimulatory (10–20 mM glucose, tolbutamide, tetrathylenammonium, and high K\(^+\)) conditions. Under both basal and depolarized states, \(\beta\)-cells were capable of Zn\(^{2+}\) uptake, and switching from basal to depolarizing conditions resulted in a marked increase in the rate of Zn\(^{2+}\) accumulation. Importantly, L-type VGCC (L-VGCC) blockers (verapamil, nifedipine, and nifedipine) as well as nonspecific inhibitors of Ca\(^{2+}\) channels, Gd\(^{3+}\) and La\(^{3+}\), inhibited Zn\(^{2+}\) uptake in \(\beta\)-cells under stimulatory conditions with little or no change in Zn\(^{2+}\) accumulation under low glucose conditions. To determine the mechanism of VGCC-independent Zn\(^{2+}\) uptake the expression of a number of ZIP family Zn\(^{2+}\) transporter mRNAs in islets and \(\beta\)-cells was investigated. In conclusion, we demonstrate for the first time that, in part, Zn\(^{2+}\) transport into \(\beta\)-cells takes place through the L-VGCC. Our investigation demonstrates direct Zn\(^{2+}\) accumulation in insulin-secreting cells by two pathways and suggests that the rate of Zn\(^{2+}\) transport across the plasma membrane is dependent upon the metabolic status of the cell.

Zinc is an important trace element in living organisms, and it plays a key role in many biological processes (1, 2). Under normal conditions the concentration of free Zn\(^{2+}\) in cells is extremely low (3). However, the total intracellular Zn\(^{2+}\) concentration can reach 150–200 \(\mu\)M (4, 5). A low concentration of Zn\(^{2+}\) in the cytoplasm is maintained by Zn\(^{2+}\)-buffering systems such as metallothioneins and by compartmentalization into intracellular vesicles (6, 7). Regulation of Zn\(^{2+}\) metabolism in cells can also include plasma membrane Zn\(^{2+}\) transporters that can have opposite roles in cellular Zn\(^{2+}\) homeostasis. The Zip (Zrt/IRT-related protein) family of metal ion transporters facilitates Zn\(^{2+}\) uptake from the extracellular space or the lumen of intracellular organelles into the cytoplasm, whereas the ZnT (zinc transporter) family of transporters promotes Zn\(^{2+}\) efflux from cells or into various intracellular compartments (8–10).

In pancreatic \(\beta\)-cells a fraction of the intracellular Zn\(^{2+}\) pool is stored with insulin in vesicles as a complex of Zn\(^{2+}\)-insulin with a stoichiometry of 2:1 (3). During exocytosis Zn\(^{2+}\) is released together with insulin into the extracellular medium. The concentration of Zn\(^{2+}\) in insulin-containing vesicles of \(\beta\)-cells is \(\sim 20 \text{mM} \) (11, 12). Thus during exocytosis Zn\(^{2+}\) is released from vesicles into the extracellular space and, because of the increase of Zn\(^{2+}\), it can be transported back into the host cell or into neighboring cells (13). Although there is a significant amount of literature regarding transport of Zn\(^{2+}\) into various types of cells (14–24), surprisingly, little is known about transport of Zn\(^{2+}\) into \(\beta\)-cells.

Studies conducted several years ago, demonstrated the possibility of differential mechanisms of Zn\(^{2+}\) uptake into rat pancreatic islets under basal and stimulatory conditions and suggested that Zn\(^{2+}\) may be required for metabolic processes in addition to insulin crystallization (25, 26). The precise subcellular localization and distribution of Zn\(^{2+}\) in islets is also under debate. Histological data in fixed cells have shown mainly granular localization of Zn\(^{2+}\) (27, 28), whereas experiments with fractionation of islets have demonstrated that only 20–30% of the total islet Zn\(^{2+}\) was associated with the granular fraction (26, 29). Given the significance of Zn\(^{2+}\) for proinsulin biosynthesis and exocytosis, understanding the mechanisms of its uptake into islet cells is important. Dysregulation of the Zn\(^{2+}\) transport system may result in secretory defects and contribute to \(\beta\)-cell dysfunction associated with type-2 diabetes (30).

It is also known that Zn\(^{2+}\) can induce oxidative damage (31–33), mitochondrial depolarization, opening of the mitochondrial permeability transition pore (34, 35), and function as a regulator of apoptosis (36).

In addition, it has been suggested that Zn\(^{2+}\) can act as a paracrine factor involved in pancreatic cell death (37) and in the suppression of glucagon secretion from \(\alpha\)-cells (13, 38).

Electrical excitability of pancreatic \(\beta\)-cells that controls the triggering phase of insulin secretion (39) depends on the coordinated activity of specific ion channels including the L-type VGCC (40, 41) and ATP-regulated potassium channels (K\(_{ATP}\) channels) (42). Here glucose metabolism increases the ATP/ADP ratio resulting in the closure of K\(_{ATP}\) channels, which in turn depolarizes the plasma membrane and

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3 The abbreviations used are: VGCC, voltage-gated calcium channel; L-VGCC, L-type VGCC; K\(_{ATP}\) ATP-regulated K\(^+\) channel; K\(_{V, Ca}\) voltage-dependent K\(^+\) channel; K\(_{V, Ca}, Ca\(^{2+}\) -sensitive voltage-dependent K\(^+\) channel; ΔΨ\(_{m}\), plasma membrane potential; DiBAC\(4(3)\), bis(1,3-dibutylbarbituric acid)trimethine oxonol; TMRE, tetramethylrhodamine; TET, tetraethylammonium; TPEN, N,N',N''-tetraakis-(2-pyridyl- methyl)-ethylenediamined; TRITC, tetramethylrhodamine isothiocyanate; qPCR, quantitative real time PCR; DIC, differential interference contrast; AMPA, \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid.
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opens L-VGCCs. This event leads to an acceleration of Ca$^{2+}$ influx through the L-VGCCs (43, 44). Insulin stored in secretory vesicles is released in response to an elevation of intracellular Ca$^{2+}$. Plasma membrane depolarization and increase in intracellular Ca$^{2+}$ also result in the opening of voltage-dependent K$^+$ (Kv) and Ca$^{2+}$-sensitive voltage-dependent K$^+$ (K$\text{Ca}$) channels, which restore an outward flow of K$^+$, thereby repolarizing of the cell membrane and closing the VGCC (39, 45).

It has been demonstrated that Zn$^{2+}$ entry into heart cells can take place via dihydropyridine-sensitive Ca$^{2+}$ channels (20), or through the N-methyl-D-aspartate- and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate-activated Ca$^{2+}$-permeable channels in neurons (18, 19). Routinely, the transport of Zn$^{2+}$ into cells is investigated by radioactive methods (16, 17, 25, 26), Ca$^{2+}$ fluorescent dyes (20, 21, 32), or the Zn$^{2+}$-sensitive fluorescent dye Newport Green (22–24). Recently a new Zn$^{2+}$-selective fluorescent dye, FluoZin-3, was introduced as a probe for Zn$^{2+}$ detection (46). FluoZin-3 binds Zn$^{2+}$ with very high affinity ($K_D = 15 \text{ nm}$), and it has much higher quantum yield than other Zn$^{2+}$-sensitive dyes previously used (46). We employed a cell-permeant form of the dye, FluoZin-3 AM, to elucidate the Zn$^{2+}$-transporting pathways in the β-cell lines MIN6 and β-TC67 (β-TC), mouse islets, and dispersed islet cells. We observed that Zn$^{2+}$ uptake into β-cells occurred through two distinct pathways, one involving the plasma membrane Zn$^{2+}$ transporter under basal (low glucose/hypoperfused) conditions and another through L-VGCC during the depolarization of the plasma membrane. Our data suggest that Zn$^{2+}$ entry into β-cells is linked to the metabolic status of the cell. Under stimulatory conditions, Zn$^{2+}$ entry is mediated through the L-type VGCC, whereas under basal conditions its uptake is independent of this channel and possibly dependent on the ZIP family of transporters. To this end we demonstrate by real time PCR analysis the expression of Zip1, Zip2, Zip3, Zip4, Zip5, Zip8, Zip9, Zip10, and Zip14 mRNA in MIN6 cells and mouse islets, with Zip1 and Zip8 being the most abundantly expressed transcripts in MIN6 cells and Zip1 in islets.

**Materials and Methods**

Reagents—Fluorescent dyes FluoZin-3 AM, bis-(1,3-dibutylbarbituric acid)trimethine oxonol (DiBAC$_4$(3)), tetramethylrhodamine methyl ester (TMRM), Hoechst 33342 were obtained from Molecular Probes (Eugene, OR). Dispase II (neutral protease) was from Roche Diagnostics. Pyrithione, N,N,N’′,N′-tetraakis-(2-pyridylmethyl)-ethylenediamine (TEA), verapamil, nifedipine, nitrobenzamide, tetrathylenommonium (TEA), Gd$^{3+}$, La$^{3+}$, collagenase, collagen, poly(L-lysine), and poly(L-ornithine) were from Sigma-Aldrich.

**MIN6 and β-TC Cells**—MIN6 insulinoma cells (passages 35–45), a gift from Dr. S. Seino (Chiba University), were cultured in high glucose Dulbecco’s modified Eagle’s medium with 10% bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 μl/500 ml β-mercaptoethanol at 37 °C and 5% CO$_2$–95% air. β-TC insulinoma cells (passages 40–45), a gift from Dr. S. Efret (Albert Einstein University), were cultured in RPMI 1640 medium with 10% bovine serum, 1% l-glutamine, 5.6 mM glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, pH 7.4. Culture medium in both cell lines was changed every 2 days.

**Pancreatic Islets**—Islets were isolated from C57BL/6 mice as described previously (47, 48) with minor modifications. Briefly, mice were anesthetized, and the pancreatic duct was perfused with RPMI 1640 containing type V collagenase (0.8 mg/ml) and bovine serum albumin (2%). The pancreas was then removed and digested for 12 min at 37 °C. Islets were subsequently picked by hand, transferred to fresh medium, and maintained at 37 °C. The islets were cultured in RPMI 1640 supplemented with 10% bovine serum, 11.1 mM glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, pH 7.4.

The islets were plated on glass coverslips coated with collagen, poly(L-lysine) or poly(L-ornithine) and maintained in culture medium for 1–2 days.

**Dispersed Islet Cells**—To obtain dispersed cells, isolated islets were incubated for 10 min in Ca$^{2+}$-free phosphate-buffered solution, supplemented with 2 mM EGTA, 3 mM glucose, 100 units/ml penicillin, and 100 μg/ml streptomycin. Islets were then centrifuged and incubated with dispase II for 10 min at 37 °C for digestion, followed by the addition of RPMI 1640 medium with 11.1 mM glucose, 10% bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES, pH 7.4. The suspension was centrifuged, and the pellet resuspended in the same medium. The cells were plated on glass coverslips coated with poly(L-lysine) and maintained in culture for 1–3 days.

**Conventional Fluorescent Measurements**—For fluorescent measurements the incubation and perfusion buffer had the following composition: 130 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 5 mM NaHCO$_3$, 200 μM TPEN, 10 mM HEPES, pH 7.4, or the same medium without Ca$^{2+}$. In selected experiments, to depolarize the cells, 50 mM NaCl was replaced with 50 mM KCl.

Fluorescent experiments were carried out using an Olympus BX51W1 fluorescent microscope fitted with 20×/0.95 water immersion objective and cooled CCD camera equipped with magnification changer (U-TVAC, Olympus). For excitation a xenon lamp-based DeltaRay high speed monochromator from Photon Technology International (PTI, Lawrenceville, NJ) was used. For the control of the monochromator and videocamera, as well as for fluorescent imaging and collection of the data, ImageMaster 3.0 software (PTI) was used.

For experiments, the cells or islets were transferred to an open chamber, placed on the microscope stage, and perfused at 1 ml/min. All time-dependent experiments were performed at 36–37 °C using TC-324B Heater Controller (Warner Instruments, Hamden, CT). In static experiments a Delta T culture dish controller (Bioptechs, Butler, PA) was used for heating. 1–30 cells or 1–2 islets were analyzed per coverslip.

**Measurement of Zn$^{2+}$ and Plasma Membrane Potential (ΔΨm)**—For Zn$^{2+}$ measurements coverslips with MIN6, β-TC, dispersed islet cells, or islets were loaded with 2 μM FluoZin-3 AM for 50 min in incubation buffer in the presence of low glucose (1 mM) at 37 °C and 5% CO$_2$–95% air. The cells or islets were subsequently incubated for 10 min in the same conditions without dye. The fluorescence of FluoZin-3 AM was excited at 480 nm and emission measured with 525 nm band pass filter using 505 nm beam splitter.

We examined the response profile of dispersed islet cells, loaded with FluoZin-3 AM and exposed to sequential perfusion with Zn$^{2+}$. To minimize background Zn$^{2+}$ level in these experiments all chemicals used for the buffer were of highest purity, and the water was of HPLS grade (Sigma). However, perfusion of cells with the Zn$^{2+}$ ionophore pyri-
Deconvolution Fluorescent Microscopy—Coverslips with MIN6 cells co-loaded with FluoZin-3 AM (2 μM) and the DNA staining reagent Hoechst 33342 (1 μM) were transferred into the chamber and plated on the stage of the Axioplan 2 microscope fitted with a 40 × 0.75 water immersion objective. For Hoechst 33342 and FluoZin-3 AM excitation and emission, 4′,6-diamidino-2-phenylindole and fluorescein isothiocyanate set of filters were used, respectively. The Zeiss Axiovision 3.0 software was used for deconvolution.

Quantitative PCR for Zinc Transporters—Total RNA was isolated from MIN6 cells, mouse islets, and whole mouse brain using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. Following extraction, total RNA was treated with rDNase I (Ambion, Houston, TX), and first strand synthesis of cDNA was carried out using Superscript II RNase H reverse transcriptase (Invitrogen) and oligo(dT) primers (Invitrogen) according to the manufacturer’s instructions. The resulting cDNA (10 ng/reaction or 2.5 ng/100 ng) was used for amplification in quantitative real-time PCR (qPCR). Serial dilutions of mouse genomic DNA were used for the generation of a standard curve (9, 3, 1, 0.33, and 0.11 ng and a nontemplate negative control). Briefly, genomic DNA or cDNA (4 μl/well) was added to a qPCR mixture (6 μl/well) containing the following components: 3.5 μl of water, 1 μl of 10× PCR buffer, 0.6 μl of 50 mM MgCl2, 0.2 μl of 50 μM Primer Mix (or 0.1 μl of forward and 0.1 μl of reverse), 0.2 μl of 10 mM dNTP mixture, 0.2 μl of ROX reference dye, 0.3 μl of SYBR green 1 (stock-diluted 1:1000 in water) and 0.025 μl (or 125 units) of platinum Taq polymerase (Invitrogen). For PCR amplification the following general protocol was employed: 95 °C (3 min); 40 cycles of PCR: 95 °C (10 s), 65 °C (15 s), 72 °C (20 s); 95 °C (15 s), 60 °C (15 s), 95 °C (15 s). qPCR was performed in an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Streetsville, ON, Canada). Gene-specific oligonucleotide primers were designed using the Primer Quest SciTool (Integrated DNA Technologies, Skokie, IL). Primer sequences are indicated in supplemental Table 1. The expression level of various Zip transcripts was calculated using the standard curve method (49). Values were normalized to thione in the absence of Zn2+ led to a considerable increase of fluorescence, due to the presence of Zn2+ traces. To avoid this initial pyrithione-induced increase in fluorescence 200–300 nM Zn2+ chelator TPEN was added in the buffer. In this case, perfusion with pyrithione in the absence of Zn2+ led to a slight increase of fluorescence. Experiments showed that perfusion even with 100 nM Zn2+ resulted in a significant increase of fluorescence (supplemental Fig. 1).

Cell ∆Ψm was measured using the negatively charged oxonol dye DiBAC4(3). Coverslips with cells or islets were loaded with 250 nM dye for 15 min at the same conditions as with FluoZin-3 AM. The perfusion solution contained the same concentration of oxonol dye. The excitation wavelength was 470 nm, and emission was measured using a 525 nm band pass filter and 505 nm beam splitter. Although excitation wavelengths for FluoZin-3 and DiBAC4(3) were not optimal, we used them to avoid possible photobleaching and photodamaging effects. Also to minimize these effects, the shutter on the monochromator was closed for 3 s between each acquisition.

Confocal Fluorescent Measurements—Confocal imaging was performed using a Zeiss LSM510 laser scanning microscope. Coverslips with islets, dispersed cells, or MIN6 cells loaded with FluoZin-3 AM (2 μM), or co-loaded with FluoZin-3 AM and TMRM (100 nM), were transferred into the chamber and plated on the stage of the microscope fitted with a 40 × 0.75 water immersion objective. For both FluoZin-3 AM and TMRM excitation the 488 nm argon laser line was used, and emissions were acquired using fluorescein isothiocyanate and TRITC set of filters, respectively. The temperature was kept at 37 °C by warming the closed box surrounding the microscope stage with air. Samples were subjected to optical sectioning by moving the focal plane along the vertical (z) axis. The images were analyzed using Zeiss LSM Image Browser software.
mouse β-actin mRNA and represent the average of three independent experiments.

Statistics—All experiments were repeated in three or more cell cultures or islets, and typical results are presented. The raw data were processed using PSI-PLOT. The time-dependent traces of MIN6, β-TC, and dispersed islet cell fluorescence are shown as mean value averaged from all investigated cells ± S.E. expressed as percentage of fluorescence compared with baseline (100%), which was taken as the mean level of fluorescence during the initial 1 min of the recording. In the case of islets the representative traces averaged from whole islet (global response) and from two randomly selected noncontiguous regions from periphery and the middle of islet are shown. In experiments shown in figures where the time-dependent traces are compared, the cells were from the same seeding, and islets were from the same isolation.

RESULTS

Intracellular Zn\(^{2+}\) Distribution and Time-dependent Kinetics—In previous studies a cell-impermeable form of the dye FluoZin-3 tetrapotassium salt was used to monitor efflux of Zn\(^{2+}\) from the cells (50). In the present study we used the membrane-permeable FluoZin-3 AM to investigate the intracellular distribution and kinetics of Zn\(^{2+}\) accumulation into β-cell lines and islets. Fig. 1 shows differential interference contrast (DIC) (Fig. 1A) and fluorescent images (Fig. 1, B–D) of a single MIN6 cell stained with 2 μM FluoZin-3 AM. The image in Fig. 1B indi-
FIGURE 3. Time-dependent kinetics of Zn\(^{2+}\) accumulation and \(\Delta \Psi_p\) changes in islets at basal and depolarizing conditions. A, upper panels depict DIC image and fluorescent images of islet loaded with FluoZin-3 AM at different times during Zn\(^{2+}\) accumulation as shown below in panel A. Zn\(^{2+}\) uptake by islet. B, the normalized values of the mean slope ± S.E. of FluoZin-3 fluorescence change after perfusion with corresponding reagents shown in A. Data show the results of three experiments. Scale bar, 50 μm. B, effect of depolarizing compounds on \(\Delta \Psi_p\) in islet. Upper panels depict fluorescent images of cells loaded with DiBAC\(_4\)(3) at different times during the perfusion with depolarizing compounds shown in the lower panel. The traces both in A and B show the global response (solid line) and responses from randomly chosen noncontiguous regions from the middle (dotted line) and from periphery (dashed line) of the islet. In both cases these regions are shown in the upper panel by numbers 1 (middle) and 3 (periphery). In both cases the traces are representative of three independent experiments.
cates a visible level of fluorescence after incubation in medium with low glucose and in Zn\(^{2+}\)-free conditions, demonstrating the presence of endogenous Zn\(^{2+}\). Further addition of 10 \(\mu\)M Zn\(^{2+}\) to cells strongly increases the fluorescence intensity (Fig. 1C). Following treatment with the membrane-depolarizing agent tolbutamide, an inhibitor of the \(K\text{\textsubscript{ATP}}\) channel, a further increase of fluorescence was observed (Fig. 1D). The corresponding profiles of fluorescence intensity along the line shown in Fig. 1A are presented under the images. Images Fig. 1, B–D and line profiles of fluorescence intensity demonstrate a nonuniform distribution of the fluorescent probe. As seen from the images, after incubation of the cells with Zn\(^{2+}\) (Fig. 1C) and then with tolbutamide (Fig. 1D), the whole-cell fluorescence was elevated, and the area of maximal fluorescence intensity was shifted to a more central point in the cell. Similar results were obtained with another insulinoma cell line \(\beta\)-TC (supplemental Fig. 2). This shift in fluorescence to the central part of the cell does not indicate that Zn\(^{2+}\) penetrates into the nuclear region. Experiments with simultaneous loading of MIN6 cells with FluoZin-3 AM and the DNA staining reagent Hoechst 33342 demonstrated the absence of Zn\(^{2+}\) within the nucleus (supplemental Fig. 3A). A series of optical sections of dispersed mouse islet cells incubated with Zn\(^{2+}\) for 10 min under depolarized conditions (30 mM KCl) also did not cause Zn\(^{2+}\) accumulation in the nucleus (supplemental Fig. 3B). Most likely, the increase in fluorescence after Zn\(^{2+}\) treatment (Fig. 1, C and D, and supplemental Fig. 2, C and D) reflects the accumulation of Zn\(^{2+}\) in the space surrounding the nucleus. It is noteworthy that a series of optical sections of MIN6 cells (supplemental Fig. 4) stained simultaneously with FluoZin-3 AM (green) and the mitochondrial membrane potential marker TMRM (red) showed a punctuate pattern of fluorescence with evidence of partial co-localization, probably because of the presence of Zn\(^{2+}\) within mitochondria. It is interesting to note the prominent location of mitochondria in the perinuclear region. Taking into account the ability of Zn\(^{2+}\) to accumulate in mitochondria (51–54) the shift and increase in fluorescence could reflect Zn\(^{2+}\) entry into the mitochondria.

For our kinetic experiments a perfusion system was employed using FluoZin-3 AM and 5–10 \(\mu\)M Zn\(^{2+}\). Fig. 2 shows the time-dependent changes in Zn\(^{2+}\) (Fig. 2A) and \(\Delta\Psi\text{m}\) (Fig. 2B) in MIN6 cells at basal and depolarizing conditions. Panel A in Fig. 2A demonstrates the averaged response ± S.E. of 17 cells perfused with 1 mM glucose followed by 250 \(\mu\)M tolbutamide, 50 mM KCl, and finally TPEN, a membrane-permeable Zn\(^{2+}\) chelator. As seen from this panel, perfusion of cells with 10 \(\mu\)M Zn\(^{2+}\)/1 mM glucose led to a distinct increase of fluorescence over basal levels reflecting accumulation of Zn\(^{2+}\). Subsequent perfusion with tolbutamide and then with KCl resulted in a further increase of Zn\(^{2+}\) influx. This effect was reversed to initial levels after the administration of 20 \(\mu\)M TPEN. The upper panels in Fig. 2A are a representative sequential set of DIC and fluorescent images from the same coverslip following the described treatments. Panel B in Fig. 2A presents the normalized values of the slopes (mean ± S.E.) of the FluoZin-3 fluorescence shown in panel A. The values of these slopes reflect the rate of Zn\(^{2+}\) accumulation, showing a significant effect of both tolbutamide and KCl on Zn\(^{2+}\) influx. In all experiments at least 60% of cells in the field responded to Zn\(^{2+}\) treatment.

To determine that the conditions used in Fig. 2A actually led to cell depolarization we performed experiments with the anionic dye DiBAC\(_4\)(3), used for monitoring \(\Delta\Psi\text{m}\). As seen in Fig. 2B in the presence of 1 mM glucose, MIN6 cells are in a polarized state (low level of fluorescence). Treatment with tolbutamide and subsequently KCl depolarized the plasma membrane (increased level of fluorescence) because of...
the closure of the $K_{ATP}$ channel. Upper panels represent fluorescent images of the same cells at corresponding times.

To determine whether $Zn^{2+}$ accumulation and influx could be monitored in intact mouse islets, they were loaded with FluoZen-3 and tested for responsiveness to tolbutamide and KCl. Panel A in Fig. 3A demonstrates representative traces of $Zn^{2+}$ accumulation in an islet during perfusion with the same compounds as in Fig. 2A. The traces show the global response of the islet and responses from peripheral and middle regions as pointed in the upper panel, representing light and fluorescent images of the islet at different times of treatment. The normalized values of the corresponding slopes (mean ± S.E., n = 3) shown in panel B indicate that the rate of $Zn^{2+}$ accumulation during the successive administration of corresponding reagents is increased. Fig. 3B shows fluorescent images (upper panels) and time-dependent traces of DiBAC$_{3}(3)$ fluorescence of an islet treated with depolarizing compounds. Traces were collected from the whole islet and from peripheral and middle regions.

As seen from Fig. 3A (upper panel) the highest level of fluorescence is localized in the periphery of the islet. It is known that the predominant cell types in islets are $\beta$-cells and $\alpha$-cells (in general 70–80% and 10–20%, respectively) (38, 55), and in mouse islets $\alpha$-cells mainly occupy the periphery (mantel) of a $\beta$-cell core (56). To determine the origin of brighter fluorescence intensity in the periphery of the islet we performed a confocal microscopic examination. A series of optical sections through the islet (supplemental Fig. 5, B and C) revealed bright fluorescence not only in the periphery of the islet, but also more centrally where most of the cells are $\beta$-cells (56). The intensity of fluorescence decreases when approaching the islet center (supplemental Fig. 5, E–G) most likely reflecting a limited diffusion of the dye to the core of the islet.

We also conducted experiments with dispersed islet cells (Fig. 4). Because we measured the FluoZen-3 fluorescence mainly from the large cells (the mean diameter of $\beta$-cells is more than 20% larger than the $\alpha$-cell diameter (57, 58)), it is highly probable that these data are representative of $\beta$-cells. Fig. 4 demonstrates that the kinetics of $Zn^{2+}$ accumulation in dispersed cells are similar to these presented in Fig. 2A (MIN6 cells) and Fig. 3A (islet).

It is known that TPEN has very high affinity for $Zn^{2+}$ (10$^{-15.58}$ M$^{-1}$) (59), much higher than its affinity for $Ca^{2+}$ (10$^{-4.4}$ M$^{-1}$) or $Mg^{2+}$ (10$^{-1.7}$ M$^{-1}$) (60). These data strongly suggest that the observed changes in FluoZen-3 fluorescence reflect $Zn^{2+}$ accumulation into cells, rather than an increase in intracellular $Ca^{2+}$ due to $Ca^{2+}$ influx, or a possible indirect effect of $Zn^{2+}$ on intracellular $Ca^{2+}$ stores. To further validate this we investigated the changes in fluorescence of FluoZen-3 AM-loaded MIN6 cells perfused in the absence of $Zn^{2+}$. Our data show that perfusion of cells with 1 mM glucose followed by 20 mM glucose, 50 mM KCl and then by 5 $\mu$M $Zn^{2+}$ ionophore pyrithione did not result in an increase of fluorescence (supplemental Fig. 6A). Experiments with islets and dispersed islet cells also demonstrated the absence of a $Ca^{2+}$ effect on the $Zn^{2+}$ dye (supplemental Fig. 6, B and C). As seen from these figures, treatment with high glucose in the absence of $Zn^{2+}$ in the medium resulted in a decrease in intracellular $Zn^{2+}$. This $Zn^{2+}$ release was not necessarily associated solely with insulin granules. It has been
previously demonstrated in islets loaded with $^{65}$Zn that Zn$^{2+}$ release can occur in the absence of detectable insulin secretion (61).

All experiments shown in these figures were conducted in buffer containing physiological concentrations of Ca$^{2+}$ and Mg$^{2+}$. To determine how Ca$^{2+}$ removal would affect Zn$^{2+}$ flux we examined Zn$^{2+}$ transport in Ca$^{2+}$-free conditions with Mg$^{2+}$. Experiments with MIN6 cells did not reveal any significant differences of Zn$^{2+}$ between these conditions (supplemental Fig. 6D). Thus, our experiments demonstrate that Zn$^{2+}$ can transport across the plasma membrane in insulinoma and primary islet cells under basal and stimulated conditions.

**Glucose-stimulated Zn$^{2+}$ Uptake**—Our initial experiments have shown that Zn$^{2+}$ can accumulate in β-cells under low glucose (1 mM glucose) and following cell depolarization, using tolbutamide or KCl, which dramatically increased the rate of Zn$^{2+}$ transport into the cytosol. The effect of a more physiological insulinotropic stimulus, glucose (15–20 mM), on FluoZin-3 AM-loaded MIN6 cells and islets, is shown in Fig. 5. Perfusion of MIN6 cells with high glucose led to significant Zn$^{2+}$ accumulation (Fig. 5A). Experiments with DiBAC$_4$(3) verified that treatment with 20 mM glucose indeed led to the depolarization of the plasma membrane (Fig. 5B). Perfusion of islets with a stimulatory amount of glucose (15 mM) also resulted in Zn$^{2+}$ accumulation (Fig. 5C). Treatment with 15 mM glucose led to depolarization of the plasma membrane in islets as well (Fig. 5D).

**The Effect of $K_v$ Channel Inhibition on Glucose-stimulated Zn$^{2+}$ Uptake**—It is well known that TEA potentiates glucose-stimulated insulin secretion via the inhibition of the delayed rectifier potassium channel ($K_v$) causing increased frequency of action potentials (45). We found that combined treatment with 10 mM TEA/high glucose caused a robust increase in the rate of Zn$^{2+}$ accumulation compared with high glucose alone both in MIN6 cells (Fig. 6A) and islets (Fig. 6C). Experiments with DiBAC$_4$(3) demonstrated the changes in ∆Ψ$_p$ after TEA administration in MIN6 cells (Fig. 6B) and in islets (Fig. 6D).

**Effect of L-type Ca$^{2+}$ Channel Inhibitors on Zn$^{2+}$ Uptake**—It is well known that all L-type channels share a common pharmacological profile and are blocked by phenylalkylamines and dihydropyridines (62). For evaluation of the role of L-VGCC in Zn$^{2+}$ transport we used verapamil (phenylalkylamine), nitrendipine, and nifedipine (dihydropyridines), as well as the nonspecific inhibitors of Ca$^{2+}$ transport, Gd$^{3+}$, and La$^{3+}$. In Fig. 7A, curve 1, is a representative trace of the mean value ± S.E. received from control experiments with MIN6 cells. Fig. 7A, curve 2 shows the results of pretreatment with 50 μM nitrendipine on Zn$^{2+}$ uptake. As seen from Fig. 7A (curve 2) nitrendipine did not inhibit Zn$^{2+}$ uptake under nonstimulatory conditions (1 mM glucose). However, it significantly inhibits KCl-induced Zn$^{2+}$ accumulation.

Verapamil, another known L-VGCC inhibitor, also blocked KCl-induced Zn$^{2+}$ uptake in MIN6 and β-TC cells (Fig. 7, B and C). Fig. 7D demonstrates the effect of the nonspecific Ca$^{2+}$ channel blocker Gd$^{3+}$ on the kinetics of Zn$^{2+}$ influx during the perfusion of β-TC cells with a stimulatory amount of glucose. Curve 1 (Fig. 7D) represents the mean value ± S.E. obtained from control cells. As seen from curve 2 (Fig. 7D) perfusion with 100 μM Gd$^{3+}$ significantly inhibits the increase in Zn$^{2+}$ accumulation caused by 10 mM glucose. Another L-type channel inhibitor (nifedipine 80 μM, not shown) or the nonspecific Ca$^{2+}$ channel...
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Figure 7. Effect of L-VGCC inhibitors on Zn²⁺ uptake by MIN6, β-TC cells, and islets. A, effect of treatment with nitrendipine on Zn²⁺ accumulation in MIN6 cells. Curve 1, MIN6 cells were perfused with low amount of glucose followed by 50 mM KCl. Curve 2, MIN6 cells were perfused under same conditions as in curve 1, except that medium additionally contains 50 μM nitrendipine. As seen from comparison of curves 1 and 2, nitrendipine maintains the ability of cells to accumulate Zn²⁺ in low glucose condition, but inhibits Zn²⁺ accumulation caused by KCl depolarization. Traces 1 and 2 represent averaged responses ± S.E. of 17 and 18 cells, respectively. B, verapamil-induced inhibition of Zn²⁺ uptake in MIN6 cells at depolarizing conditions. Traces 1 and 2 represent the averaged responses ± S.E. of 15 and 20 cells, respectively. C, verapamil-induced inhibition of Zn²⁺ uptake in β-TC cells at depolarizing conditions. Traces 1 and 2 represent the averaged responses ± S.E. of 9 and 11 cells, respectively. D, inhibition of Zn²⁺ accumulation by Gd³⁺ in β-TC cells. In both cases traces represent averaged responses ± S.E. of 15 cells. E, inhibition of Zn²⁺ uptake by verapamil in islets. Verapamil maintains the ability of Zn²⁺ accumulation in basal hyperpolarized conditions but inhibits Zn²⁺ accumulation caused by KCl depolarization. The global response of islet is shown. In all cases the traces are representative of three to six independent experiments.

Expression of Zinc Transporters in MIN6 Clonal Pancreatic β-Cells, Mouse Islets, and Mouse Brain—To begin to understand the mechanism of glucose-dependent Zn²⁺ transport, we used quantitative real-time PCR to probe for Zn²⁺ transporter gene expression in MIN6 cells and islets. Our analysis indicated that PCR products for Zip1, Zip2, Zip3, Zip4, Zip5, Zip8, Zip9, Zip10, and Zip14 are all expressed in MIN6 cells as well as pancreatic islets as shown in Fig. 8A. qPCR primer pair efficiencies were verified from the slope of the standard curve generated as well as the dissociation curve for each gene-specific primer. qPCR demonstrated that Zip1 and Zip8 are the most abundantly expressed zinc transporter transcripts in MIN6 cells, followed by Zip9 and Zip14. Furthermore, all other zinc transporter transcripts were expressed in MIN6 cells but to a lesser extent, especially Zip5. In islets the most abundantly expressed transporters were Zip1 and Zip9.

It has long been recognized that Zn²⁺ is an important element in brain function (63, 64). For this reason we used brain tissue as a positive control for the expression of various Zip transcripts. Our experiments in whole brain showed that the most robustly expressed Zn²⁺ transporter transcripts in brain were Zip1, Zip9, Zip10, and Zip14 (Fig. 8B). The high expression of Zip1 mRNA has previously been reported in the human brain (14).

Discussion

In the β-cell Zn²⁺ has an important role in the stabilization of insulin molecules and serves as a cofactor for many vital enzymes. It is also possible that Zn²⁺ can regulate Ca²⁺ influx in cells and thereby modulate insulin secretion in an alternative way. This possibility is supported by the observation that Zn²⁺ can regulate Ca²⁺ and KATP channels, as demonstrated in pheochromocytoma and kidney cell lines as well as hippocampal mossy fibers (21, 65). The mechanism of Zn²⁺ transport into β-cells through the plasma membrane has not been extensively examined. One study suggested that a facilitated mechanism of Zn²⁺ influx exists at low Zn²⁺ (0.5–7 μM) concentrations and a diffusion mechanism at higher Zn²⁺ (10–3000 μM) concentrations (25). Our investigation is the first to demonstrate, both spatially and temporally, direct Zn²⁺ accumulation in insulin-secreting cells. The main finding of our study was that transport of Zn²⁺ into MIN6 and β-TC insulinoma cell lines and mouse islets occurs by two pathways, likely via a plasma membrane Zn²⁺ transporter(s) in basal state and through the L-type of Ca²⁺ channels (VGCCs) under depolarizing conditions. These results therefore suggest that the rate of Zn²⁺ transport and accumulation is in part dependent upon the metabolic status of the β-cell.

It appears that Zn²⁺ entry into neuronal cells may occur via three


FIGURE 8. Real time quantitative PCR analysis for zinc transporter transcript expression in MIN6 cells, islets, and brain. Expression of zinc transporter transcripts in MIN6 cells and islets (A) and brain (B) was calculated using mouse β-actin as the normalizing gene. Results represent the average ± S.E. of three to four independent experiments.

FIGURE 9. Schematic representation of sequence of events resulting in Zn2+ transport into β-cells using different pathways. The numbers 1–7 indicate the sequence of events. 1, Zn2+ transport into β-cells at basal conditions using transporter(s); 2, increase of ATP/ADP ratio because of stimulation of mitochondrial metabolism by glucose; 3, closure of KATP channel and depolarization of plasma membrane; 4, activation of L-VGCC and Ca2+ entry; 5, release of Zn-insulin granules into extracellular space; 6, Zn2+ entry through L-VGCC; 7, repolarization of plasma membrane by K+ channels. The symbols ○ or ◦ indicate activation or inhibition, respectively.

pathways: VGCCs, N-methyl-D-aspartate receptor-gated channels, and the Ca2+-permeable AMPA-kainate receptor-gated channels (18, 19, 23, 66–68). Zn2+ influx through VGCCs is also observed in bovine chromaffin cells (69) and myocytes (20). Activation of VGCCs was caused by high K+ solution or AMPA/kainate in neuronal cells (19, 68) and by high K+ or 1,1-dimethyl-4-phenylpiperazinium in chromaffin cells (69). In myocytes VGCCs were activated by electrical stimulation (20). In all aforementioned cells Zn2+ entry was inhibited by the Ca2+ channel antagonist dihydropyridine. Verapamil, another L-VGCC inhibitor had no effect in neuronal cells (68). The unique feature of pancreatic β-cells is depolarization of the plasma membrane and activation of VGCCs by stimulatory glucose concentrations (10–20 mM) because of an inhibition of KATP channels (42–44). Data presented in Fig. 5 show that perfusion with stimulatory amounts of glucose causes Zn2+ entry into β-cells. The rate of Zn2+ accumulation is significantly enhanced with inhibition of KATP and KCa channels (Fig. 6), which repolarize the plasma membrane. Fluorescent images of an individual cell shown in Fig. 1, and the time-dependent kinetics of FluoZin-3 and DiBAC4(3) fluorescence in insulinoma cells (Fig. 2), islets (Fig. 3), and dispersed islet cells (Fig. 4) also confirm that Zn2+ accumulation takes place following depolarization of cells by the KATP channel inhibitor tobutamide or by KCl. Experiments with specific inhibitors of VGCCs (verapamil, nitrendipine, and nifedipine) (Fig. 7) indicate that one of the pathways of Zn2+ entry into β-cells is the L-type VGCC.

The results presented in Figs. 2 and 3 show that at low glucose Zn2+ can accumulate in both MIN6 cells and mouse islets. Importantly, Ca2+ channel blockers nitrendipine and verapamil (Fig. 7) do not inhibit this entry, whereas KCl-stimulated Zn2+ influx is almost completely blocked by these compounds. A possible explanation of these results is that Zn2+ can enter into β-cells using not only L-VGCC, but also other transport mechanisms. Plasma membrane Zn2+ influx transporter(s), which are present in some types of cells (8–10), may serve as an alternative Zn2+ transporting pathway.

The balance between Zn2+ accumulation and Zn2+ efflux is crucial for cell survival. The zinc efflux transporter (ZnT-5) that is abundantly expressed in the human pancreas was recently cloned and characterized (70). Moreover, a β-cell-specific zinc transporter, ZnT-8, localized on insulin secretory granules, was identified and cloned (71). ZnT-8 apparently plays an important role for Zn2+ transport into secretory vesicles of pancreatic β-cells. However, as stated, little is known about Zn2+ influx transporter(s) at the level of the β-cell plasma membrane. Detectable levels of Zip1 mRNA were previously demonstrated in human (14), but not mouse, pancreas (72). Two recent studies (17, 73) showed expression of Zip5 mRNA transcripts in the whole mouse pancreas by means of Northern blotting, although in the first case (17) the expression was much higher than in the latter (73). Using immunofluorescent methods the presence of Zip4 in pancreatic β-cells was also detected (73).

Our quantitative real time PCR experiments for identification of genes of the ZIP family of Zn2+ transporters in MIN6 cells and islets
demonstrated that multiple isoforms are present with various levels of expression. These include Zip1, Zip3, Zip4, Zip5, Zip8, Zip9, Zip10, and Zip14 with Zip1 and Zip8 showing the highest level of expression in MIN6 cells, and with Zip1 and Zip9 in islets (Fig. 8A). This lack of convergence with Dufner-Beatie et al. (72) regarding the expression of mouse Zip1 transcripts is probably explained by the fact that islets typically represent only a small fraction of the total mass of the pancreas. We also detected Zip4 with one order of magnitude higher in islets than in MIN6 cells (Fig. 8A).

The necessity for such a broad spectrum of zinc transporters in β-cells is not clear. The mechanism of action of these transporters, as well as which of them plays a predominant role in Zn\(^{2+}\) uptake in basal conditions has not been defined. It is possible that accumulation of Zn\(^{2+}\) through this transporter(s) is driven by changes in plasma membrane potential. Further studies are needed to clarify the specific localization of these transporters and regulation of their expression. Zip4, which is induced and recruited to the apical surface of enteroctyes and endoderm cells during zinc deficiency, is an example of zinc transporter gene regulation (73). It has also been demonstrated that in zinc-repleted embryonic kidney cells, Zip1 and Zip3 transporters can rapidly translocate between the plasma membrane and intracellular compartments (74).

It is possible that Zn\(^{2+}\) influx into β-cells is primarily regulated by the activities of the plasma membrane transporter(s) and the L-VGCCs. Under our model (Fig. 9), during low glucose conditions plasma membrane Zn\(^{2+}\) transporter(s) compensate for any possible Zn\(^{2+}\) deficiency. However, after glucose-stimulated insulin secretion, when the concentration of Zn\(^{2+}\) in the cytoplasm is rapidly depleted, the capacity of Zip1 and Zip3 transporter(s) is insufficient for fast uptake of Zn\(^{2+}\) into cells. Under this condition, Zn\(^{2+}\) can also accumulate in cells via L-type VGCC. Apparently, switching from one type of transport to another would depend on the metabolic status of the cell.

As we mentioned above, the predominant cell types in pancreatic islets are β- and α-cells. Because our methods do not distinguish between primary β- and α-cells we cannot rule out that a similar or related transport system exists in α-cells. This possibility is supported by the presence of L-VGCC in α-cells (75). In fact, the cells on the periphery of the islet, part of which are α-cells (56), do increase Zn\(^{2+}\) accumulation after depolarization (Figs. 3A and 5C). Given that Zn\(^{2+}\) release from β-cells may influence glucagon secretion from α-cells (13, 38), such putative transport mechanisms warrant further study in this cell type.

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