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Zinc influx and physiological consequences in the β-insulinoma cell line, Min6

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Abstract

In the mammalian pancreas, high concentrations of Zn^{2+} are co-secreted with insulin, which may then permeate via abundant L-type Ca^{2+} channels (LTCC) present on the β-cells. Neither the mechanisms utilized by these cells to lower cytosolic Zn^{2+} nor the implications of increased intracellular Zn^{2+} on β-cell survival are well understood. To address this, we employed cell imaging of Zn^{2+} and Ca^{2+} in the β-insulinoma cell line, Min6. Depolarization induced an intense zinc influx that was blocked by nifedipine and verapamil, indicating that Zn^{2+} permeates via the LTCC. Both Ca^{2+} and Zn^{2+} permeated concomitantly, yet while Ca^{2+} was subsequently removed from the cytosol, Zn^{2+} was retained in the cells. Fluorescent staining of vesicular Zn^{2+} using ZP1 demonstrated that Zn^{2+} could be slowly sequestered following a brief exposure to low concentration of Zn^{2+}. In contrast, cells were unable to sequester Zn^{2+} following application of high concentrations, which was followed by massive cell death. Our results demonstrate homeostatic crosstalk between the plasma membrane and intracellular zinc transporters and suggest that attenuating zinc influx may enhance β-cell survival.

Keywords: Zinc; Zinc toxicity; Pancreatic β-cells; L-type calcium channels

Zn^{2+} ions are essential for the packing of insulin in the secretory vesicles of pancreatic β-cells and are co-released with insulin [1,2]. Although earlier studies suggested that Zn^{2+} is merely a structural component in the pancreatic cells, more recent work suggests that Zn^{2+} acts as signaling molecule, e.g., down-regulating glucagon secretion [3,4]. Similarly, in the brain, Zn^{2+} is packed and released together with glutamate at excitatory synapses, where it participates in cell signaling [5,6]. Among the permeation pathways for Zn^{2+} ions into cells in the CNS, the L-type Ca^{2+} channel (LTCC) is a major pathway [7]. In the pancreas, released Zn^{2+} may also permeate through these channels, which are abundantly expressed on islet cells, open in the presence of glucose, and are involved in regulation of insulin secretion [8]. On the other hand, reduction in the uptake of Zn^{2+}, as occurs during general Zn^{2+} deficiency, has been associated with the progression of type II diabetes [9].

Despite the emerging importance of Zn^{2+} in pancreatic islets, the implications of its permeation to β-cell function and survival have not been adequately explored. Permeation of Zn^{2+} ions is associated with cellular toxicity in many tissues [10]. Free zinc in the cytosol of neurons, for example, induces cell death [5] and is, therefore, maintained at extremely low levels by a variety of regulatory mechanisms. These include specific transporters and intracellular proteins such as glutathione and metallothioneins [11]. It seems likely that zinc toxicity is relevant to pancreatic islet cells as well [12], though it is not clear if β-cells, like neurons, possess mechanisms that rapidly lower cytosolic Zn^{2+} [13]. If such mechanisms are functional in β-cells, candidates for the removal of cytosolic Zn^{2+} are likely to be proteins from the ZnT and Zip transporter protein families [14,15]. Several members of these families have been identified previously in β-cells and may be involved in sequestration of Zn^{2+} into intracellular compartments.

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Prominent among these are ZnT-5 and ZnT-8, the latter being exclusively expressed in β-cells [16,17].

In the present study, we assessed Zn^{2+} permeation and the subsequent homeostatic events in Min6, β-insulinoma cells. We demonstrate that Zn^{2+}, at extracellular concentrations lower than 5μM, permeates through the LTCC even in the presence of physiological Ca^{2+} concentrations. However, while Ca^{2+} is rapidly removed from the cytosol, the clearance of Zn^{2+} is much slower. We also demonstrate that Zn^{2+} sequestration from the cytosol is inefficient at high concentrations, attesting to the serious challenge presented by excess Zn^{2+}. Our results further indicate that Zn^{2+} influx through the LTTC triggers cell death in cultured β-cells, and that cells can be rescued upon selective blockade of the LTCC.

Materials and methods

Cell culture and fluorescent imaging. Min6 cells (insulinoma cell line) were grown in DMEM as previously described [18]. Cells were seeded on glass coverslips 24 h prior to experiments. Imaging was performed using an inverted microscope equipped with a Polychrome II monochromator (TILL Photonics, Germany) and SensiCam cooled CCD (PCO, Germany). Measurements were acquired using AIW2 (Axon Instruments, Foster City, CA). Cells were loaded with 5 μM Newport green AM (Molecular Probes) with 0.01% Pluronic acid or 5 μM Fura-2 AM (TEF-Lab) in 0.1% BSA in Ringer's solution and washed for at least 20 min. Coverslips were mounted in a chamber that allowed the superfusion of cells and imaging was performed using a 10× objective [19]. Newport green was excited at 480 nm and Fura-2 at 340 and 380 nm. Both were imaged with a 510 nm long-pass filter. All results shown are averaged over at least 30–50 cells from four independent experiments. Intracellular Zn^{2+} concentrations were calibrated by using the Zn^{2+} ionophore pyrithione and the Zn^{2+} chelator TPEN as described previously for determination of zinc permeation via the LTCC [20,21]. To monitor cytoplasmic and vesicular zinc, cells were incubated in depolarizing Ringer's solution containing 3.7 or 75 μM Zn^{2+} for 1 min. Cells were then stained for 1 min with the Zn^{2+}-sensitive dye, 2 μM ZP1 (according to the manufacturer's instructions, NeuroBiotex, TX, USA). The cellular distribution of Zn^{2+} was determined using ZP1 and imaged with an LSM 510, Zeiss confocal microscope under a 100× objective. Images were acquired at an excitation wavelength of 488 nm.

Determination of free zinc (pZn) in the experimental solutions. Free concentrations of zinc ions often differ from nominal concentrations because of the chelating effects of ions in the physiological solutions and contaminants. Hence, direct measurements of free Zn^{2+} are required. Free Zn^{2+} concentrations in the Ringer's solution were determined using the pZn method described previously [22]. We first calibrated the pZn ($-\log_{10}$[free Zn^{2+}]) using 200 nM ZP1 (according to the manufacturer's instructions, NeuroBiotex, TX, USA) in 50 mM Hepes solution, adding nominal Zn^{2+} at concentrations between 1 and 100 nM. Using the calibration curve we then monitored free Zn^{2+} in the physiological solutions. This analysis indicated that Zn^{2+} contaminations in the Ringer's solutions were of the order of 90 nM, pZn approximately 7. We then assessed the amount of free Zn^{2+} upon the addition of varying concentrations of $ZnSO_4$. Our results indicate that free Zn^{2+} in the Ringer's solution is about 15% lower than in the Hepes solution. Therefore, the concentrations indicated are calibrated to the free Zn^{2+} and not the nominal values added, unless otherwise indicated.

Cell viability assay. Viability assays were performed using Min6 cells seeded on 96-well plates. Zinc was added at a concentration of 150 μM (free Zn^{2+} 113 μM, pZn 3.95) while depolarizing the cells (NaCl replaced with 50 mM KCl) in the presence or absence of the LTCC blocker nimodipine. Subsequently, a MTT assay (Sigma–Aldrich) was performed according to the manufacturer's instructions. Each graph represents an average of at least three independent experiments. Statistical analysis was performed using unpaired Student's t test assuming unequal variance, comparing each treatment to zinc.

Results

In the brain, kainate receptors and the LTCC are the major routes of Zn^{2+} permeation [7]. Because of its abundance, we hypothesized that the LTCC is the route of Zn^{2+} influx in β-cells. To assess this, Min6 cells, a β-insulinoma cell line, were loaded with Fura-2 AM and depolarized in high-K^+ Ringer's solution (replacing 50 mM Na^+ with K^+) in the presence of 155 μM free Zn^{2+} (pZn 3.8), in nominally Ca^{2+}-free Ringer's. As shown in Fig. 1a, in the presence of Zn^{2+}, the depolarizing Ringer's solution induced a rapid rise in Fura-2 fluorescence. Because Fura-2 is sensitive to both Zn^{2+} and Ca^{2+} ions, we employed TPEN (50 μM), a cell-permeable Zn^{2+} chelator, to distinguish between signals resulting from these two cations. Application of TPEN was followed by a rapid decline of the fluorescent signal to resting level, indicating that the signal is related to intracellular Zn^{2+}. To determine that L-type Ca^{2+} channels mediate Zn^{2+} influx, cells loaded with Fura-2 were treated with the Ca^{2+} channel blocker, verapamil [23], and the same experimental paradigm as in Fig. 1a was used. Complete inhibition was apparent in the presence of 100 μM (Fig. 1b). Finally, to determine whether the fluorescent signal reflects Zn^{2+} permeation via the LTCC, cells loaded with a Zn^{2+}-specific dye, Newport green (5 μM), were depolarized in the presence or absence of the LTCC blocker, nifedipine (0.5 μm). Newport green has a lower affinity to Zn^{2+} than Fura-2, thereby avoiding saturation of the fluorescent signal [24]. The depolarization-induced fluorescent rise was completely blocked in the presence of the LTCC blocker (Fig. 1c). These results are in agreement with previous studies indicating that Zn^{2+} permeation in β-cells is mediated through the LTCC [25].

Zinc release accompanying insulin secretion has been estimated to be in the micromolar range [2]; we therefore, sought to determine whether influx of Zn^{2+} via LTCC can be monitored at that range. Min6 cells loaded with Fura-2 were first perfused with nominal 1–100 μM Zn^{2+} in Ringer's solution, and then cells were depolarized in the presence of the same Zn^{2+} concentrations. As shown in Fig. 2a, no Zn^2 influx was apparent prior to depolarization even in the presence of 75 μM free Zn^{2+} (pZn 4.12). In contrast, Zn^{2+} permeation was clearly observed following depolarization, already at a concentration of less than 5 μM Zn^{2+}. Dose response analysis of the Zn^{2+} influx rate (Fig. 2b) shows that Zn^{2+} influx reached saturation at approximately 75 μM. The apparent K_m for Zn^{2+} is 37 ± 5 μM, which is well within the physiological range. Thus, our results suggest that the LTCC is the major pathway for Zn^{2+} permeation in β-cells.

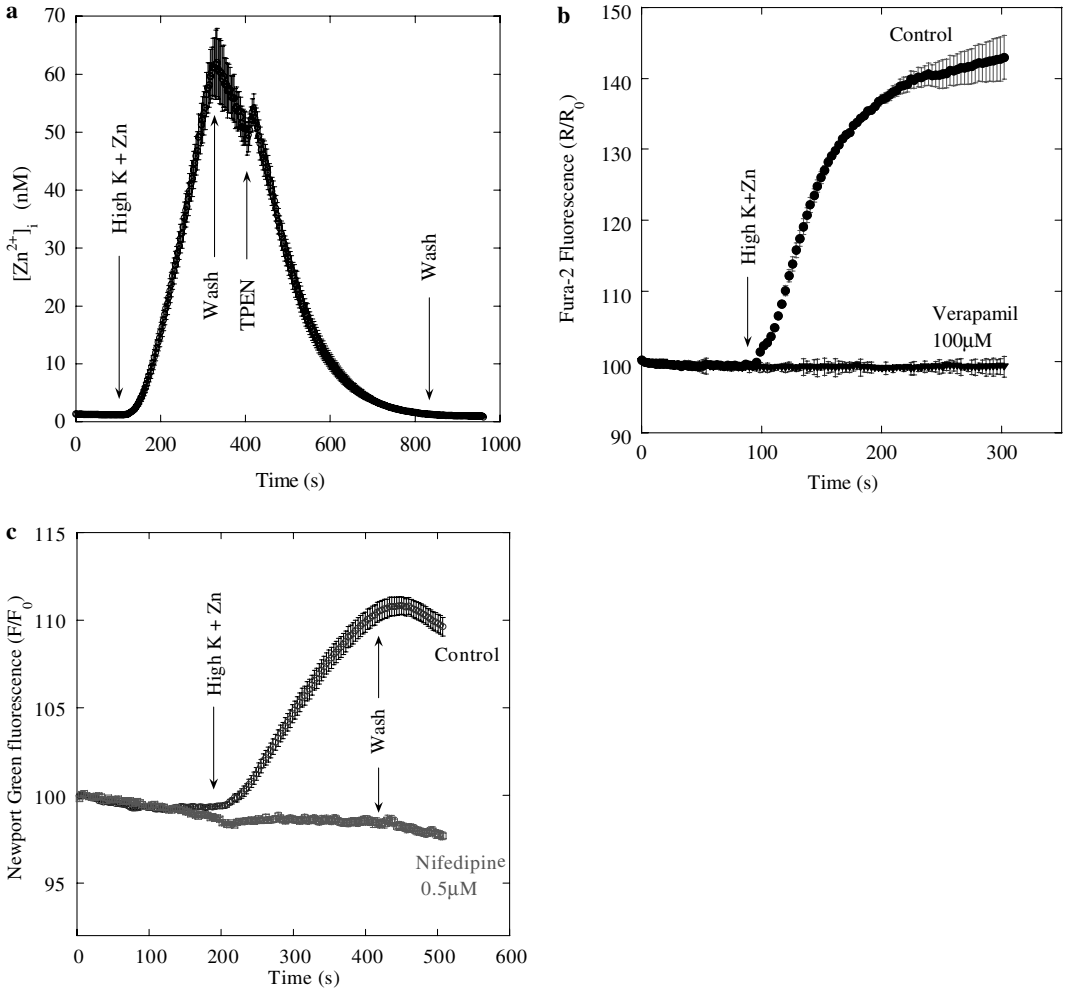

Fig. 1. Zinc transport in Min6 cells. (a) Min6 insulinoma cell line, grown on coverslips, was loaded with Fura-2 AM ($K_{d_{Zn}} \sim 5$ nM) and changes in fluorescence monitored. A rise in fluorescence resulting from cation permeation following depolarization by high-K^+ (50 mM KCl replacing NaCl) Ca^{2+}-free Ringer's solution is apparent. The cell-permeable Zn^{2+} chelator, TPEN, reduced the fluorescence to baseline levels, indicating that it was a Zn^{2+}-dependent signal. (b) Cells were loaded with Fura-2 and Zn^{2+} (75 μM) permeation was monitored in Ca^{2+}-free Ringer's solution. The application of 100 μM verapamil completely blocked Zn^{2+} influx. (c) Zn^{2+} influx was monitored in cells loaded with the Zn^{2+}-sensitive dye (Newport green, $K_{d_{Zn}} \sim 1$ μM) in the presence or absence of the LTCC blocker, nifedipine (in Ca^{2+}-free Ringer's). Application of nifedipine completely blocked Zn^{2+} influx. Our results indicate that the Zn^{2+} influx was mediated by LTCC.

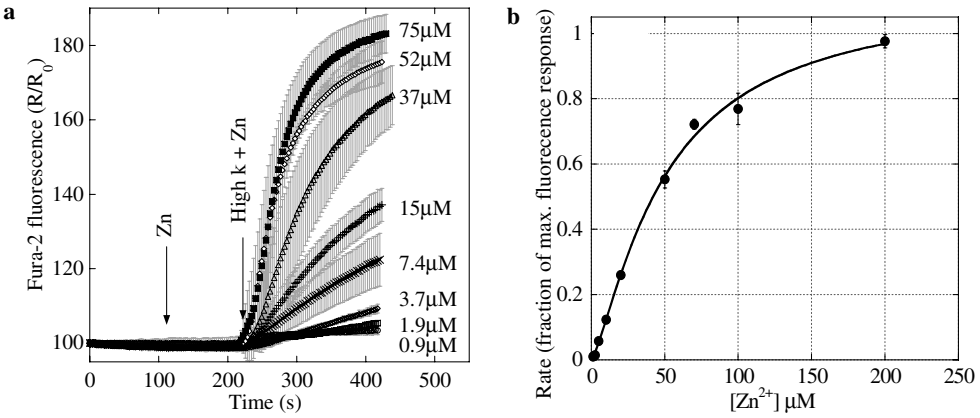

Fig. 2. Zinc, at physiological concentrations, permeates via the L-type Ca^{2+} channels into Min6 cells. (a) Cells were depolarized in the presence of the indicated Zn^{2+} concentrations, in Ca^{2+}-free Ringer's. The calibrated pZn ($-\log_{10}$[free Zn^{2+}]) values corresponding to the concentrations added are: 6.05, 5.73, 5.43, 5.13, 4.82, 4.43, 4.28, and 4.12. Note that when Zn^{2+} was added prior to depolarization, no change in the fluorescence signal was observed. Zinc influx was observed at low zinc concentration (less than 5 μM). (b) The initial rate of the fluorescence response was calculated and is plotted against the applied Zn^{2+} concentration. Fitting the data to the Michaelis–Menten equation yielded a K_m for Zn^{2+} of 37 ± 5μM (free Zn^{2+}).

The above experiments were performed in Ca^{2+}-free solution and were intended to identify the Zn^{2+} permeation pathway in β-cells. To assess the physiological relevance of Zn^{2+} entry into these cells, the same influx paradigm was adopted, albeit in the presence of extracellular Ca^{2+}. Cells were again loaded with Fura-2 and depolarized in the presence of physiological concentrations of Ca^{2+} (1.8 mM), and in the presence or absence of Zn^{2+} (75 μM). The membrane-permeable Zn^{2+} chelator, TPEN, was used to distinguish between Zn^{2+} and Ca^{2+} signals. The rates of fluorescent signal rise following depolarization were similar in the presence or absence of Zn^{2+}. Subsequently, when the cells were perfused with Na^{+}-containing Ringer's solution, there was a significant difference in the rate of fluorescence decline. In the presence of Zn^{2+}, the rate of decrease of the fluorescent signal was 0.5 ± 0.1 ΔR/s, while in the Zn^{2+}-free Ringer's, the rate was significantly faster, 0.9 ± 0.1 ΔR/s ($p < 0.02$, Fig. 3a). In addition, in the absence of Zn^{2+}, the fluorescent signal returned to 'resting level', while with Zn^{2+} in the medium, the fluorescence reached an 'elevated plateau'. Application of TPEN (100 μM) during this plateau phase resulted in a rapid drop in fluorescence to the resting level, indicating that this residual fluorescence represented accumulation of cytoplasmic Zn^{2+}. To determine if the Zn^{2+} permeation rate was affected by the presence or absence of Ca^{2+}, we used the Ca^{2+}-insensitive Zn^{2+} dye, Newport green, applying the same experimental paradigm shown in Fig. 3a. The change observed in fluorescent signal following depolarization was indistinguishable (0.047 ± 0.01 and 0.051 ± 0.01 ΔR/s, in the presence or absence of Ca^{2+}, Fig. 3b). Again, prior to depolarization, Zn^{2+} permeation was insignificant (see Fig. 2a). Taken together, these results indicate that Zn^{2+} readily permeates into β-cells in the presence or absence of Ca^{2+}. They further show that free Zn^{2+} is retained in the cytoplasm for much longer times than Ca^{2+}.

We next sought to assess if Min6 cells have the capacity to reduce intracellular Zn^{2+} following its permeation. Cells were loaded with Fura-2 and exposed to high or low concentrations of Zn^{2+} (75 or 3.7 μM, pZn of 4.12 and 5.43, respectively) under depolarizing conditions. Rates of Zn^{2+}-induced fluorescence change were then determined. As shown in Fig. 4a, a brief, 1 min, exposure to 3.7 μM Zn^{2+} resulted in an increase in fluorescence. Subsequently, the Zn^{2+} was removed from the medium and cells were perfused with Ca^{2+}-free Ringer's solution, leading to a decrease in the Fura-2 signal, at a rate of 0.002 ± 0.0005 ΔR/s. Within 18 min, the fluorescence level returned to baseline, suggesting that following these Zn^{2+} loading conditions, Min6 cells are able to reduce cytosolic free Zn^{2+}. Note that the rate of Zn^{2+} clearance was two orders of magnitude lower than the rate of Ca^{2+} clearance (Fig. 3a). In contrast, following an initial decrease, no further reduction of the Zn^{2+}-induced fluorescence was observed when cells were loaded by using either 75 μM Zn^{2+} for 1 min or even 3.7 μM Zn^{2+} for 5 min. The Zn^{2+}-dependent, elevated, fluorescent signal was monitored for as long as 120 min following initial exposure (data not shown). Taken together, our results indicate that the β-cells were able to mediate slow removal of cytosolic Zn^{2+}. Only after a brief exposure to a low zinc concentration was the cytosolic Zn^{2+} removed. The cells were overwhelmed, however, by the permeation of higher, albeit physiological, Zn^{2+} concentrations.

Several intracellular ZnT family members are expressed by β-cells and are able to sequester Zn^{2+} into vesicles. In

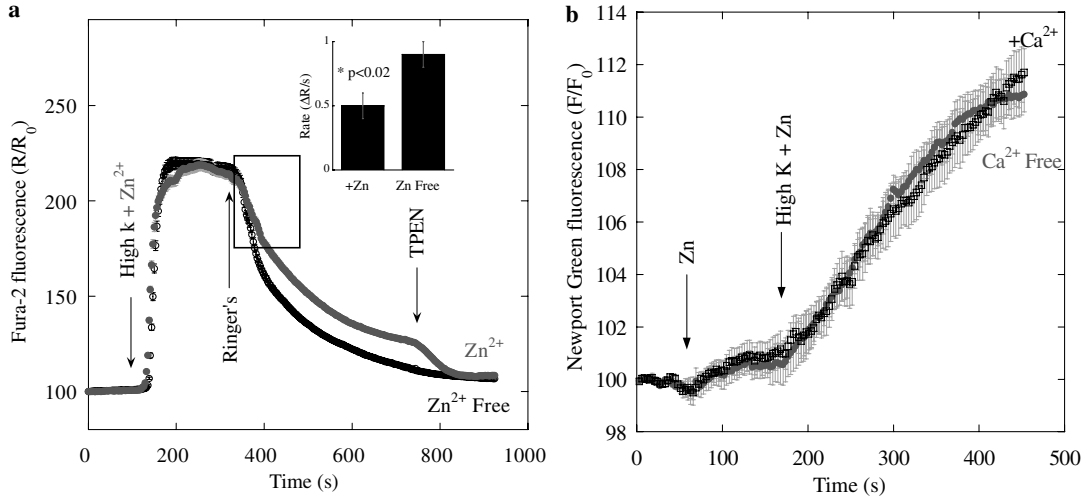

Fig. 3. Zn^{2+} is retained in the cytosol of Min6 cells, while Ca^{2+} is rapidly removed. (a) Ca^{2+} influx was monitored in Zn^{2+}-free or Zn^{2+}-containing Ringer's solution using cells loaded with Fura-2, which is sensitive to both Zn^{2+} and Ca^{2+}. Following depolarization in Ca^{2+}-containing Ringer's, the fluorescent signal rise is similar in the presence or absence of Zn^{2+}. Subsequently, the cells were washed, and a decrease in fluorescence observed. The rate of fluorescence decrease in the marked region is shown in the inset and is significantly smaller in the presence of Zn^{2+} ($p < 0.02$). In the Zn^{2+}-containing Ringers' solution, the fluorescent signal decreased to an elevated plateau which was eliminated only by the application of TPEN. (b) Zinc influx was monitored in cells loaded with the Zn^{2+}-specific dye, Newport green, in the absence or presence of extracellular Ca^{2+} in the Ringer's solution. The similar rates of increase in fluorescence suggest that Zn^{2+} influx through LTCC is maintained in the presence of physiological concentrations of Ca^{2+}.

contrast, there is no evidence for the expression of plasma membrane Zn^{2+} regulating proteins such as ZnT-1 or the Na^{+}/Zn^{2+} exchanger. We therefore asked whether removal of cellular Zn^{2+} following permeation through the LTCC is mediated by sequestration of Zn^{2+} into discrete intracellular compartments. To address this issue, cells were treated with 50 mM KCl-Ringer's containing either low (3.7 μM) or high (75 μM) concentrations of Zn^{2+} for 1 min, or depolarized in the absence of Zn^{2+} (control), and then stained with ZP1 (2 μM). The advantage of this dye is that it stains not only cytoplasmic but also vesicular Zn^{2+} [26]. As shown in Fig. 4b, ZP1 staining in cells exposed to high Zn^{2+} concentration (75 μM) was strong and highly diffuse, and was not associated with specific cellular compartments. In contrast, when cells were treated with a low Zn^{2+} concentration for a short duration, sufficient for cytoplasmic clearance (Fig. 4a), the resulting fluorescence was greatly reduced but strongly associated with distinct vesicular structures within the cells (Fig. 4b). This experiment demonstrated that permeation of low Zn^{2+} concentrations is followed by slow sequestration of this ion into intracellular organelles, and that this process is significantly less efficient in the presence of a larger Zn^{2+} load.

Finally, we sought to determine the effect of Zn^{2+} permeation via the LTCC on cell viability. Min6 cells were depolarized with 50 mM KCl-Ringer's containing 113 μM Zn^{2+} (pZn 3.95) for 1–10 min, and the MTT assay was performed 24 h later. An exposure of only 1 min was sufficient to reduce cell viability to 60%, while treatment for 5 min resulted in a reduction to only 10% cell viability (Fig. 5a). To determine if zinc toxicity is mediated in these cells by the permeation of Zn^{2+} through the LTCC, the same experimental paradigm was applied in the presence of an LTCC blocker or a Zn^{2+} chelator (Fig. 5b). Applica-

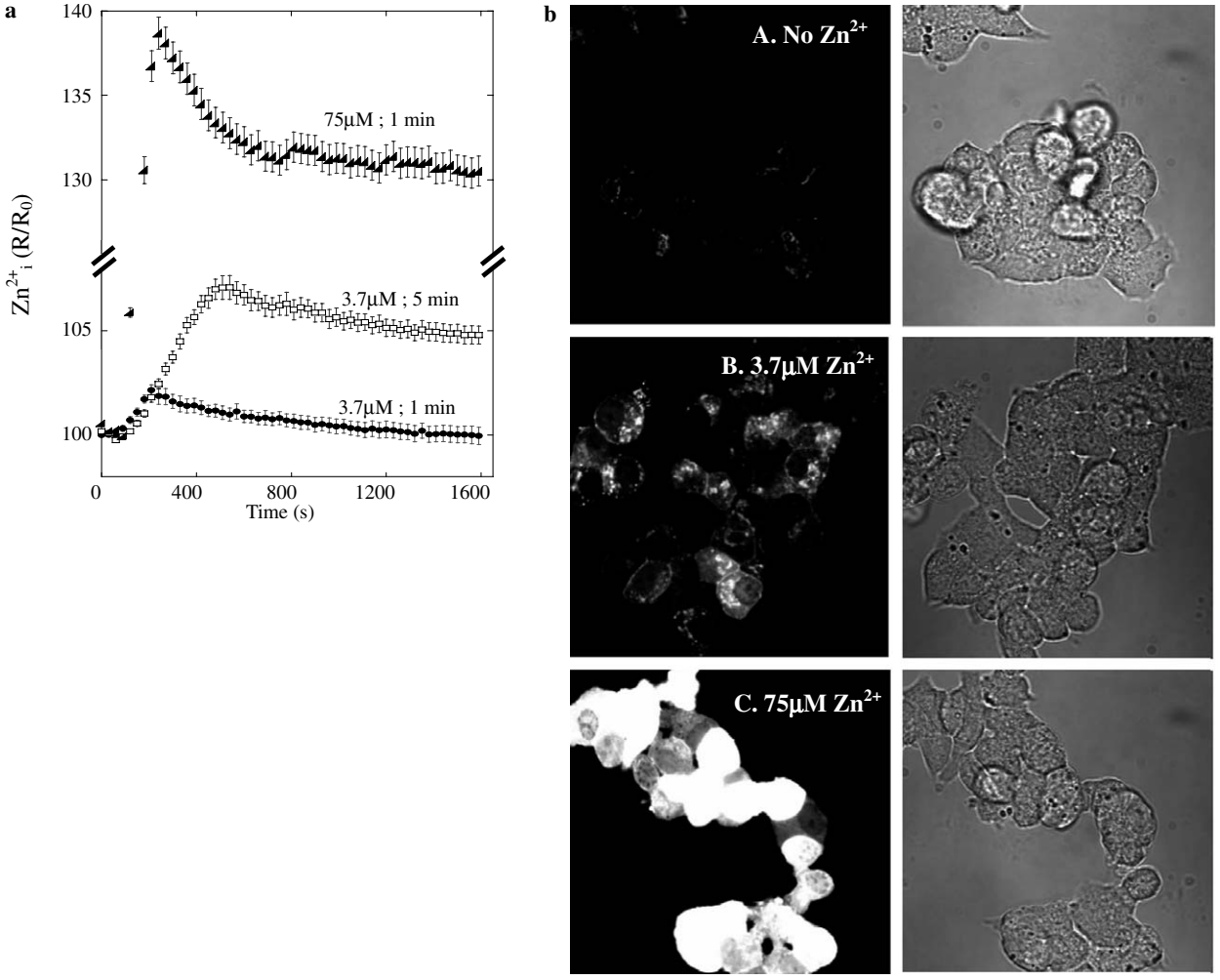

Fig. 4. Sequestration of Zn^{2+} into vesicles in Min6 cells. (a) Min6 cells, loaded with Fura-2, were depolarized in the presence of Zn^{2+} (free Zn^{2+} concentrations 3.7 or 75 μM) for 1 or 5 min (in Ca^{2+}-free Ringer's), and the fluorescent signal monitored. A rise in the fluorescent signal, indicating Zn^{2+} permeation, is apparent throughout the duration of the depolarization. Zn^{2+} accumulation in cells exposed for 5 min to 3.7 μM Zn^{2+}, was approximately threefold larger than in cells exposed to the same concentration for only 1 min. Fluorescence in cells exposed for 5 min to 3.7 μM Zn^{2+} or for 1 min to 75 μM Zn^{2+} remained at an elevated level following a small initial decrease. (b) Cells were exposed for 1 min to 3.7 or 75 μM Zn^{2+} (in Ca^{2+}-free Ringer's) and then loaded with ZP1. The right panel shows the fluorescent image while in the left panel, the same image is overlaid on a brightfield image of the cells, images were acquired using a 100× objective. In cells loaded with low concentrations of Zn^{2+}, fluorescent signal is localized to vesicle-like structures within the cells, while the high Zn^{2+} concentration resulted in diffuse fluorescence within the cytosol.

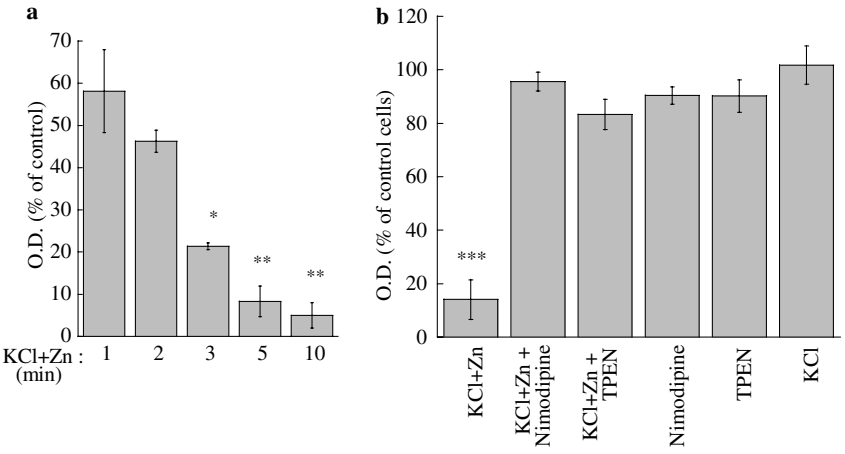

Fig. 5. Permeation of Zn^{2+} via the LTCC into Min6 cells induces β-cell death. (a) Min6 cells were depolarized with 50 mM KCl-Ringer's containing 113 μM Zn^{2+} for 1–10 min (in Ca^{2+}-free Ringer's), and MTT assay was performed 24 h later. *$p < 0.01$, **p 0.005, versus 1 min exposure to Zn^{2+}. (b) The same experimental paradigm was applied in the presence of the LTCC blocker, nimodipine 1 μM, or was followed by application of TPEN (100 μM). Cell viability was similar in cells treated with the LTCC blocker, the Zn^{2+} chelator or in the non-treated controls. ***$p < 0.0005$.

tion of Zn^{2+} for 5 min in the presence of 1 μM nimodipine resulted in cell viability that did not differ from the non-treated cells. Similarly, the addition of TPEN, following removal of extracellular Zn^{2+}, attenuated Zn^{2+}-induced cell death. Thus, our results indicate that intracellular Zn^{2+}, permeating into the cells via the LTCC, induces death of β-cells.

Discussion

We have studied Zn^{2+} homeostasis in a β-cell line and monitored its effects on cell survival. Our results support the hypothesis that the L-type Ca^{2+} channel is the major route of Zn^{2+} entry into these cells. Influx of Zn^{2+} was observed in the presence of Ca^{2+}; but when Ca^{2+} was rapidly removed from the cytosol, Zn^{2+} levels remained elevated. Efficient sequestration of Zn^{2+} into intracellular vesicles occurred only after a short exposure to low concentrations of this ion. Finally, we demonstrated that Zn^{2+}-induced β-cell death following exposure to extracellular Zn^{2+} occurred via the LTCC.

It has long been known that L-type Ca^{2+} channels are required for the Ca^{2+} permeation regulating insulin secretion in pancreatic β-cells [27]. Among Ca^{2+} channels, the L-type channels are relatively non-specific and have been shown to mediate transport of a variety of divalent and even trivalent cations; e.g., Zn^{2+}, Cd^{2+}, and Fe^{3+} [28,29]. Because of their abundance in the pancreas relative to these other ions, however, only Zn^{2+} and Ca^{2+} are relevant to islet physiology. The affinity of the L-type Ca^{2+} channel for Zn^{2+} transport measured in this study in cultured β-cells, moreover, is well within the concentrations of Zn^{2+} known to be released during insulin secretion [2]. Furthermore, because secretion of insulin involves opening of the LTCC, the permeation of Zn^{2+} that is co-secreted with it through this pathway is of particular physiological relevance [9]. Our findings indicate that Zn^{2+} permeation is followed by a prolonged rise of cytosolic Zn^{2+}. Although we do not know, at present, the precise effect of this Zn^{2+} accumulation, it is tempting to speculate that it will activate the metal transcription factor, MTF, which is acutely regulated by intracellular Zn^{2+} [30,31]. This will then induce the expression of zinc homeostatic proteins, including metallothioneins and zinc transporters [32]. A prolonged rise in cytosolic Zn^{2+} may also lead to accumulation of this ion in mitochondria, thereby affecting their function [33,34].

It has recently been demonstrated in Min6 cells that Zn^{2+} influx is mediated by the L-type Ca^{2+} channel at Zn^{2+} concentrations above 10 μM; and by an as-yet-unknown, high affinity transport mechanism that is active also under basal, non-depolarizing, conditions [25]. Based on the demonstrated presence of ZIP transcripts, it has been suggested that these ZIP proteins may conduct the high affinity basal Zn^{2+} transport in β-cells. While our results agree with a role for the LTCC, we did not observe basal Zn^{2+} permeation, indicating that the LTCC is the major permeation pathway even under conditions of low zinc concentrations. In fact, we report here that transport via the LTCC is apparent already at approximately 5 μM Zn^{2+}. Thus, these channels have a high enough affinity to mediate Zn^{2+} transport across the large range of physiological concentrations. Although the reason for the discrepancy in basal Zn^{2+} transport is not clear, a possible explanation may be that slight changes in concentrations of Zn^{2+} in the growth medium may induce changes in the expression or cellular localization (plasma membrane or intracellular compartments) of ZIP which is highly regulated by Zn^{2+} [35,36].

Another important finding of this study is that although Ca^{2+} is removed from the cytosol within minutes, Zn^{2+} levels may remain high for as long as 2 h. In neurons and glial cells, a variety of mechanisms for lowering intracellular Zn^{2+} via the plasma membrane have been demonstrated,

including the Na^+/Zn^{2+} exchanger and the zinc transporter, ZnT-1 [21,37,38]. Our results in β-cells, however, suggest that the removal of cytoplasmic Zn^{2+} may be mediated by sequestration of Zn^{2+} into intracellular organelles. Indeed, β-cells express an unusually high number of intracellular proteins involved in the sequestration of this ion into organelles, including ZnT-5 and -8 [16,17]. Such mechanisms are essential for uptake of the released Zn^{2+} into secretory vesicles where it is required for the proper packaging of insulin, and may also confer some protection from Zn^{2+} toxicity. The intracellular Zn^{2+} sequestration machinery appears to be much slower than plasma membrane transport mechanisms, such as those found in neurons [37]. The reason for the slow rate as well as the mechanism of action of the intracellular ZnTs are, at present, not clear. It may be related to the relatively slow turnover rate of these transporters. Alternatively, it has been suggested that ZnTs are unable to directly bind Zn^{2+} and require intermediate molecules acting as metal chaperones to facilitate their activity. Such a mechanism has been demonstrated for Cu^{2+} transport [39].

Regardless of the mechanism limiting the removal of Zn^{2+}, such a slow rate can lead to toxic Zn^{2+} accumulation. This may be particularly critical during early stages of diabetes when signaling pathways that are activated may further increase the burden by releasing Zn^{2+} from intracellular pools. Among these pathways, are cytokine-induced NO production and mitochondrial dysfunction, both linked to enhanced Zn^{2+} release [18,40]. Zinc stress in the islet may also be aggravated by the acidosis that often accompanies inflammation. For example, acidic shifts rapidly destabilize the interaction between MTs and Zn^{2+} [41] and promote intracellular Zn^{2+} mobilization. This, in turn, can lead to a marked increase in intracellular Zn^{2+} generated by oxidation of MT [42]. Although the transport mechanism by which mammalian intracellular ZnTs mediate Zn^{2+} transport is unknown, studies of their yeast and bacterial homologues indicate that they may mediate H^+/Zn^{2+} exchange [43]. Such a mode of transport is potentially sensitive to small changes in intracellular pH. It may further imply that the acidic cellular environment encountered during inflammatory processes may attenuate the activity of Zn^{2+} transporters, thereby preventing the efficient removal of toxic accumulations of cytoplasmic Zn^{2+}.

Endogenous mechanisms may not be sufficient to reduce the burden of Zn^{2+} permeation. Our results, showing that an LTCC blocker attenuated Zn^{2+}-induced β-cell death, suggest that a pharmacologic approach to block the major Zn^{2+} permeation pathway, the LTCC, might be a useful intervention to prevent or delay the onset of diabetes. In a study conducted in type I diabetes patients, activation of the LTCC by apolipoprotein CIII was shown to trigger islet destruction [44]. Although this effect has been attributed to elevation of $[Ca^{2+}]_i$, our data suggest that an accelerated Zn^{2+} influx via the LTCC may further enhance islet cell death. Blockade of the LTCC pathway may have the additional benefit of controlling excessive rise in Ca^{2+}. It has been shown previously that chronic elevation of $[Ca^{2+}]_i$ induces unresponsiveness of Min6 cells to glucose [45]. This has led to the suggestion that lowering Ca^{2+} influx via the LTCC may represent an effective therapeutic approach to impaired insulin secretion and prevent islet destruction. Taken together, our results support the conclusion that blocking the LTCC may inhibit permeation of both ions, thereby attenuating the Ca^{2+} burden as well as Zn^{2+} toxicity in β-cells.

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