Glucose-dependent increase in mitochondrial membrane potential, but not cytoplasmic calcium, correlates with insulin secretion in single islet cells

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Both mitochondrial and cytoplasmic metabolism play crucial roles in fuel-stimulated insulin secretion. Fuel metabolism-dependent increases in the ATP-to-ADP ratio close ATP-dependent K⁺ (KATP) channels, leading to membrane depolarization and Ca²⁺ influx through voltage-gated Ca²⁺ channels. A rise in the cytosolic free Ca²⁺ is a necessary component in the process of insulin secretion in response to elevated glucose concentrations (26, 36). Cytosolic free Ca²⁺ oscillations, which depend on periodic depolarization of the plasma membrane, occur in parallel with oscillations in insulin secretion during glucose stimulation (2, 38). Imposed Ca²⁺ pulses caused by repetitive depolarization with extracellular K⁺ are accompanied by synchronous pulses in insulin secretion (38), but insulin secretion also occurs when elevated cytoplasmic Ca²⁺ is maintained by sulfonylureas or high K⁺ in the presence of the KATP channel activator diazoxide (13, 39). It has been shown that this so-called amplifying pathway operates independently of imposed intracellular Ca²⁺ oscillations in intact islets (31). In the latter study, Ca²⁺ pulses were imposed by alternating K⁺ concentration between 4.8 and 30 mM in the presence of diazoxide. Increasing glucose concentrations augmented the amplitude of insulin pulses with no effect on the amplitude of Ca²⁺ pulses.

Questions arise as to what parameters correlate and therefore are likely determinants of full-scale fuel-dependent insulin secretion, when cytoplasmic Ca²⁺ does not. We therefore examined two responses [cytoplasmic Ca²⁺ and mitochondrial membrane potential (ΔΨm)] in single islet cells and with physiological stimulatory glucose concentrations. There are few studies examining markers of mitochondrial metabolism at the single cell level. In addition, most studies addressing the relationship between the Ca²⁺ rise and insulin secretion have been done in intact islets where results may be influenced by intercellular interactions (1, 2, 8). It is well established that various functions of β-cells, including insulin release, vary with glucose concentration in a concentration-dependent manner. Several studies have suggested that the graded response of a whole population might represent concentration-dependent recruitment of individual β-cells, which have different levels of sensitivity to glucose (17, 29). A limited number of studies have addressed this issue on the single cell level and under physiologically relevant glucose concentrations (17). In the present study, we compared single-cell cytoplasmic Ca²⁺ and ΔΨm responses to glucose in the range of 4 to 16 mM and compared these responses with insulin secretion under the same conditions.

METHODS

Animals, Swiss-Webster mice (Charles River) were killed with CO₂ asphyxiation. All procedures were performed in accordance with the Institutional Guidelines for Animal Care at Boston University (IACUC no. AN-14071) in compliance with United States Public Health Service regulations.

Islet isolation and primary cell culture. Pancreatic islets were isolated from 3- to 4-mo-old animals by collagenase (Roche Diagnostics, Penzberg, Germany) digestion, as previously described (21). Islets were picked by hand four times under a dissecting microscope and after overnight culture dispersed by incubation in Ca²⁺- and Mg²⁺-free PBS containing 3 mM EGTA and 0.05 mg/ml trypsin for 10 min at 37°C with occasional agitation. Isolated cells were centrifuged, washed, and suspended in RPMI 1640 culture media (Sigma) supplemented with 5 mM glucose, 10% FCS (HyClone), 100 IU/ml penicillin, and 100 µg/ml streptomycin. A 100-µl aliquot containing an estimated 1 × 10⁶ cell suspension was plated on a poly-d-lysine-coated cover slip in a 35-mm petri dish (MatTek, Ashland, MA). Cells were allowed to adhere for 2 h before replenishment with 2 ml RPMI 1640 media containing 5 mM glucose and 10% FCS. Experiments were performed after a minimum of 2 h. All experiments were performed in ad libitum-fed animals.
were performed on individual cells not in contact with other cells after culture for 2–3 days. Under these conditions, β-cells constituted ~78% of the total cell population, as determined by infecting cells with insulin promoter-directed green fluorescent protein (6). Relatively large cells were selected for study, since mouse β-cells are known to be larger than α- and δ-cells (5).

Fura loading and Ca²⁺ measurement. Cells were loaded for 30 min at 37°C with 0.5 μM fura 2-AM (Molecular Probes, Eugene, OR) in Krebs-Ringer-bicarbonate (KRB) buffer containing 5 mM glucose, 140 mM NaCl, 30 mM HEPES, 4.6 mM MgCl₂, 1 mM MgSO₄, 0.15 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 5 mM NaHCO₃, 2 mM CaCl₂, and 0.1% BSA, pH 7.4. After being loaded, cells were washed and incubated in this buffer for 15 min to allow cleavage of intracellular fura 2-AM by cytosolic esterases. The intracellular Ca²⁺ was measured by placing the dish containing cells on the stage of a Zeiss IM 35 microscope using a ×40 glycerin objective in a temperature-controlled cage heated to 37°C. Intracellular fura 2 was excited by a xenon lamp and a dual-wavelength Ionoptix synchronized chopped mirror (340 and 380 nm). Fluorescence excitation intensity at 380 nm was equalized with that at 340 using a neutral density filter. The fura 2 emission signal at 510 nm was recorded by an intensified charge-coupled detector Ionoptix camera (IonOptix, Milton, MA). Images were collected at 4-s intervals. Fluorescence data were acquired and analyzed using Ion Wizard software (IonOptix). The free Ca²⁺ was calculated from the fluorescence ratio R as described by Grynkiewicz et al. (11). The ratios of maximum and minimum fluorescence were obtained by exposing cells to ionomycin or EGTA, respectively (30). A dissociation constant of 224 nM for Ca²⁺ binding to free fura was used in calculations (11, 30).

ΔΦm. Mitochondria were labeled with the mitochondria-specific dye tetramethylrhodamine ethyl ester perchlorate (TMRE; Molecular Probes). Cells were incubated for 45 min before visualization in KRB buffer containing 10 nM TMRE. Confocal microscopy was performed on living cells using a Leica Confocal microscope (TCS SP2) with the Kr (568/20 mW) and Ar (488/20 mW) lasers. The ΔΦm-dependent component of TMRE will accumulate in a Nernstian fashion that can be described by the intensity of its fluorescence (28). The non-ΔΦm-dependent component of TMRE, also known as the binding component, was ignored, since it is fixed and voltage independent (28). A change in ΔΦm is in linear relationship to log(F₆₃₀/F₅₁₀), where F₆₃₀ and F₅₁₀ represent the fluorescence intensities of TMRE in two time points. This formula is based on the Nernst equation and is a modified version for ion concentrations across a potentiated membrane used in experiments that included the use of TMRE (28).

Insulin secretion. Glucose-stimulated insulin secretion was determined by static incubation. Dispersed islet cells were cultured in 48-well plates and stimulated with various glucose concentrations under the same conditions as described for Ca²⁺ and ΔΦm studies. The amount of released insulin was determined using an RIA kit (Linco Research, St. Charles, MO) using rat insulin as the standard.

Materials. Unless otherwise indicated, all reagents were obtained from Sigma Chemicals (St. Louis, MO).

RESULTS

Ca²⁺ responses. Cells responded to increases in glucose concentration with transient elevations of Ca²⁺ that peaked at levels three- to fourfold above the basal value, with an average oscillatory period of 3.4 ± 1.1 min and an average lag of 3.2 ± 0.6 min (n = 59). These responses were heterogeneous, and, depending on applied glucose concentration, included single transient responses and regular and irregular oscillatory responses (Fig. 1). Regular oscillatory responses became more prevalent with increasing glucose concentration (7 mM glucose and above). No sustained elevations of Ca²⁺ were observed at any applied glucose concentration. Consistent with previous reports, in the majority of responding cells, the Ca²⁺ elevation was preceded by a brief initial decrease, most likely because of enhanced Ca²⁺ uptake by endoplasmic reticulum (ER) as a consequence of the increase in the ATP-to-ADP ratio (33, 41).

Recruitment of Ca²⁺ responses. The number of responsive cells was dependent on the concentration of glucose (recruitment), with ~18% of cells responding to 6 mM glucose, and ~78% responding to 16 mM glucose. We evaluated Ca²⁺ responses to stimulatory glucose in the following two ways: single step-jumps and gradual glucose challenge. These two protocols did not yield significant differences, consistent with the fact that, in contrast to rat islet cells (9), mouse islet cells are not primed by stimulatory glucose to yield a subsequent increase in the response to the second glucose stimulus (3). The percentage of cells that responded to different glucose concentrations with any type of response (single transient, irregular and regular oscillatory) and only regular oscillatory responses are presented in Fig. 2. Maximal recruitment in both cases was reached by 8–10 mM glucose.
Digitality of the amplitude of the Ca$^{2+}$ response. Figure 3A shows the effect of different glucose concentrations on the amplitude (peak value less average basal) of individual Ca$^{2+}$ transients. Although there was some variation among cells, the mean of the amplitude of the Ca$^{2+}$ rise was not affected by the glucose concentration. The digital character of this aspect of the oscillatory response was clearly apparent when individual cells were challenged with stepwise increases in glucose concentration (Fig. 3C). As apparent from Fig. 3, neither the amplitude (Fig. 3A) nor the frequency (Fig. 3B) of the oscillations changed between 8 and 16 mM glucose. To ensure that this “digital” effect was not the result of saturation of the fura signal, ionomycin was applied at the end of the trace recording. Ionomycin caused a two- to threefold increase in Ca$^{2+}$ above the observed rise (data not shown). Illustrations of single-cell Ca$^{2+}$ and Δψ_m responses to increasing glucose concentrations are presented in Fig. 3, C and D, respectively.

Average value of the Ca$^{2+}$ response. The mean intracellular Ca$^{2+}$ was calculated from all (responsive and nonresponsive) cells at each glucose concentration and is presented in Fig. 4A. The mean intracellular Ca$^{2+}$ rise, in contrast to the earlier described amplitude (peak) of the oscillatory wave, is the average intracellular Ca$^{2+}$ value during the recording period and reflects recruitment (how many cells responded at each glucose concentration), frequency of the oscillatory response, and the amplitude (peak) of this oscillatory response. Average Ca$^{2+}$ reached saturation at 8 mM glucose, as apparent from single cell analysis, where 88% of cells reached their saturation of Ca$^{2+}$ response at 8 mM glucose, 7% at 12 mM glucose, and 5% at 16 mM glucose (Fig. 4A).

Δψ_m Response. Changes in Δψ_m occurred within 30 s after stimulatory glucose addition. In agreement with previously published data (20, 27), the glucose-dependent increase in Δψ_m reached a steady-state level with slow oscillations on the top of this rise. Amplitude of these oscillations accounted for <8% of the total intensity value of this rise (data not shown). In contrast to the Ca$^{2+}$ signal, Δψ_m in individual cells showed a graded response to stimulatory glucose concentrations (Fig. 3D) in 77% of cells. Only 5% of all cells reached their maximal Δψ_m response at 8 mM glucose and 18% of cells at 12 mM glucose (Fig. 4B). These data are in agreement with data in a different strain of mice (CD-1) in which 69% of 52 cells exhibited linear increments to increasing glucose concentration, 29% cells had nonlinear increments in the response, and 7% were nonresponsive. Only 20% of cells that exhibited nonlinear responses (6% of the total population) exhibited saturation of the Δψ_m response at 8 mM.

The relationship between the Ca$^{2+}$ responses, Δψ_m responses, and insulin secretion in a population of single islet cells at each stimulatory glucose concentration was assessed. The mean levels of Ca$^{2+}$ rise and Δψ_m at each glucose concentration was calculated from all (responsive and nonre-
responsive) cells in that set for the entire recording period and correlated with the level of glucose-stimulated insulin secretion, which was measured under the same conditions (Fig. 5). Consistent with previous reports, dispersed islet cells responded to glucose with a lower magnitude of insulin secretion than intact islets (16, 22), but followed the same concentration-dependent pattern. The mean $\Delta \psi_{m}$ reached saturation at 8 mM glucose, whereas insulin secretion and $\Delta \psi_{m}$ did not. Lack of correlation between the mean $\Delta \psi_{m}$ response and insulin secretion was apparent at 8 mM stimulatory glucose, and this difference increased as the concentration of glucose increased (Fig. 5A). In contrast, the linearity of the glucose-dependent rise in $\Delta \psi_{m}$ and the linear correlation between the level of insulin secretion and $\Delta \psi_{m}$ is shown in Fig. 5, B and C, respectively.

**DISCUSSION**

The principal differences between the responses produced by individual $\beta$-cells compared with intact islets lies in their heterogeneity (12, 14, 29, 37). Recently, it has been shown that a similar situation applies to the $Ca^{2+}$ rise (17). Less attention has been paid to effects of glucose on recruitment and graded responses of mitochondrial metabolism. We evaluated both cytosolic $Ca^{2+}$, $\Delta \psi_{m}$, and insulin secretion in response to a wide range of physiological glucose concentrations. Our results showed that the $Ca^{2+}$ response, expressed as the average $Ca^{2+}$ elevation during the glucose stimulation, was reached by $\sim$8 mM glucose, similar to the earlier study (17). It has been shown in intact islet studies that increasing glucose concentration can augment insulin secretion pulses without affecting $Ca^{2+}$ (31). That implies that the magnitude of insulin secretion and the level of $Ca^{2+}$ cannot correlate under these conditions and support a role for the amplifying pathway to explain this phenomenon.

It has been reported in systems other than islet cells that increases in agonist concentrations augment the frequency rather than the amplitude of the $Ca^{2+}$ oscillations, suggesting that the cell converts the original continuously graded (analog) signal into a frequency-encoded (digital) signal (4, 40). Although in islet cells we did not observe a frequency change in the single cell, consistent with studies in intact islets, we have shown that the glucose concentration had no effect on the average amplitude of the oscillatory peak of $Ca^{2+}$ rise in responding cells, suggesting a digital (all or none) type of this aspect of $Ca^{2+}$ response. This contrasts with a mixed model of cellular activation in insulin-secreting cells (35), according to which both dose-dependent amplification of the individual cell responses and recruitment of individual cells are responsible for the graded response of the cell population. This difference can be explained by the fact that clonal cells (BRIN-BD11) used in the study (35) are derived from rats, which have been shown to exhibit a priming effect during a single-stimulation protocol (9). Our finding is supported by the observation that subsequent application of higher (up to 20 mM) glucose to the same cell already displaying oscillations at a lower stimulatory glucose, did not change the amplitude or frequency of the $Ca^{2+}$ response. The $Ca^{2+}$ response was not saturated under our experimental conditions, since the application of ionomycin further increased the intracellular $Ca^{2+}$ in these cells.

To explain how the amplitude of $Ca^{2+}$ oscillations remains constant over a range of stimulatory glucose concentrations, it has recently been suggested that mitochondria regulate the amplitude of $Ca^{2+}$ oscillations (4, 10, 25, 40). Mitochondria,
located in close proximity to the ER release sites (7, 32, 34), are capable of fast Ca\(^{2+}\) uptake, which becomes activated if the average concentration of the cytosolic Ca\(^{2+}\) rises above a level of ~500 nM (15, 19). Mitochondria therefore act as buffers, with the majority of mitochondrial Ca\(^{2+}\) bound to macromolecules, thus limiting the amplitude of Ca\(^{2+}\) oscillations and therefore keeping them constant. Recently, Ca\(^{2+}\) amplitude regulation was demonstrated for several types of mathematical models of Ca\(^{2+}\) oscillations, including receptor-operated and store-operated models, by incorporating a mitochondrial component in the equations. In these simulations, the amplitude of Ca\(^{2+}\) oscillations remains fairly constant over a broad range of stimulus concentrations (19). Similar findings were documented by Magnus and Kaiser (23, 24) in their model of Ca\(^{2+}\) handling in pancreatic \(\beta\)-cells. The Ca\(^{2+}\) ionophore, ionomycin, which equilibrates Ca\(^{2+}\) across the membranes of intracellular organelles, including ER and mitochondria, disrupts this type of regulation and can therefore increase Ca\(^{2+}\) levels above those we observed after addition of glucose.

Although we did not observe changes in the frequency of Ca\(^{2+}\) oscillations with increased glucose concentration, consistent with whole islet studies, we have noticed that more and more cells seemed to be recruited in the regular oscillatory state (in contrast to the single transient response) as the glucose concentration increased. In our single cell preparations, up to 67% of cells displayed regular oscillations in response to glucose. It has been reported that whole islets or islet clusters readily display oscillations in contrast to single cell preparations (18). It is generally believed that loss of cell-to-cell contact is responsible for the difference, but other explanations might be plausible, such as enzymatic, mechanical, and metabolic stress to which cells are exposed during the isolation and subsequent dissociation protocol, resulting in ~50% cell death during the procedure (12, 22). Based on our experience, harsher dissociation conditions (higher concentrations of trypsin, longer dissociation periods than used for these studies) resulted in more cell death, and the remaining live cells had a tendency to display more irregular oscillations.

We did not find a good correlation between the intracellular Ca\(^{2+}\) rise and insulin secretion in a population of single islet cells, in contrast to the previously published data (17) in which authors compared clusters and individual cells and found a good correlation between insulin secretion and glucose-stimulated Ca\(^{2+}\) rise. The latter finding is surprising and not consistent with our observations. It is particularly puzzling, since the amplification pathway has been shown to operate at the single islet level, and an explanation is lacking as to why this does not apply to the single islet cell.

Mitochondrially derived ATP represents the major portion of ATP production in the cell. Because the determinant of mitochondrially derived ATP synthesis is largely the \(\Delta \Psi_\text{m}\), under normal physiological conditions, where uncoupling is minimal, we wanted to assess how different cells responded to glucose with changes in \(\Delta \Psi_\text{m}\). As in the case of average Ca\(^{2+}\), \(\Delta \Psi_\text{m}\) response was heterogeneous in terms of the percentage of additional increase in individual cells. In contrast to Ca\(^{2+}\), the single cell \(\Delta \psi_\text{m}\) response to glucose was graded and continued to rise up to 16 mM glucose. These data also suggest that only a portion of the glucose-derived rise in \(\Delta \psi_\text{m}\), and consequently ATP produced, is used to close \(KATP\) channels. The concentration of ATP required to close channels represents a fixed value, whereas any excess ATP may play a role as a component in the amplification pathway.

In conclusion, in this work, we described the digitality of the glucose-induced amplitude of Ca\(^{2+}\) oscillatory responses and showed that the different levels of sensitivity to glucose in individual cells can account for the glucose-induced Ca\(^{2+}\) response in a population. Although the Ca\(^{2+}\) signal reached saturation at 8 mM glucose, \(\Delta \psi_\text{m}\) and insulin secretion increased beyond this concentration. These findings correlate with studies in intact islets and are consistent with the role of a \(KATP\) channel-independent pathway operating at the single cell level. The correlation between \(\Delta \psi_\text{m}\) and insulin secretion suggests that ATP or other mitochondrial products may have other roles in addition to \(KATP\) channel closure in insulin secretion. Alternatively, other aspects of mitochondrial metabolism may also play a role.

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