Voltage-dependent Intracellular Calcium Release from Mouse Islets Stimulated by Glucose*

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Glucose-activated β-cell insulin secretion depends upon elevation of intracellular calcium concentration, [Ca\(^{2+}\)]. This is thought to arise from Ca\(^{2+}\) influx through voltage-dependent calcium channels. Using fura-2-loaded mouse islets, we demonstrate, in fact, that the major component of the glucose-activated [Ca\(^{2+}\)]\(_i\) rise represents voltage-dependent intracellular Ca\(^{2+}\) release. Furthermore, the Ca\(^{2+}\) release pool possesses a novel pharmacology in that it is caffeine-sensitive but ryanodine-insensitive. In the absence of external Ca\(^{2+}\), glucose still caused intracellular Ca\(^{2+}\) release, an effect blockable by tetrodotoxin. However, depolarization of the islet with KCl in low Ca\(^{2+}\)-containing solutions induced intracellular Ca\(^{2+}\) release, which was resistant to tetrodotoxin. We conclude that glucose release of intracellular Ca\(^{2+}\) is dependent upon depolarization alone, possibly through increasing inositol 1,4,5-trisphosphate production.

An increase in β-cell intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) is required for glucose-stimulated insulin secretion (1–4). Electrophysiological recordings from islets of Langerhans have demonstrated that glucose-stimulated insulin secretion is associated with the appearance of phasic depolarizations which, in the physiological range of glucose concentrations, consist of bursts of calcium-dependent action potentials (5). Other observations have indicated that glucose also induces phasic increases in [Ca\(^{2+}\)]\(_i\) in islets of Langerhans, primary β-cells, and cultured β-cell lines (6–10). In the current model, this glucose-evoked [Ca\(^{2+}\)]\(_i\) rise is thought to originate from membrane depolarization, secondary to the closure of ATP-dependent K\(^+\) channels (K\(_{ATP}\)). Voltage-dependent Ca\(^{2+}\) channels then open, leading to a further depolarization, an increase in [Ca\(^{2+}\)]\(_i\), and exocytosis of insulin-containing granules (5, 11). In studies characterizing the depolarization-activated calcium transients in β-cells and glucose-activated [Ca\(^{2+}\)]\(_i\) increases in islets, the phasic increases in [Ca\(^{2+}\)]\(_i\) were attributed solely to Ca\(^{2+}\) channel activation (6–10). However, other β-cell insulin secretagogues including acetylcholine induce [Ca\(^{2+}\)]\(_i\) transients and insulin secretion, not by depolarization but instead by mobilizing intracellular Ca\(^{2+}\) stores via stimulating inositol 1,4,5-trisphosphate (IP\(_3\)) production (12). Such an intracellular Ca\(^{2+}\) pool could likewise be mobilized secondary to depolarization via a charge-, Na\(^+\) current-, or Ca\(^{2+}\) current-coupled mechanism, as has been described in other cell types (13–15). In this report, utilizing primary cultures of intact mouse islets of Langerhans, we provide evidence that glucose in addition to activating Ca\(^{2+}\) influx also induces voltage-dependent release of Ca\(^{2+}\) from intracellular stores.

**MATERIALS AND METHODS**

Intact islets of Langerhans were obtained from the pancrea of 3- to 7-month-old C57BL/6J mice by collagenase digestion. After separation from acinar tissue on a discontinuous Ficoll gradient, the islets were individually hand-picked and cultured on uncoated coverslips in RPMI-1640 medium supplemented with fetal bovine serum, 11.6 mM glucose, 100 μmol/l L-arginine, and 100 μg/ml streptomycin for periods of 6–12 days before use. Medium was changed every 2–3 days. No studies on acutely isolated islets were possible, since the adhering process to the coverslips took a number of days. However, microelectrode recordings of electrical bursting activity from islets exposed to glucose were identical in islets maintained in culture and in acutely microdissected islet preparations.

Islets were loaded with fura-2 by a 25-min incubation at 37 °C in Krebs-Ringer buffer (in mM) 119 NaCl, 4.7 KCl, 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), 25 NaHC\(_3\)O\(_3\), 2 glucose) containing 5 μM acetoxymethyl ester of fura-2 (Molecular Probes Inc.). The specimen chamber (volume, 1 ml) was mounted on a temperature-controlled stage (Medical Systems Inc.) of an inverted microscope (Diaphot, Nikon Inc.) equipped with a × 40 Fluor objective (Nikon Inc.), and was continuously perfused with Krebs-Ringer buffer at 37 °C and pH 7.4 at a rate of 2.5 ml/min. Fura-2 dual excitation (340 and 380 nm) and fluorescence detection (510 nm) were accomplished using a Photocan-2 ratio fluorescence photometry system (Nikon Inc.); the 340, 380, and 340/380 nm ratio signals were acquired continuously at 2 Hz. Estimations of free [Ca\(^{2+}\)] were made using the methodology described elsewhere (16), where the maximum and minimum ratio calibrations were achieved by exposing a fura-2-loaded islet to 30 nM ionomycin and 20 μM EGTA, pH 8.5, respectively. A value of 224 nM for the Ka of Ca\(^{2+}\) for the dye was assumed.

**RESULTS AND DISCUSSION**

Increasing the external glucose concentration from 2 to 12 mM caused highly reproducible alterations in [Ca\(^{2+}\)]\(_i\). (Fig. 1). The initial response (phase 0), evident 60–90 s after increasing the glucose concentration, was a 30–50% decrease in [Ca\(^{2+}\)]\(_i\), that was maintained for 3–5 min. Immediately thereafter, a rapid and transient 4–6-fold increase in [Ca\(^{2+}\)]\(_i\) was seen.

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1 The abbreviations used are: [Ca\(^{2+}\)]\(_i\), intracellular free Ca\(^{2+}\) concentration; IP\(_3\), inositol 1,4,5-trisphosphate; K\(_{ATP}\), ATP-dependent K\(^+\) channels; TTX, tetrodotoxin.

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**Fig. 1.** Typical (n = 51) effect of 12 mM glucose on mouse islet [Ca\(^{2+}\)]\(_i\). Open bar indicates perfusion of islet with 12 mM glucose. Three phases of islet responsiveness to glucose are indicated (0, 1, and 2; see text for details).
(phase 1), peak values being achieved within 30-50 s of onset, and remained at these levels for periods ranging from 2 to 5 min before declining. After this period, phasic sinusoidal oscillations of [Ca\(^{2+}\)](i) developed (phase 2) with a frequency of 0.5-3/min. The time course of these oscillations mirrors the phasic bursts of Ca\(^{2+}\)-dependent action potentials recorded from islets exposed to glucose (8-12), supporting the idea that the phase 2 Ca\(^{2+}\) oscillations at least arise secondary to depolarization-driven Ca\(^{2+}\) influx.

We treated islets with caffeine in order to investigate the role of mobilization of intracellular Ca\(^{2+}\) stores in mediating glucose-induced alterations of [Ca\(^{2+}\)](i). Caffeine has complex actions on intracellular Ca\(^{2+}\) stores in a variety of cell types; rapid exposure induces a transient release from intracellular storage sites, whereas prolonged incubation disables the store from releasing further Ca\(^{2+}\) (17-19). In the presence of 2 mM glucose, rapid (<2 s) exposure of an islet to 5 mM caffeine caused a phasic increase in [Ca\(^{2+}\)](i) (Fig. 2A). This increase was not blocked either by short incubations (1 min) with the Ca\(^{2+}\) channel blocker CoCl\(_2\) (1 mM) or by low external Ca\(^{2+}\) (<100 \muM), indicating that caffeine releases Ca\(^{2+}\) from intracellular stores in mouse islets. Rapid exposure to caffeine during 12 mM glucose-induced phase 2 [Ca\(^{2+}\)](i), oscillations suppressed the Ca\(^{2+}\) transients for a period of 30-60 s (Fig. 2B), thereafter being replaced by lower amplitude, higher frequency oscillations. Continuous superfusion with 5 mM caffeine caused a smaller and more slowly developing rise in [Ca\(^{2+}\)](i), in the 2 mM glucose-containing solution (Fig. 2C). Elevating glucose to 12 mM in the continued presence of caffeine resulted in a blunted or completely absent phase 1 Ca\(^{2+}\) transient. Furthermore, the phase 2 Ca\(^{2+}\) oscillations were greatly attenuated and of higher frequency (Fig. 2C, a). Upon removal of caffeine in the presence of 12 mM glucose, there was a large and sustained phase 1 Ca\(^{2+}\) transient (Fig. 2C, b) consistent with the notion that this event represents augmented intracellular Ca\(^{2+}\) release, since caffeine promotes the refilling of intracellular Ca\(^{2+}\) reservoirs (20). Re-exposure to 12 mM glucose following a further period in 2 mM glucose produced responses identical to the initial control (Fig. 2C, c). The related xanthine compound, theophylline, which also mobilizes [Ca\(^{2+}\)](i), stores (21, 22), produced qualitatively similar changes in [Ca\(^{2+}\)](i), in the mouse islet to caffeine (n = 3). On the other hand, ryanodine, which usually blocks intracellular Ca\(^{2+}\) release in the same systems where xanthines are effective (23, 24), failed to block the glucose-activated Ca\(^{2+}\) transient, either when applied during phase 2 [Ca\(^{2+}\)](i), oscillations (Fig. 2D) or following pretreatment prior to glucose exposure (n = 3). These data indicate that xanthine-specific intracellular Ca\(^{2+}\) mobilization and sequestration play a critical role in glucose-activated phase 1 [Ca\(^{2+}\)](i), transients and an important contributory role in phase 2 [Ca\(^{2+}\)](i), oscillations.

The trigger mechanism for glucose-induced Ca\(^{2+}\) release was investigated in the following experiments. Exposure of the islet to 500 \muM diazoxide during the glucose-induced phase 2 [Ca\(^{2+}\)](i), oscillations totally suppressed the Ca\(^{2+}\) transients (Fig. 3A), an effect consistent with the phase 2 oscillations arising from phasic depolarization-induced Ca\(^{2+}\) influx, since diazoxide, by opening K\(_{ATP}\) channels, will suppress glucose-activated depolarization (25). However, pretreatment with diazoxide before glucose exposure prevented the phase 1 Ca\(^{2+}\) transient as well (Fig. 3B). Since our results suggest that the phase 1 Ca\(^{2+}\) transient primarily represents Ca\(^{2+}\) release, this indicated that the trigger mechanism for release was voltage-dependent. Whether the depolarization itself or the secondary influx of Ca\(^{2+}\) was the trigger for Ca\(^{2+}\) release was examined by lowering external Ca\(^{2+}\) below 50-100 \muM. A variety of additional experiments was tried. If the concentration of EGTA was too high or the incubation time in the low Ca\(^{2+}\)-containing solutions was too long (cf. Ref. 10), then depletion of intracellular Ca\(^{2+}\) occurred. In the presence of 2.5 mM Ca\(^{2+}\), 2.5 mM EGTA-containing perfusion solution, raising external glucose to 12 mM still induced a phase 1 [Ca\(^{2+}\)](i)
Phase 2 \([Ca^{2+}]_i\), oscillations were not observed, indicating that external \(Ca^{2+}\) was too low to support \(Ca^{2+}\) current-driven \(Ca^{2+}\) transients. In accordance with this idea, EGTA addition during the glucose-activated phase 2 \([Ca^{2+}]_i\) oscillations arrested the \(Ca^{2+}\) transients (Fig. 3C). Substitution of external \(Na^+\) with \(Li^+\), which can also permeate through \(Na^+\) channels, had no effect on the glucose-induced \(Ca^{2+}\) transient in the low \(Ca^{2+}\)-containing solution, ruling out \(Na^+\)/\(Ca^{2+}\) exchange as being responsible for the increase in \([Ca^{2+}]_i\) (26) (Fig. 3D). The glucose-induced phase 1 \([Ca^{2+}]_i\) transient, was however, totally suppressed by application of 1 \(\mu M\) TTX, a blocker of voltage-dependent \(Na^+\) channels (Fig. 3D). Furthermore, TTX was without effect on the glucose-activated \([Ca^{2+}]_i\) changes in normal \(Ca^{2+}\)-containing solutions.

The most likely explanation for these results was that both \(Na^+\) and \(Ca^{2+}\) influx triggered release of the \(Ca^{2+}\) store by a common mechanism by virtue of causing membrane depolarization; the failure of TTX to block release in normal \(Ca^{2+}\)-containing solutions would then relate to the greater importance of \(Ca^{2+}\) influx in bringing about \(\beta\)-cell depolarization (27). We tested this notion further by depolarizing the islet independently of glucose using 40 mM KCl. In the presence of 0.5 mM EGTA (0 mM \(Ca^{2+}\) added), KCl produced a similar rise in \([Ca^{2+}]_i\) to glucose, due to intracellular \(Ca^{2+}\) release (Fig. 3F). In contrast to the effects of glucose, addition of up to 5 \(\mu M\) TTX had no effect on the KCl-induced intracellular \(Ca^{2+}\) mobilization (Fig. 3F). These observations support the notion that the glucose-induced release of intracellular \(Ca^{2+}\) is dependent only upon membrane depolarization, the combined block of voltage-dependent \(Ca^{2+}\) and \(Na^+\) influx being sufficient to inhibit complete glucose-dependent depolarization and thereby prevent intracellular \(Ca^{2+}\) release.

Our data therefore indicate that glucose induces release of intracellular \(Ca^{2+}\) from a xanthine-sensitive but ryanodine-insensitive store via a depolarization-dependent mechanism. Since caffeine has been reported to block IP_3 synthesis (28) and IP_3 production itself may be a depolarization-activated process (29, 30), it is conceivable that glucose might mobilize \(Ca^{2+}\) stores by this mechanism. This effect would clearly differ from the voltage-independent \(Ca^{2+}\)-mobilizing effect of muscarinic agonists on \(\beta\)-cell (25) but would be consistent with reported elevations of IP_3 by glucose (31) and the observations that IP_3 receptors can mediate \(Ca^{2+}\)-induced \(Ca^{2+}\) release (32). Furthermore, the putative involvement of IP_3 in synchronizing electrical oscillations in the islet (33) may explain the faster, low amplitude \(Ca^{2+}\) oscillations in the presence of caffeine (Fig. 2, B and C). Obviously, measurements of changes in islet IP_3 production upon depolarization are warranted to fully test this hypothesis. A recent report has challenged the notion that IP_3 is a significant intracellular \(Ca^{2+}\) mobilizer in the islet (34), their findings suggesting that cyclic adenosine diphasphate-ribose, and not IP_3, induces \(Ca^{2+}\) release from islet-derived microsomes. The applicability of their data to glucose-dependent \([Ca^{2+}]_i\), mobilization is unclear since high concentrations of ryanodine (100 \(\mu M\)) were able to inhibit completely their microsomal \(Ca^{2+}\) release, whereas we were unable to detect any effect of ryanodine on the glucose-activated \(Ca^{2+}\) release process (Fig. 2D).

Whatever second messenger is involved, glucose mobilization of intracellular \(Ca^{2+}\) stores plays a vital role in determining both the nature and magnitude of the glucose-induced \(Ca^{2+}\) increases in the islet, in particular as it relates to the phase 1 \([Ca^{2+}]_i\), transient. One of the earliest detectable defects in patients with type II (maturity onset) non-insulin-depend-
ent diabetes mellitus is a loss of early (first phase) insulin secretion in response to a glucose challenge (35). In perfused mouse islets, there seems to be a close temporal relationship between the first phase and later second phase of glucose-dependent insulin secretion, and the phase 1 and phase 2 Ca\(^{2+}\) responses that we have observed (36, 37). This suggests that defects in intracellular Ca\(^{2+}\) release mechanisms may play an important role in the pathogenesis of this disease.

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