

Atypical Ca^{2+} -induced Ca^{2+} release from a sarco-endoplasmic reticulum Ca^{2+} -ATPase 3-dependent Ca^{2+} pool in mouse pancreatic β -cells

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The contribution of Ca^{2+} release from intracellular stores to the rise in the free cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) triggered by Ca^{2+} influx was investigated in mouse pancreatic β -cells. Depolarization of β -cells by 45 mM K^+ (in the presence of 15 mM glucose and 0.1 mM diazoxide) evoked two types of $[\text{Ca}^{2+}]_c$ responses: a monotonic and sustained elevation; or a sustained elevation superimposed by a transient $[\text{Ca}^{2+}]_c$ peak (TCP) (40–120 s after the onset of depolarization). Simultaneous measurements of $[\text{Ca}^{2+}]_c$ and voltage-dependent Ca^{2+} current established that the TCP did not result from a larger Ca^{2+} current. Abolition of the TCP by thapsigargin and its absence in sarco-endoplasmic reticulum Ca^{2+} -ATPase 3 (SERCA3) knockout mice show that it is caused by Ca^{2+} mobilization from the endoplasmic reticulum. A TCP could not be evoked by the sole depolarization of β -cells but required a rise in $[\text{Ca}^{2+}]_c$ pointing to a Ca^{2+} -induced Ca^{2+} release (CICR). This CICR did not involve inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) because it was resistant to heparin. Nor did it involve ryanodine receptors (RyRs) because it persisted after blockade of RyRs with ryanodine, and was not mimicked by caffeine, a RyR agonist. Moreover, RyR1 and RyR2 mRNA were not found and RyR3 mRNA was only slightly expressed in purified β -cells. A CICR could also be detected in a limited number of cells in response to glucose. Our data demonstrate, for the first time in living cells, the existence of an atypical CICR that is independent from the IP_3R and the RyR . This CICR is prominent in response to a supraphysiological stimulation with high K^+ , but plays little role in response to glucose in non-obese mouse pancreatic β -cells.

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Insulin secretion induced by physiological secretagogues depends on an elevated free cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) in pancreatic β -cells (Henquin, 2000; Rorsman *et al.* 2000; Satin, 2000). In these electrically excitable cells, Ca^{2+} influx is a major determinant of the $[\text{Ca}^{2+}]_c$ rise that glucose triggers through the following sequence of events (Ashcroft & Rorsman, 1989; Rorsman *et al.* 2000; Satin, 2000; Henquin, 2000). Acceleration of glucose metabolism increases the ATP/ADP ratio, which closes ATP-sensitive K^+ channels (K_{ATP} channels) in the plasma membrane (Ashcroft & Rorsman, 1989; Henquin, 2000). The resulting decrease in K^+ conductance leads to depolarization of

the plasma membrane, with subsequent opening of voltage-dependent Ca^{2+} channels and stimulation of Ca^{2+} influx. In addition to this undisputed mechanism, it has been suggested that release of Ca^{2+} from intracellular stores might contribute to the $[\text{Ca}^{2+}]_c$ rise elicited by plasma membrane depolarization, but this remains highly controversial (Satin, 2000; Islam, 2002).

In various cell types, two major classes of receptors can trigger release of Ca^{2+} from intracellular stores upon plasma membrane depolarization and Ca^{2+} influx: inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) and ryanodine (Ry) receptors (RyRs) (Berridge *et al.* 2003), of which different isoforms have been identified ($\text{IP}_3\text{R1}$ –3 and RyR1 –3). These receptors are thought to be mainly located in the membrane of the sarco-endoplasmic reticulum because the Ca^{2+} release that they induce is

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abrogated by thapsigargin (TG), a potent blocker of the sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) that depletes the organelle of Ca^{2+} . IP_3 Rs and RyRs can mediate two release processes referred to as Ca^{2+} -induced Ca^{2+} release (CICR) and depolarization-induced Ca^{2+} release (DICR). Generally, CICR is attributed to the activation of IP_3 Rs or RyRs by Ca^{2+} itself. Such a mechanism is well documented in cardiomyocytes where a small Ca^{2+} influx through voltage-dependent Ca^{2+} channels triggers a large Ca^{2+} release through RyRs (Bers & Perez-Reyes, 1999). DICR is well characterized in skeletal muscle cells (Berridge, 1997; Murayama & Ogawa, 2002), in which RyR1 is activated by the depolarization of the plasma membrane alone; this activation results from a change in the conformation-coupling between voltage-dependent Ca^{2+} channels in the plasma membrane and the RyR1 in the sarcoplasmic reticulum. Activation of phospholipase C by an increase in $[\text{Ca}^{2+}]_c$ (Biden *et al.* 1987) or by depolarization alone (Gromada *et al.* 1996; Liu *et al.* 1996) may also explain indirect CICR and DICR. It is also worth noting that receptors for nicotinic acid-adenine dinucleotide phosphate (NAADP) are insensitive to divalent cations and cannot trigger CICR (Chini & Dousa, 1996; Bak *et al.* 1999; Bak *et al.* 2002; Galione & Churchill, 2002).

The possible role of CICR and DICR in pancreatic β -cells remains unclear. Whereas several studies have identified such mechanisms in insulin-secreting cells (Islam *et al.* 1998; Holz *et al.* 1999; Lemmens *et al.* 2001; for review see Islam, 2002; Johnson *et al.* 2004), no consensus has been reached about the underlying mechanisms and functional significance. Moreover, other reports do not support the existence of these Ca^{2+} release processes in β -cells (Rutter *et al.* 1994; Tengholm *et al.* 1998). Several reasons might explain these discrepancies: the use of different animal species, cell types (cell lines *versus* primary β -cells), or experimental approaches. This controversy prompted us to reappraise the possible contribution of Ca^{2+} release from intracellular Ca^{2+} stores to the $[\text{Ca}^{2+}]_c$ rise evoked by depolarization of the plasma membrane in mouse pancreatic β -cells. The results show that non-obese mouse β -cells display an atypical CICR that does not involve the RyR or IP_3 R. This CICR significantly contributes to the $[\text{Ca}^{2+}]_c$ rise elicited by large and prolonged Ca^{2+} influx, but hardly influences glucose-induced $[\text{Ca}^{2+}]_c$ oscillations.

Methods

Solutions and drugs for islet cell preparation and $[\text{Ca}^{2+}]_c$ experiments

The medium used was a bicarbonate-buffered solution containing (mM): NaCl 120, KCl 4.8, CaCl_2 2.5, MgCl_2 1.2, NaHCO_3 24 and glucose 10 (for islet cell preparation) or 15 (for $[\text{Ca}^{2+}]_c$ experiments). It was supplemented

with 1 mg ml⁻¹ bovine serum albumin (BSA; fraction V, Boehringer-Mannheim, Mannheim, Germany) and gassed with 94% O_2 –6% CO_2 to maintain pH 7.4 at 37 °C. When the concentration of KCl was increased, that of NaCl was decreased accordingly to keep the osmolarity of the medium unchanged. Ca^{2+} -free solutions were prepared by replacing CaCl_2 with MgCl_2 , and were supplemented with 2 mM EGTA.

Diazoxide was a gift from Schering-Plough Avondal (Rathdrum, Ireland), ryanodine was obtained from RBI (Natick, MA, USA) or Alomone (Jerusalem, Israel), forskolin from Calbiochem (San Diego, CA, USA) and caffeine from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland). All other chemicals were from Sigma (St Louis, MO, USA).

Preparation of cells

Single cells and cell clusters of pancreatic islets. Mice were killed by cervical dislocation and decapitation, in accordance with the guidelines of the Commission d'Ethique d'Expérimentation Animale of the University of Louvain School of Medicine. Islets of Langerhans were aseptically isolated after collagenase digestion of the pancreas of fed female NMRI mice, *ob/ob* mice or their lean littermates (*ob/+* or *+/+*) (from the Umea colony, gift from J. Sehlin), SERCA3 knockout (*SERCA3*^{-/-}) mice (Liu *et al.* 1997) or their control homozygous C57BL/6J wild-type littermates (*SERCA3*^{+/+}). Islets were dispersed into single cells in a Ca^{2+} -free medium (Jonkers *et al.* 1999). The cells were then cultured on glass coverslips for 1–4 days in RPMI 1640 culture medium containing 10% heat-inactivated fetal calf serum, 100 IU ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 10 mM glucose. To separate β - from non- β -cells, islets from NMRI mice were dissociated with trypsin and sorted by flow cytometry on a Facstar + (Becton Dickinson, Sunnyvale, CA, USA) as previously described for rat β -cells (Pipeleers *et al.* 1985).

Skeletal muscle fibres. Single cells from flexor digitorum brevis (FDB) muscles of NMRI mice were prepared as previously described (De Backer *et al.* 2002). Isolated fibres were plated on glass coverslips covered with the Extracellular Matrix Basement Membrane (Harbour Bio-Products, Norwood, MA, USA), which permitted fibre attachment within 2 h. They were used after overnight culture at 30 °C in (DMEM/HAM F12) containing 10% heat-inactivated fetal calf serum, 100 IU ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin.

Cardiac myocytes. Single cardiomyocytes from NMRI mice were prepared as described (Macianskiene *et al.* 2002), and maintained for up to 12 h at room temperature in a Tyrode solution containing (mM): NaCl 137, KCl 5.4, MgCl_2 0.5, CaCl_2 0.8, Hepes 11.8 and glucose 10; pH adjusted to 7.4 with NaOH.

[Ca²⁺]_c measurements

In most experiments, including patch-clamp experiments in perforated mode (see below), cultured islet cells were loaded for 40–60 min with 1 μ M of the acetoxymethyl ester of fura2 (fura2 AM; Molecular Probes, Eugene, OR) at 37 °C. Some control experiments were performed on cells loaded for a shorter time (10 min) or with a lower fura2 concentration (0.1 μ M) (enough to have a measurable fluorescence signal) and yielded similar results. Skeletal muscle cells and cardiomyocytes were loaded, respectively, with 200 nM and 1 μ M fura2 AM for 60 min at room temperature. The loading solution was a bicarbonate-buffered solution containing 10 mM glucose for all cell types. When needed, 1 μ M TG and 10 or 100 μ M ryanodine was added to the loading solution. In one series of experiments, single islet cells were pressure-injected with a 5242 Eppendorf microinjector (Hamburg, Germany). The injected solution contained 18 mM fura2 K⁺ salt (Molecular Probes) dissolved in H₂O, and it was supplemented or not with 200 mg ml⁻¹ heparin (molecular weight 3000, Sigma) (Gilon *et al.* 1999) and 10 mM ryanodine. The estimated injected volume was ~1% of the volume of the cell. To ensure fast changes of the solutions, a 200- μ l perfusion chamber maintained at 37 °C was used. A different system was used for patch-clamp experiments (see below).

[Ca²⁺]_c was measured by dual wavelength excitation microspectrofluorimetry, using a photometric-based system (Photon Technologies International Ltd, Princeton, NJ, USA), a Quanti-Cell system (VisiTech International Ltd, Sunderland, UK) or a Photometrics Cascade:650 camera (Roper Scientific Inc., Trenton, NJ, USA) driven by Metafluor (Universal Imaging Corporation, Downingtown, PA, USA). The sampling rate was 5 ratio points per second with the photometric-based system and 0.83 ratio point per second with the imaging systems. Measurements on single cells and clusters of cells were performed with, respectively, a Zeiss Fluor 40x or 100x objective (Zeiss, Jena, Germany). [Ca²⁺]_c was calculated as previously described (Gilon & Henquin, 1992). For [Ca²⁺]_c measurements in islet cells, only large cells were used to exclude as much as possible non- β -cells. Their mean diameter was 14.6 ± 0.2 μ m, which is in the range of the size of isolated mouse β -cells (14.8 μ m) and above that of isolated α - and δ -cells (respectively, 10.6 and 11.8 μ m) (Barg *et al.* 2000).

Electrophysiology

Voltage-clamp experiments were performed on single β -cells at 31–33 °C using the perforated-whole-cell configuration and an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA, USA) as previously described (Rolland *et al.* 2002). The extracellular solution contained (mM): NaCl 110, KCl 4.8,

CaCl₂ 2.5, MgCl₂ 1.2, TEA 10, Hepes 5, NaHCO₃ 24 and glucose 15; pH adjusted to 7.4 with NaOH. The pipette solution contained (mM): Cs₂SO₄ 76, NaCl 10, KCl 10, MgCl₂ 1 and Hepes 5; pH adjusted to 7.15 with CsOH. Electrical contact with the cell interior was established by adding 0.3 mg ml⁻¹ amphotericin B to the pipette solution.

RT-PCR experiments

Radioactive RT-PCR. Total RNA was extracted, quantified and reverse transcribed into cDNA exactly as described (Jonas *et al.* 1999). Pairs of primers were designed using HybSimulator 4.0 software (Advanced Gene Computing Technologies, Irvine, CA, USA). The sense and anti-sense primers were chosen in the coding region of gene mRNA sequences and their specificity was checked by BLAST search on GenBank database (Table 1). Polymerization reactions were performed with a Perkin Elmer 9700 Thermocycler in a 25 μ l reaction volume containing 3 μ l cDNA (20 ng total RNA equivalents), 80 μ M cold deoxynucleosides 5'-triphosphate (dNTPs), 1.25 μ Ci [α -³²P] deoxycytidine 5'-triphosphate (dCTP) (3000 Ci mmol⁻¹), 5 pmol of appropriate oligonucleotide primers, GeneAmp Gold PCR buffer and 1.25 U of AmpliTaq Gold DNA polymerase (Perkin Elmer, Foster City, CA, USA). The thermal cycle profile was a 10 min denaturing step at 94 °C followed by the amplification cycles (1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C each), and a final extension step of 10 min at 72 °C. Amplification of the ubiquitously expressed TATA-box binding protein (TBP) was performed to check the quality of cDNAs. The amplimers were then separated on a 6% polyacrylamide gel in Tris borate EDTA buffer, in parallel with 100 bp DNA ladder. The gel was dried and [α -³²P]dCTP-labelled amplimers were revealed with a Cyclone Storage Phosphor System (Packard, Meriden, CT, USA). The size of amplicons corresponded to the expected ones (published sequences).

RyR3 sequencing. After PCR amplification of RyR3, the PCR products were checked by agarose gel electrophoresis before being labelled by cycle sequencing (Big Dye Terminator Cycle Sequencing Kit – Applied Biosystems, Foster City, CA, USA) both in forward and reverse direction using the PCR primers. After removal of unincorporated dye-labelled terminators, the products were sequenced on a ABI Prism 310 Genetic analyser (Applied Biosystems).

Presentation of the results

The experiments are illustrated by traces that are means $\pm$ s.e. or representative traces of results obtained with the indicated number of cells or clusters of cells from at least three different cultures. The statistical significance

Table 1. Sequences of oligonucleotide primers

Gene name	Size (bp)	5' oligonucleotide	3' oligonucleotide	GenBank accession no.
RyR1	263	GGC.CGT.AGT.GGT.CTA.CTT.GTA.TAC	TGA.TAG.CCA.GCA.GAA.TGA.CGA.TAA.C	NM_009109 (14662–14924)
RyR2	434	TGT.TGG.CTT.ATT.GGC.TGT.TGT.TG	GGT.TGT.GTT.CCT.GTA.AAG.TAT.GGG	AF295105 (14310–14743)
RyR3	338	GGA.CAA.AAA.TGC.CCT.TGA.CTT.TAG.C	TGA.AAG.CCA.CCA.CAG.TAT.AGA.GG	X83934 (499–836)
TBP ^a	190	ACC.CTT.CAC.CAA.TGA.CTC.CTA.TG	ATG.ATG.ACT.GCA.GCA.AAT.CGC	D01034

^aOligonucleotide primer sequences from Jensen *et al.* (1996). All RyR amplicons are located in the mRNA region coding for the transmembrane domains (last 1000 AA) of RyRs.

between means was assessed by unpaired Student's *t* test. Differences were considered significant at $P < 0.05$.

Results

Depolarization with high K^+ elicits two types of $[Ca^{2+}]_c$ response in β -cells

The contribution of intracellular Ca^{2+} stores to the $[Ca^{2+}]_c$ rise evoked by depolarization of the plasma membrane was evaluated in single β -cells (using a photometric-based system) or islet cells within clusters (using digital image analysis) of NMRI mice. The cells were perfused with 15 mM glucose, a concentration that stimulates sequestration of Ca^{2+} by intracellular organelles, in particular by the endoplasmic reticulum (ER) (Tengholm *et al.* 1999). To prevent $[Ca^{2+}]_c$ oscillations resulting from glucose-induced depolarization, the perfusion medium was supplemented with diazoxide which, by opening ATP-sensitive K^+ channels (Trube *et al.* 1986), clamps the plasma membrane at the resting potential and keeps $[Ca^{2+}]_c$ at low and basal levels (beginning of trace of Fig. 1A and B). $[Ca^{2+}]_c$ was then increased by depolarizing β -cells for at least 200 s with 45 mM K^+ in the continuous presence of diazoxide. Two patterns of $[Ca^{2+}]_c$ responses were observed: a monotonic rise followed by a sustained elevation (Fig. 1A), or a biphasic rise characterized by a single large and transient $[Ca^{2+}]_c$ peak (TCP) superimposed on a sustained elevation (Fig. 1B). The TCP occurred with a variable delay after the onset of depolarization – usually between 40 and 120 s – in different cells. This occurrence of a TCP did not depend on the magnitude of the initial $[Ca^{2+}]_c$ rise. Thus, $[Ca^{2+}]_c$ just before the TCP (825 ± 47 nM, $n = 22$) was not different from the maximal $[Ca^{2+}]_c$ rise in cells without a TCP (951 ± 66 nM, $n = 10$). However, the $[Ca^{2+}]_c$ peak during the TCP (1961 ± 164 nM, $n = 22$), was much higher ($P < 0.001$) than the maximum reached in the absence of a TCP, indicating that the TCP amplifies the $[Ca^{2+}]_c$ rise induced by the depolarization. The TCP did

not require the presence of glucose as it could be elicited after 10 min of perfusion without glucose. For unknown reasons, the percentage of β -cells displaying a TCP in response to 45 mM K^+ was larger in cells within clusters (84%, 103/123 cells from 45 clusters; only 2 clusters without TCPs) than in isolated cells (47%, 89/189 cells). There was no difference in the diameter of single cells showing a TCP (14.9 ± 0.2 μ m) or not (14.1 ± 0.7 μ m).

The TCP corresponds to a release of Ca^{2+} from the ER

When the ER was emptied by TG, a potent SERCA inhibitor, all cells stimulated by 45 mM K^+ responded with a $[Ca^{2+}]_c$ rise characterized by a very rapid upstroke phase (maximum $[Ca^{2+}]_c$ peak reached in 24 ± 4 s, $n = 7$) and an initial amplitude larger than that in control cells: for a 40 s-depolarization with K45, maximum $[Ca^{2+}]_c$ averaged 2147 ± 350 nM in seven TG-treated cells *versus* 789 ± 39 nM in 32 control cells with or without a TCP, $P < 0.0001$. After pre-treatment with TG, no cell displayed a TCP (Fig. 1C), suggesting that the TCP observed in control cells corresponds to Ca^{2+} release from the ER. It is interesting that the maximal $[Ca^{2+}]_c$ increase during a TCP (1961 ± 164 nM, $n = 22$) was not larger than the $[Ca^{2+}]_c$ rise induced by Ca^{2+} influx after inhibition of the SERCA by TG (2147 ± 350 nM, $n = 7$), indicating that, during the TCP, the ER does not release enough Ca^{2+} to increase $[Ca^{2+}]_c$ to a larger extent than that achieved by Ca^{2+} influx through voltage-dependent Ca^{2+} channels alone.

To ascertain that the TCP results from a mobilization of Ca^{2+} from the ER and not from an abrupt acceleration of Ca^{2+} influx through voltage-dependent Ca^{2+} channels, we simultaneously measured $[Ca^{2+}]_c$ and the voltage-dependent Ca^{2+} current in the perforated mode of the patch-clamp technique. In the first series of experiments, β -cells were submitted to a 3-min train of 50 ms-depolarizations (from -70 to 0 mV) applied at 4 Hz (Fig. 2A). This protocol of depolarization induced a rapid increase in $[Ca^{2+}]_c$ that slowly stabilized at a sustained level, and was accompanied by a progressive decrease of the Ca^{2+} current, reflecting rundown. In 3/7 cells,

$[\text{Ca}^{2+}]_c$ showed a second phase of increase corresponding to a TCP, that was accompanied by a decrease of the current, probably reflecting a Ca^{2+} -mediated acceleration of rundown. This demonstrates that the TCP does not result from a larger Ca^{2+} current, but from a mobilization of intracellular Ca^{2+} . No TCP was observed in voltage-clamped TG-pre-treated cells ($n = 4$).

Next we tested whether a mobilization of Ca^{2+} might also occur immediately after the onset of the depolarization and escape detection because of its masking by the concomitant Ca^{2+} influx. To separate Ca^{2+} influx and Ca^{2+} release, a second series of patch-clamp experiments was performed in the perforated mode. Single β -cells were sequentially submitted to depolarizing pulses (from -70 to 0 mV) of increasing duration (10, 25, 50, 100, 250, 500, 1000, 2500, 5000, 10000 ms) but separated by a recovery period permitting complete return of $[\text{Ca}^{2+}]_c$ to basal levels. As illustrated in Fig. 2B and C (filled circles), depolarization-induced $[\text{Ca}^{2+}]_c$ rises ($\Delta[\text{Ca}^{2+}]_c$) increased in amplitude with the duration of the depolarization. In Fig. 2D, $\Delta[\text{Ca}^{2+}]_c$ is plotted as a function of the charge density carried by Ca^{2+} through voltage-dependent Ca^{2+} channels. This type of representation is commonly used to disclose a possible contribution of Ca^{2+} mobilization from intracellular Ca^{2+} stores to the $[\text{Ca}^{2+}]_c$ rise (Llano *et al.* 1994; Shmigol *et al.* 1995). Indeed, the relationship between $\Delta[\text{Ca}^{2+}]_c$

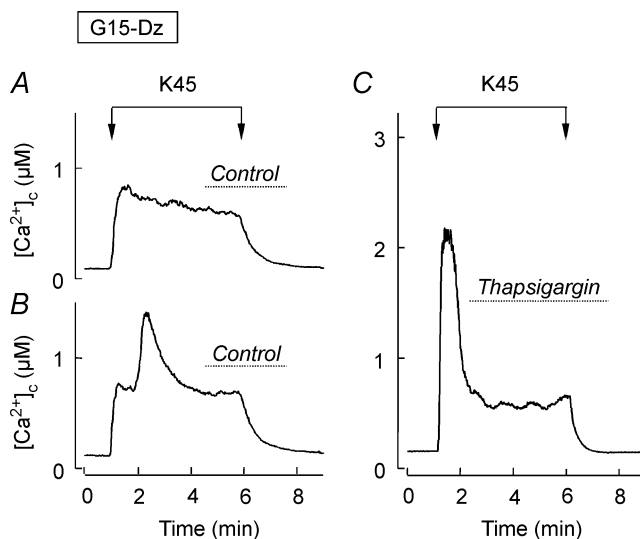


Figure 1. Depolarization with high K^+ elicits two patterns of $[\text{Ca}^{2+}]_c$ increase in NMRI mouse pancreatic β -cells

Single β -cells or islet cells within clusters were pre-treated (C) or not (A and B) with $1 \mu\text{M}$ TG during the loading with fura2. They were perfused with a medium containing 15 mM glucose (G15) and $100 \mu\text{M}$ diazoxide (Dz). The K^+ concentration of the medium was increased from 4.8 to 45 mM (K45) as indicated on top of the figure. A and B, illustrate $[\text{Ca}^{2+}]_c$ changes in two neighbouring cells of a doublet. C, shows $[\text{Ca}^{2+}]_c$ changes in a single β -cell. The traces are representative of results obtained in 120 (A), 192 (B) and 37 (C) cells.

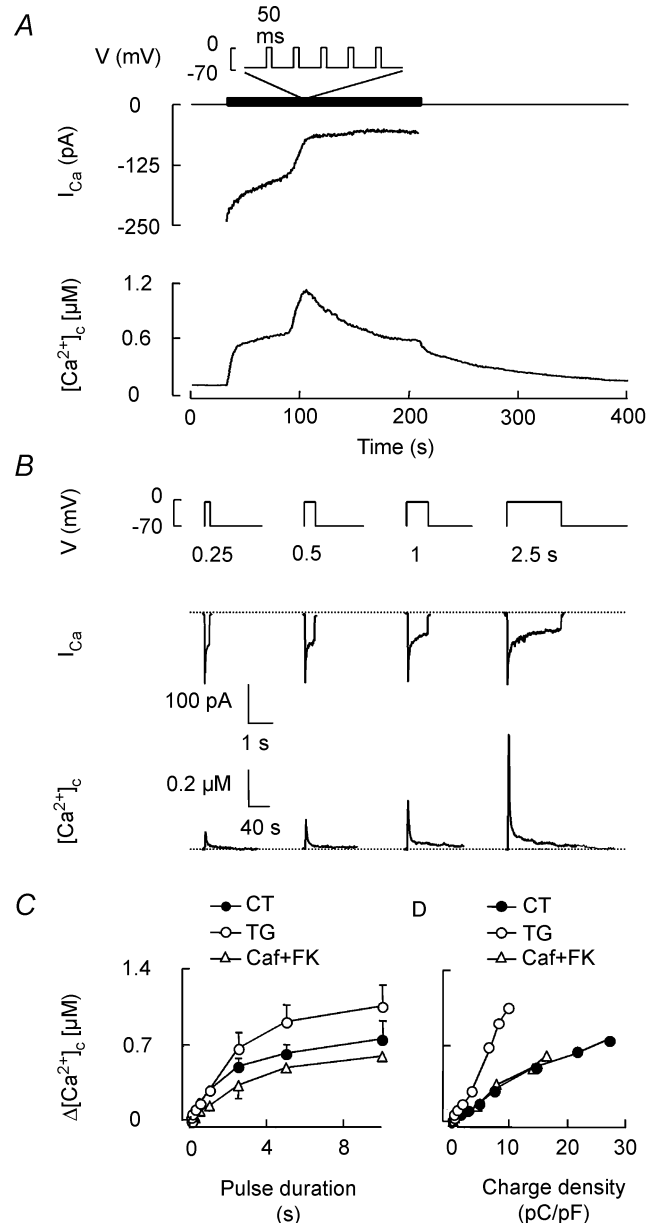


Figure 2. The ER takes up Ca^{2+} during short depolarization and releases it during prolonged depolarization

The voltage-dependent Ca^{2+} current and $[\text{Ca}^{2+}]_c$ changes were recorded simultaneously in voltage-clamped (perforated mode) single β -cells perfused with a medium containing 15 mM glucose. A, a single β -cell was submitted to a 3-min train of 50-ms depolarizations applied at 4 Hz from -70 to 0 mV. B, a single β -cell was submitted to depolarizing pulses from -70 to 0 mV of increasing durations. C and D, after loading with fura2, β -cells were perfused with a control medium (CT) or a medium containing 10 mM caffeine (Caf) and $5 \mu\text{M}$ forskolin (FK). One group of cells (TG) was pre-incubated with TG and then perfused with a control medium. $[\text{Ca}^{2+}]_c$ changes were plotted versus pulse duration (C) or charge density (D). The data are derived from experiments similar to those shown in B. The traces in A and B are representative of results obtained in three (A) and six (B) cells, and the traces in C and D are means of results obtained in six (filled circles), six (open circles) and three (open triangles) cells.

and the charge density should become supralinear as soon as $[\text{Ca}^{2+}]_c$ reaches the adequate concentration to trigger Ca^{2+} release from intracellular stores. However, as seen in Fig. 2D (filled circles), the relationship between these two parameters was linear, which indicates that the depolarization did not elicit Ca^{2+} mobilization.

Similar experiments were then performed in cells pre-treated with TG. The amplitude of $\Delta[\text{Ca}^{2+}]_c$ was larger in TG-pre-treated than in control cells (Fig. 2C: compare curves with filled and closed circles). The slope of the relationship between $\Delta[\text{Ca}^{2+}]_c$ and the charge density was also steeper in TG-pre-treated than in control cells (Fig. 2D). This indicates that, when the SERCA is not inhibited by TG, the ER does not amplify but rather buffers the rise in $[\text{Ca}^{2+}]_c$ elicited by short depolarizations as already reported (Gilon *et al.* 1999).

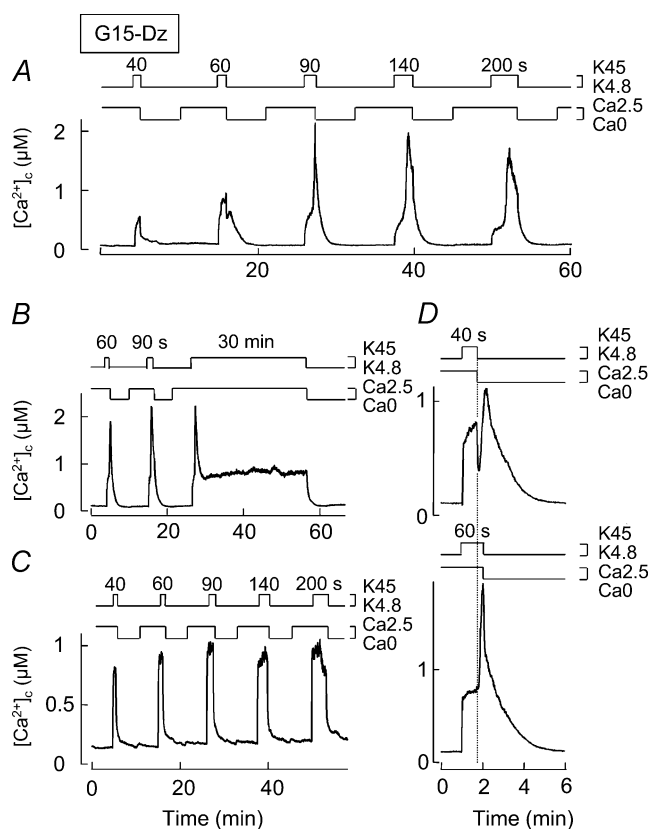


Figure 3. Characteristics of the TCP elicited by 45 mM K^+

Single β -cells were perfused with a medium containing 15 mM glucose (G15) and 100 μM diazoxide (Dz). The Ca^{2+} concentration in the medium was repeatedly changed between 0 and 2.5 mM, and that of K^+ between 4.8 and 45 mM, as indicated on the top of the panels. EGTA (2 mM) was added to the Ca^{2+} -free medium. D, shows two successive depolarizations of the same cell (taken from an experiment performed with the protocol illustrated on top of panel A), but the onsets of depolarization are aligned to show that the $[\text{Ca}^{2+}]_c$ rebound after the 40 s-depolarization and the TCP during the 60 s-depolarization occur with a similar delay after the beginning of the $[\text{Ca}^{2+}]_c$ increase. The traces are representative of results obtained in 7 (A), 4 (B), 5 (C) and 20 (D) cells.

It has been suggested that forskolin (a cAMP-producing agent) and caffeine (an agonist of RyRs) enhance RyR activity in pancreatic β -cells from *ob/ob* mice (Lemmens *et al.* 2001). We therefore repeated the patch-clamp experiments in cells continuously perfused with these two agents and not pre-treated with TG. The amplitude of $\Delta[\text{Ca}^{2+}]_c$ was slightly smaller than in control conditions (Fig. 2C: compare curves with filled circles and open triangles). This may result from an inhibition of voltage-dependent Ca^{2+} current by caffeine (Islam *et al.* 1995). Plotting $\Delta[\text{Ca}^{2+}]_c$ as a function of the charge density corrects the effects of caffeine on the Ca^{2+} current and results in a relationship with a similar slope to that of control cells (Fig. 2D). All these experiments suggest that no mobilization of Ca^{2+} from intracellular stores contributes to the $[\text{Ca}^{2+}]_c$ rise elicited by depolarizations that do not exceed 10 s.

Characteristics of the TCP

The experiments depicted in Fig. 3A were designed to explore the temporal and Ca^{2+} requirements for the development of a TCP. Single β -cells were repetitively depolarized by high K^+ pulses of increasing durations (from 20 to 300 s) separated by 10 min intervals. The medium contained 2.5 mM Ca^{2+} during the 5 min preceding the depolarization and the depolarization itself, but no Ca^{2+} (+ 2 mM EGTA) during the 5 min following the end of the depolarization (to immediately stop Ca^{2+} influx). A TCP could be elicited only when the depolarization was long enough (90 s in the cell illustrated in Fig. 3A). The TCP could then be repeatedly activated by cycles of repolarization/depolarization. Although the depolarization time required to trigger a TCP was variable between different cells, it was similar (maximal variation of 10 s) when the same cell was submitted to repetitive depolarizations. In contrast, sustained depolarization lasting 30 min did not induce regenerative TCPs (Fig. 3B).

Interestingly, most cells (20/31 cells) that developed a TCP when the depolarization was of sufficient duration, also displayed a Ca^{2+} mobilization in a Ca^{2+} -free medium when the depolarization was shorter. This is manifested as a $[\text{Ca}^{2+}]_c$ rebound, sometimes of large amplitude, after the end of the 40 s- (Fig. 3D: upper panel) or 60 s-depolarization (Fig. 3A), thus after $[\text{Ca}^{2+}]_c$ already had started to decrease. Similar observations were made when the repolarization (decrease in K^+ concentration) occurred in a medium containing Ca^{2+} (not shown, $n = 3$). Comparison of two successive depolarizations of the same cell (Fig. 3D) by aligning their onsets indicates that the post-depolarization rebound occurred at approximately the same time (± 6 s, $n = 20$) after the onset of the preceding depolarization as did a TCP after the onset of a longer depolarization. Like

the TCP, the post-depolarization rebound was abolished by thapsigargin ($n=4$). When a TCP had occurred during the depolarization, no $[\text{Ca}^{2+}]_c$ rebound ever occurred after repolarization and extracellular Ca^{2+} withdrawal. Cells that did not develop a TCP even during long depolarization, never displayed Ca^{2+} mobilization after depolarization (Fig. 3C). All these characteristics indicate that the post-depolarization $[\text{Ca}^{2+}]_c$ rebound and the TCP are two modes of expression of the same phenomenon.

The preceding experiments have characterized the influence of depolarization time on the induction of a TCP. We next evaluated the impact of the amplitude of depolarization. When β -cells were sequentially depolarized with 25, 30, 35 and 45 mM K^+ for 6 min (Fig. 4A), the $[\text{K}^+]$ at which a TCP occurred varied between cells (TCP detected in 63, 89, 95 and 95% of the cells stimulated with, respectively, 25, 30, 35 and 45 mM, $n=19$) and was 30 mM in the cell illustrated in Fig. 4A. The amplitude of the TCP and the speed of its ascending phase increased with $[\text{K}^+]$. By contrast, the delay (duration of depolarization) before development of a TCP decreased with $[\text{K}^+]$ (peak of the TCP occurring 159, 148, 134 and 103 s after the addition of, respectively, 25, 30, 35 and 45 mM) (Fig. 4A). Although this delay was previously shown to be similar during repetitive depolarizations with the same $[\text{K}^+]$ (Fig. 3A), it was modified by pre-conditioning the cell as illustrated in Fig. 4B. Thus, the time required for 45 mM K^+ to elicit a TCP was reduced by pre-exposure to a $[\text{K}^+]$ that did not elicit a TCP (compare period 1 and 2 in Fig. 4B). This experiment is compatible with the idea that the filling state of the ER is an important determinant triggering the TCP. However, when the pre-conditioning $[\text{K}^+]$ (35 mM in this example) elicited even a small TCP, 45 mM K^+ no longer triggered a TCP (Fig. 4C).

High K^+ -induced Ca^{2+} release in insulin-secreting cells other than β -cells from NMRI mice

In β -cells, Ca^{2+} is taken up in the ER by two SERCA isoforms, SERCA2b and SERCA3, the latter being operative only at high $[\text{Ca}^{2+}]_c$ (Arredouani *et al.* 2002a). To evaluate whether SERCA3 plays a role in the TCP, we compared the $[\text{Ca}^{2+}]_c$ response to 45 mM K^+ in β -cells from SERCA3 knockout (SERCA3^{-/-}) mice and control C57BL/6J mice from the same colony expressing SERCA3 (SERCA3^{+/+}). Whereas a TCP occurred in 40/45 cells from control mice (14 clusters), it was never observed in 37 cells from SERCA3 knockout mice (12 clusters) (data not illustrated).

A contribution of Ca^{2+} release from intracellular Ca^{2+} stores to the depolarization-induced $[\text{Ca}^{2+}]_c$ increase has been documented in INS-1 cells (Gamberucci *et al.*

1999) and β -cells from the leptin-deficient *ob/ob* mouse (Liu *et al.* 1996). We therefore characterized the $[\text{Ca}^{2+}]_c$ response of these two cell types to 45 mM K^+ . Depolarizing INS-1 cells with high K^+ in the presence of diazoxide elicited a monotonic $[\text{Ca}^{2+}]_c$ increase in 59% of the cells (73/123), whereas regenerative $[\text{Ca}^{2+}]_c$ spikes occurred on top of the sustained increase in the other 41% (Fig. 5A). These spikes with a very rapid upstroke phase are ascribed

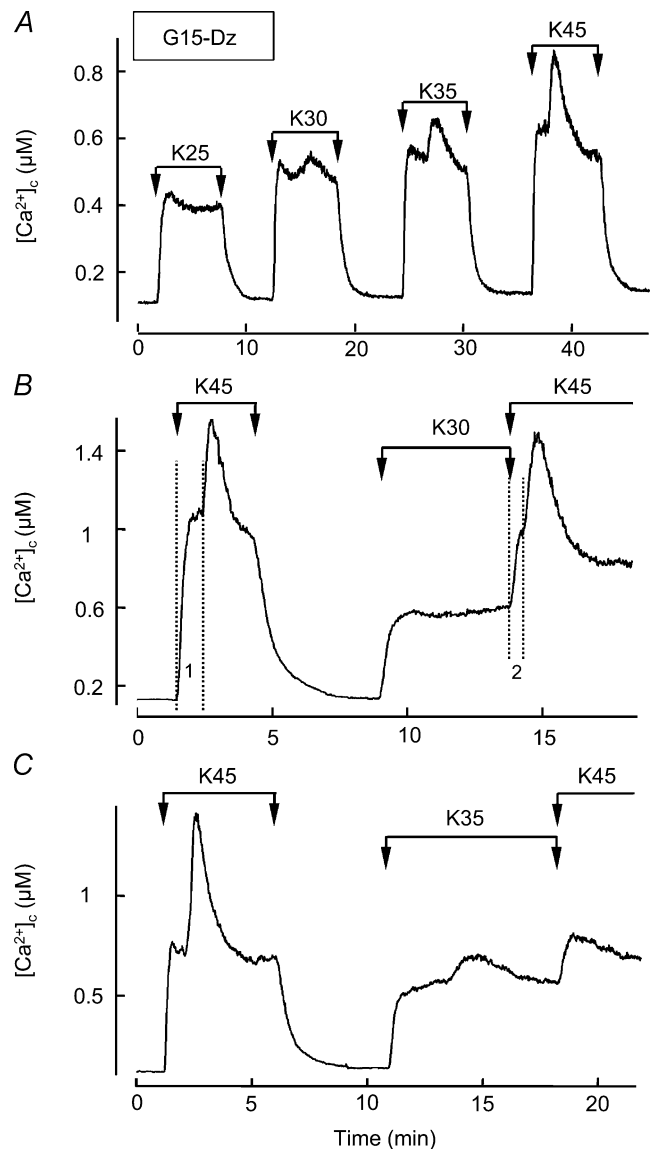
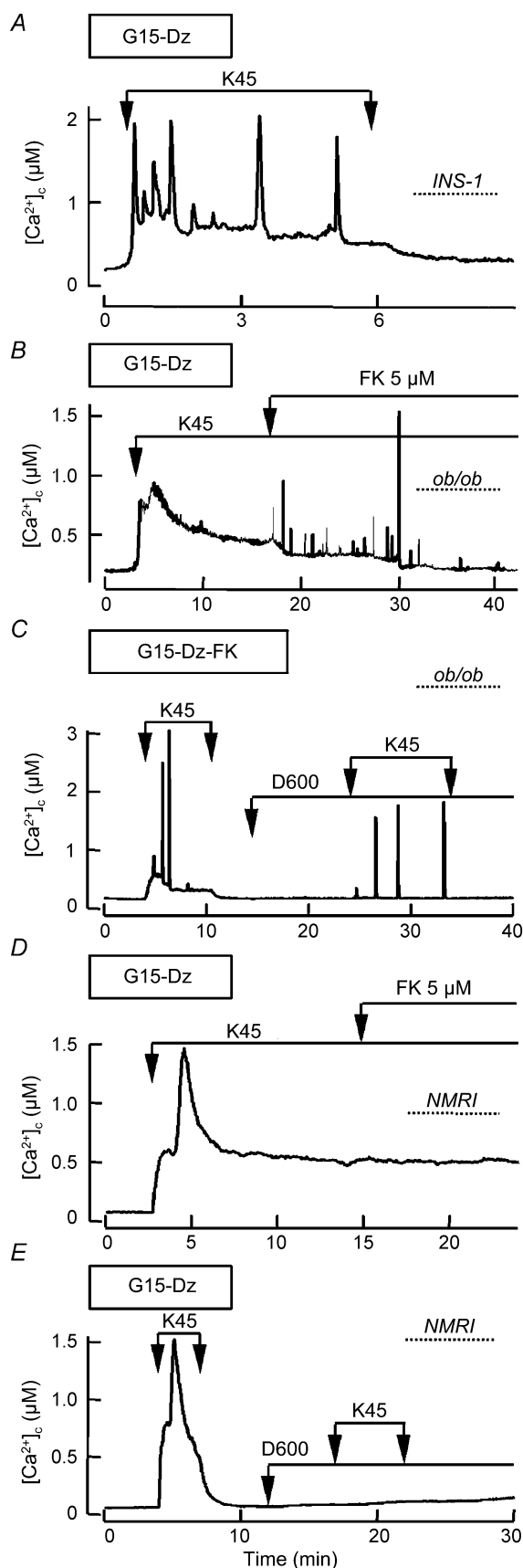


Figure 4. The characteristics of the TCP depend on the K^+ concentration

Single β -cells were perfused with a medium containing 15 mM glucose (G15) and 100 μM diazoxide (Dz). The K^+ concentration of the medium was 4.8 mM unless indicated by K25, K30, K35 or K45 which correspond to, respectively, 25, 30, 35 or 45 mM. B, 1 and 2 between the dashed lines represent the periods of time between the beginning of the depolarization with K45 and the TCP. The traces are representative of results obtained in 19 (A), 10 (B) and 9 (C) cells.



to periodic release of Ca^{2+} from the ER because they were abolished by TG (not shown, 61 cells).

Depolarization of β -cells from ob/ob mice with 45 mM K^+ rarely elicited a single broad $[Ca^{2+}]_i$ peak reminiscent of the TCP found in NMRI mouse β -cells (Fig. 5B). Subsequent addition of forskolin, a potent cAMP-producing agent, triggered large and rapid $[Ca^{2+}]_i$ transients in 54% of cells (57/105) (Fig. 5B). Blockade of voltage-dependent Ca^{2+} channels with D600 abolished the sustained elevation of $[Ca^{2+}]_i$ elicited by high K^+ but did not suppress the transients (Fig. 5C); however, they were prevented by TG-pre-treatment (not shown, 4 cells). These observations are in agreement with previous reports (Grapengiesser *et al.* 1991; Liu *et al.* 1996). In pancreatic β -cells of control lean ($ob/+$ or $+/+$) and NMRI mice, addition of forskolin to a medium containing 45 mM K^+ never elicited $[Ca^{2+}]_i$ spikes (Fig. 5D), and high K^+ was totally ineffective in the presence of D600 (Fig. 5E). $[Ca^{2+}]_i$ spikes are thus a peculiarity of β -cells from obese ob/ob mice (Fournier *et al.* 1994).

The TCP reflects an atypical CICR

Two known mechanisms could induce the TCP: a DICR or a CICR. Because activation of DICR only requires depolarization (Nabauer *et al.* 1989), it is most convincingly identified in a Ca^{2+} -free medium. In skeletal muscle fibres, where DICR is mediated by RyR1 (Berridge, 1997; Murayama & Ogawa, 2002), plasma membrane depolarization with 100 mM K^+ in a Ca^{2+} -free medium induced a transient $[Ca^{2+}]_i$ rise (Fig. 6A). Subsequent application of caffeine, an activator of RyRs (Ehrlich *et al.* 1994), triggered a large $[Ca^{2+}]_i$ mobilization. Both effects involve activation of RyRs because they were abrogated in myocytes pre-treated with ryanodine at a concentration (10 μM) that inhibits RyRs (Bers *et al.* 1987, 1989; Ehrlich *et al.* 1994; Sutko & Airey, 1996) (Fig. 6A). In β -cells displaying a TCP in response to two successive pulses of high K^+ in a medium containing Ca^{2+} , plasma membrane depolarization with 100 mM K^+ in a Ca^{2+} -free medium never induced a $[Ca^{2+}]_i$ rise (Fig. 6B), although the ER still contained Ca^{2+} as shown by the transient $[Ca^{2+}]_i$ increase induced by acetylcholine

Figure 5. Regenerative Ca^{2+} spikes induced by depolarization in INS-1 cells and β -cells from ob/ob mice have different characteristics than the TCP in NMRI β -cells

INS-1 cells (A), β -cells from ob/ob mice (B and C) or NMRI mice (D and E) were perfused with a medium containing 15 mM glucose (G15) and 100 μM diazoxide (Dz). The K^+ concentration of the medium was changed between 4.8 and 45 mM (K45), and 100 μM D600 was applied when indicated. Forskolin (FK; 5 μM) was present throughout the whole experiment (C) or applied as shown on the top of panels (B and D). The traces are representative of results obtained in 50 (A), 13 (B), 3 (C), 7 (D) and 8 (E) cells.

(ACh). These experiments indicate that the TCP does not result from a DICR, but rather from a CICR.

CICR can be triggered by the activation of two types of receptors: RyRs and IP₃Rs. The effect of caffeine was first tested in cardiomyocytes where CICR is mediated by RyRs (mainly RyR2) (Smith *et al.* 1988; Bers & Perez-Reyes, 1999). Addition of caffeine to a Ca²⁺-free medium induced a transient [Ca²⁺]_i rise that was abolished by pre-treatment of the cells with 10 μ M ryanodine (Fig. 7A). Similar results were obtained in INS-1 cells (Fig. 7B). However, in β -cells displaying a TCP, addition of caffeine to a Ca²⁺-free medium was without effect on [Ca²⁺]_i whereas subsequent application of acetylcholine mobilized Ca²⁺ from the ER (Fig. 7C). In another series of experiments, β -cells were first pre-treated with 100 μ M ryanodine, a concentration more than sufficient to block RyRs

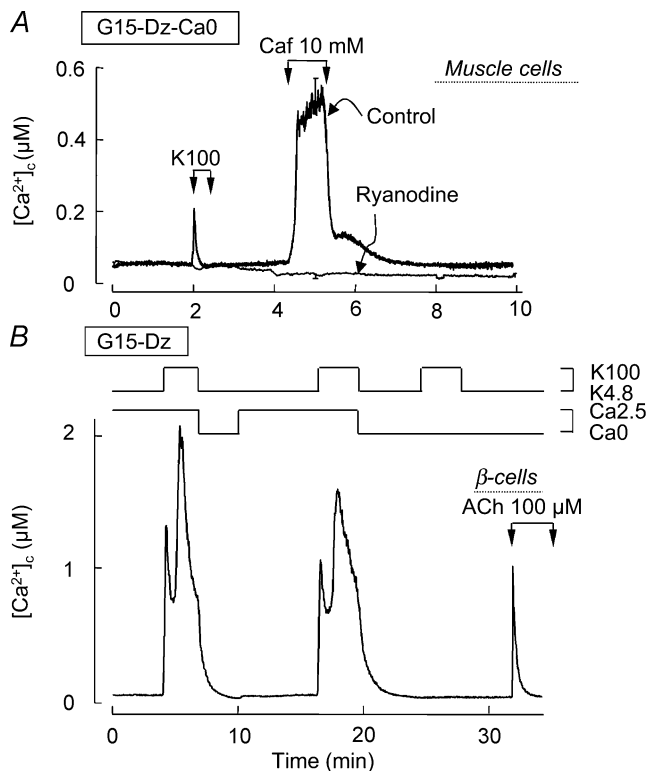


Figure 6. The TCP elicited in β -cells does not result from a depolarization-induced Ca²⁺-release

A, single FDB muscle fibres pre-treated or not with 10 μ M ryanodine during the loading with fura2 were perfused with a Ca²⁺-free medium containing 2 mM EGTA, 15 mM glucose (G15) and 100 μ M diazoxide (Dz). The K⁺ concentration was increased from 4.8 to 100 mM (K100), and 10 mM caffeine (Caf) was applied when indicated. B, a single β -cell was perfused with a medium containing 15 mM glucose (G15) and 100 μ M diazoxide (Dz). The Ca²⁺ concentration in the medium was changed between 2.5 and 0 mM, and that of K⁺ between 4.8 and 100 mM, as shown on top of the panel. EGTA (2 mM) was added to the Ca²⁺-free medium. 100 μ M acetylcholine (ACh) was applied when indicated. The traces in A are means of results obtained in three (control) and four (ryanodine) cells. The trace in B is representative of results obtained in three cells.

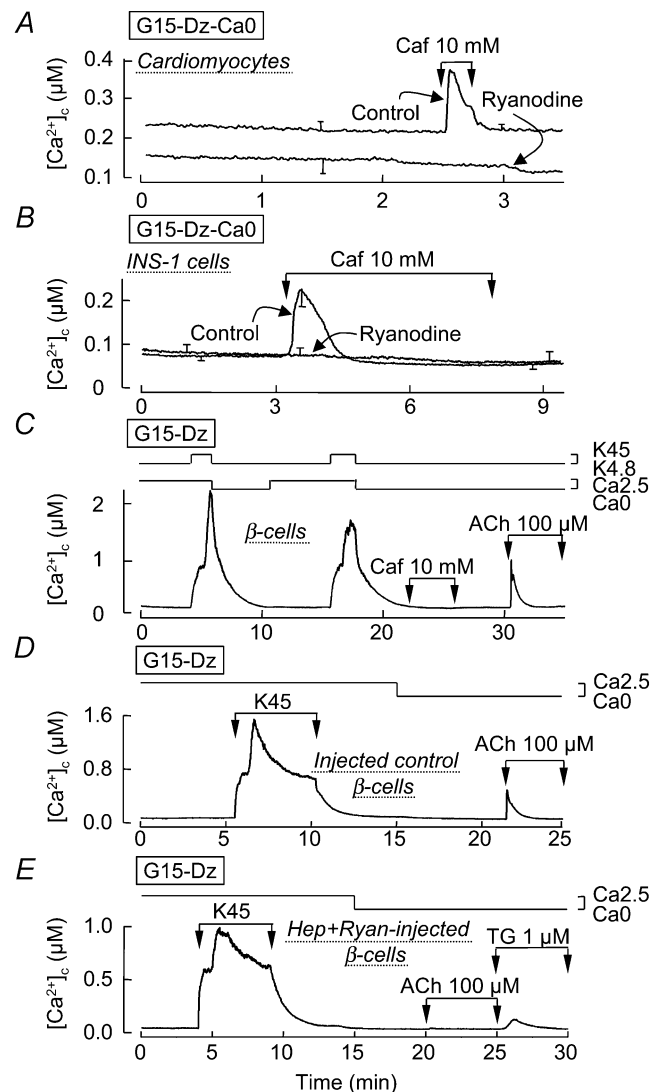


Figure 7. Ca²⁺-induced Ca²⁺ release underlying the TCP in β -cells does not involve RyRs or IP₃Rs

A and B, cardiomyocytes (A) or INS-1 cells (B) pre-treated or not with 10 μ M (A) or 100 μ M (B) ryanodine during the loading with fura2 were perfused with a Ca²⁺-free medium containing 2 mM EGTA, 15 mM glucose (G15) and 100 μ M diazoxide (Dz). Caffeine (10 mM) was applied when indicated (Caf). C, single β -cells were perfused with a medium containing 15 mM glucose (G15) and 100 μ M diazoxide (Dz). The Ca²⁺ concentration in the medium was changed between 0 (+ 2 mM EGTA) and 2.5 mM, and that of K⁺ between 4.8 and 45 mM, as shown on top of the panel. Caffeine (Caf; 10 mM) and 100 μ M acetylcholine (ACh) were applied when indicated. D and E, single β -cells microinjected with fura2 alone (D, control), or with fura2, heparin (200 mg ml⁻¹) and ryanodine (10 mM) (E, Hep + Ryan) were perfused with a medium containing 15 mM glucose (G15) and 100 μ M diazoxide (Dz). When shown on the top of the panels, the cells were perfused with a Ca²⁺-free medium supplemented with 2 mM EGTA (Ca0). When indicated, the K⁺ concentration was changed from 4.8 to 45 mM (K45), and 100 μ M acetylcholine (ACh) and 1 μ M thapsigargin (TG) were applied to the medium. The traces in A and B are means of results obtained in three (A, control and ryanodine), seven (B, control) and four (B, ryanodine) cells. The traces in C–E are representative of results obtained in three (C) and four (D and E) cells.

in muscles cells, before being submitted to a 5-min depolarization with 45 mM K^+ . The percentage of cells displaying a TCP was $\sim 50\%$, and similar in control (11/21 cells) and ryanodine-pre-treated cells (12/23). All these results exclude the involvement of RyRs in the TCP.

To evaluate whether the CICR underlying the TCP results from IP_3R activation, we used 2-aminoethoxydiphenyl borate (2-APB), a cell permeant drug that has been reported to inhibit IP_3Rs (Maruyama *et al.* 1997). A concentration of 500 μM 2-APB was required to fully suppress Ca^{2+} mobilization induced by 100 μM ACh. However, this concentration of the drug could not be used to evaluate the role of IP_3Rs in the TCP because it exerted strong non-specific effects as reported elsewhere (Missiaen *et al.* 2001). Thus, it induced a sustained $[Ca^{2+}]_c$ elevation in β -cells whose $[Ca^{2+}]_c$ was low (in the presence of 15 mM glucose plus diazoxide), whereas it decreased $[Ca^{2+}]_c$ in β -cells depolarized with high K^+ ($n = 3$, not shown). Caffeine, used at high concentrations has been reported to inhibit IP_3Rs (Ehrlich *et al.* 1994; Lund & Gylfe, 1994). In our hands, 50 mM of the drug was required to abolish ACh-induced Ca^{2+} mobilization. However, in keeping with previous reports (Islam *et al.* 1995; Islam, 2002), this caffeine concentration produced non-specific effects when tested in a medium containing Ca^{2+} ($n = 3$, not shown). We therefore used heparin, an established inhibitor of IP_3Rs (Nilsson *et al.* 1988; Ehrlich *et al.* 1994). Because IP_3Rs and RyRs can both trigger CICR, their individual blockade might not be sufficient to establish their participation. β -cells were thus microinjected with fura2 alone or with fura2 plus heparin and ryanodine, and their $[Ca^{2+}]_c$ responses to various stimuli were compared. Control cells microinjected with fura2 free acid alone displayed a TCP in response to high K^+ (1131 ± 256 nM, $n = 4$) and a subsequent large Ca^{2+} mobilization upon addition of 100 μM ACh to a Ca^{2+} -free medium (Fig. 7D). The combination of heparin plus ryanodine did not prevent the TCP elicited by high K^+ (846 ± 128 nM, $n = 4$) but inhibited IP_3Rs (heparin alone produced the same effect) as shown by the abrogation of ACh-induced $[Ca^{2+}]_c$ rise (Fig. 7E). The lack of effect of ACh was not due to the absence of Ca^{2+} in the ER as application of TG triggered a transient $[Ca^{2+}]_c$ increase.

All these experiments show that the TCP results from an atypical CICR that is independent of RyRs and IP_3Rs .

Does atypical CICR occur in response to stimuli other than high K^+ ?

We tested whether CICR occurs during glucose-induced $[Ca^{2+}]_c$ oscillations. Digital image analysis was used to compare the $[Ca^{2+}]_c$ responses to glucose and high K^+ in individual cells within clusters of 2–8 islet cells. We hypothesized that, because of the electrical coupling, all

$[Ca^{2+}]_c$ changes induced by oscillations of the membrane potential should be synchronized between neighbouring β -cells within a cluster (Jonkers *et al.* 1999), whereas $[Ca^{2+}]_c$ changes induced by CICR might be asynchronous in neighbouring cells. As illustrated in Fig. 8A, the oscillations of $[Ca^{2+}]_c$ induced by 15 mM glucose were well synchronized between the two cells of a doublet. Depolarization with 45 mM K^+ in the presence of diazoxide induced first a rapid and steep $[Ca^{2+}]_c$ increase that was also synchronized in the two cells. However, after a small period of sustained elevation of $[Ca^{2+}]_c$, a TCP occurred with different lags (shown by the dashed lines). This asynchrony was also observed in adjacent cells from larger clusters (8 cells), and explains why no TCP can be discerned when the $[Ca^{2+}]_c$ response to high K^+ is integrated over large clusters or whole islets.

Figure 8B shows a similar experiment performed in a cluster of five cells. K^+ at 45 mM triggered a TCP that occurred sooner in cell 1 than in the other four cells (see dashed lines). The changes in $[Ca^{2+}]_c$ induced by 15 mM glucose were synchronized and parallel between cells 2–5. However, during each glucose-induced $[Ca^{2+}]_c$ oscillation, cell 1 also displayed one transient $[Ca^{2+}]_c$ rise that was not observed in the other cells (shown by arrows in Fig. 8B and C), and that was short-lived because Ca^{2+} influx spontaneously stopped soon after the onset of the TCP. It is remarkable that cell 1 was the first cell to develop a TCP in response to high K^+ . These observations suggest that the TCP observed during glucose stimulation corresponds to a Ca^{2+} mobilization similar to that triggered by high K^+ .

However, we wish to emphasize that, within the clusters, only 12% of the cells (10/81) displayed a TCP in response to glucose alone whereas 84% of the cells (61/81) developed a TCP in response to high K^+ . Only 9/30 clusters contained at least one cell showing a TCP during glucose-induced $[Ca^{2+}]_c$ oscillations. When a cell displayed a TCP during glucose-induced $[Ca^{2+}]_c$ oscillations, the phenomenon was not constant, occurring in about 2/3 of the oscillations.

We also tested whether the incretin hormones, GLP-1 and GIP, could trigger a CICR. As shown in Fig. 8D, sequential application of GLP-1 and GIP transformed $[Ca^{2+}]_c$ oscillations induced by glucose into a sustained elevation but failed to induce a TCP in cells that subsequently displayed a TCP in response to high K^+ .

Expression of RyRs in β -cells

The presence of RyRs in β -cells is highly debated. RyR mRNAs from islets and control tissues (FDB skeletal muscle fibres for RyR1, cardiomyocytes for RyR2 and spleen for RyR3) were therefore amplified by RT-PCR (25 cycles for control tissues and 30 cycles for islets). Under these conditions of amplification, all RyR mRNAs were strongly expressed in the respective control tissues

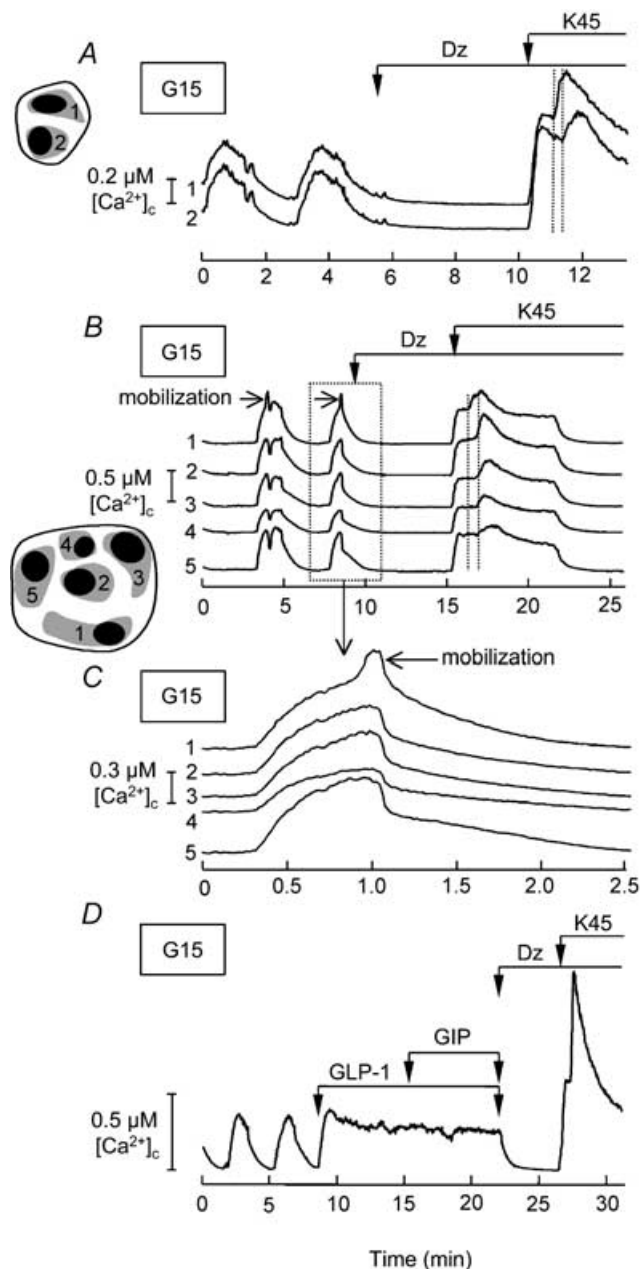


Figure 8. Atypical Ca^{2+} -induced Ca^{2+} release occurs in some β -cells stimulated with glucose

Clusters from two (A) or five (B and C) β -cells were perfused with a medium containing 15 mM glucose (G15). Diazoxide (Dz; 100 μM) was added, and the K^+ concentration of the medium was increased from 4.8 to 45 mM (K45) when indicated. In each cell, $[\text{Ca}^{2+}]_i$ was monitored in the shaded regions in the diagram, including the nucleus (in black). The numbers in front of the traces refer to the individual cells. C, shows, on a larger time-scale, the glucose-induced $[\text{Ca}^{2+}]_i$ oscillation shown by the dotted box in B. The arrows in B and C point to $[\text{Ca}^{2+}]_i$ changes induced by intracellular Ca^{2+} mobilizations. The two dashed lines in A and B help to show the different delays in onset of the TCP elicited by K45 in the cells within the cluster. The traces are representative of results obtained in 21 clusters of cells without TCPs in G15 (A), and nine clusters of cells of which one or two display a TCP in G15 (B and C). D, β -cells within clusters of two cells were perfused with a medium containing 15 mM glucose (G15). GLP-1 (25 nM),

(Fig. 9A), whereas RyR1 mRNA was undetectable and RyR2 and RyR3 mRNAs were weakly expressed in islets.

When the number of PCR cycles was increased further, the three RyR mRNAs could be detected in fresh and overnight-cultured islets (Fig. 9B). Because islets are composed of several cell types, fresh β - and non- β -cells were separated by fluorescence-activated cell sorting. This revealed that non- β -cells express all RyR mRNAs, whereas purified β -cells express only RyR3 mRNA. The sequence of RyR3 PCR products (290 bp within primers) from both spleen and purified β -cells showed 100% identity with oligonucleotides 524–813 of mouse RyR3 mRNA (GenBank Accession no. X83934.1).

Discussion

A decade ago, it was generally accepted that Ca^{2+} influx is the only mechanism underlying the $[\text{Ca}^{2+}]_i$ increase induced by depolarization of the plasma membrane of β -cells. Then, several studies suggested that intracellular Ca^{2+} stores could also play a role during this $[\text{Ca}^{2+}]_i$ rise, by either buffering (Gilon *et al.* 1999) or amplifying the $[\text{Ca}^{2+}]_i$ increase (for review see Satin, 2000; Islam, 2002). These controversies generated a confusion that prompted us to re-evaluate the situation in mouse β -cells. The present study throws new light on some of the previous results and shows that, upon Ca^{2+} influx through voltage-dependent Ca^{2+} channels, the ER rapidly takes up Ca^{2+} that can then be released by an atypical CICR if Ca^{2+} influx is sufficiently large and sustained.

During the initial period of depolarization, the ER does not release but takes up Ca^{2+}

We previously suggested that the ER buffers the initial $[\text{Ca}^{2+}]_i$ rise elicited by Ca^{2+} influx (Gilon *et al.* 1999). Our proposal is supported here by two sets of experiments using TG, a blocker of the SERCA. First, upon depolarization, the $[\text{Ca}^{2+}]_i$ increase was faster and larger in cells pre-treated with TG than in control cells. Second, simultaneous measurements of $[\text{Ca}^{2+}]_i$ and voltage-dependent Ca^{2+} current showed that the slope of the relationship between $\Delta[\text{Ca}^{2+}]_i$ and the charge density was steeper in TG-pre-treated than in control cells. Thus, there is no evidence that Ca^{2+} mobilization occurs during depolarizations that do not exceed 10 s. On the contrary, the $[\text{Ca}^{2+}]_i$ rise is attenuated by Ca^{2+} pumping into the ER.

GLP (25 nM) and diazoxide (Dz; 100 μM) were added, and the K^+ concentration of the medium was changed between 4.8 and 45 mM (K45) when indicated. The traces are representative of results obtained in six cells (D).

During prolonged depolarization, the ER releases Ca^{2+} from a pool replenished by SERCA3

Prolonged and strong depolarization of the plasma membrane of β -cells from NMRI and C57BL/6J mice often triggered a TCP on top of a sustained elevation of $[\text{Ca}^{2+}]_c$. A TCP could also occur after repolarization in a Ca^{2+} -free medium (thus appearing as a $[\text{Ca}^{2+}]_c$ rebound) provided the preceding depolarization in a Ca^{2+} -containing medium was long enough (≥ 40 s). This suggests that, once the ER has taken up enough Ca^{2+} , Ca^{2+} is mobilized.

The TCP was much more frequent in cells within clusters (84%) than in isolated cells (47%) suggesting that it is probably present in most β -cells within islets. Because this TCP is not accompanied by a concomitant increase in voltage-dependent Ca^{2+} current, can occur in a Ca^{2+} -free medium ($[\text{Ca}^{2+}]_c$ rebound), and is suppressed by TG, we attribute it to Ca^{2+} mobilization. The TG-sensitive stores correspond to the ER and, possibly, the Golgi apparatus (Pinton *et al.* 1998; Wuytack *et al.* 2002). The

lack of TCP in β -cells from SERCA3 knockout mice indicates that SERCA3 refills the Ca^{2+} pool from which the TCP occurs.

Ca^{2+} mobilization induced by high- K^+ reflects CICR independent from RyRs and IP_3Rs

Several arguments rule out the existence of a DICR in non-obese mouse β -cells. Depolarization with high K^+ in a Ca^{2+} -free medium did not increase $[\text{Ca}^{2+}]_c$ in β -cells, whereas the same manoeuvre evoked a typical DICR in skeletal muscle used as a control. Voltage-clamp depolarizations failed to increase $[\text{Ca}^{2+}]_c$ in fura2-loaded β -cells perfused with a Ca^{2+} -free medium (Rolland *et al.* 2002). Molecular biology experiments also showed that RyR1, that mediates DICR in skeletal muscle cells, is not expressed in NMRI mouse β -cells, whereas very low levels of RyR1 mRNA have been observed in the insulin-secreting βTC3 cell line (Holz *et al.* 1999). To produce DICR, RyR1 can only be activated by the α_{1S} subunit of L type Ca^{2+} channels (Schneider, 1994), which is also not present in β -cells that mainly express α_{1C} and α_{1D} subunits (Seino *et al.* 1992; Yang *et al.* 1999; Satin, 2000; Barg *et al.* 2001).

The second mechanism that could underlie the TCP is CICR, a phenomenon that is generally mediated by RyR activation. Previous studies (Islam *et al.* 1998; Takasawa *et al.* 1998; Holz *et al.* 1999) using RNase protection assays and RT-PCR experiments have reported that RyR2 mRNA is the main isoform expressed in islets of some species. However, its expression was found to be very low, at least 1000-fold less than in the heart (Islam *et al.* 1998). Our RT-PCR experiments confirmed the very low level of RyR mRNA in islet cells compared to control tissues. Moreover, analysis of purified β - and non- β -cells demonstrated that the pattern of RyR mRNA expression is very different between both cell types. Thus, after strong amplification, all RyR mRNA was detected in non- β -cells, whereas only RyR3 mRNA was found in β -cells. In addition to this low expression, functional experiments exclude the participation of RyRs in the TCP. Thus caffeine, a potent activator of all types of RyRs (Ehrlich *et al.* 1994) did not induce Ca^{2+} mobilization in NMRI β -cells, in contrast to skeletal muscle cells, cardiomyocytes and INS-1 cells, three cell types expressing RyRs (Berridge, 1997; Bers & Perez-Reyes, 1999; Gamberucci *et al.* 1999; Murayama & Ogawa, 2002). Moreover, pre-incubation of β -cells with a concentration of ryanodine that effectively blocked RyRs in muscle cells and INS-1 cells, did not suppress the TCP. It is unclear whether the low expression of RyR3 mRNA in β -cells permits production of physiologically relevant amounts of RyR3 protein, and if so, what function RyR3 serves in β -cells.

The alternative mechanism of CICR from the ER involves activation of IP_3Rs (Dyachok *et al.* 2004),

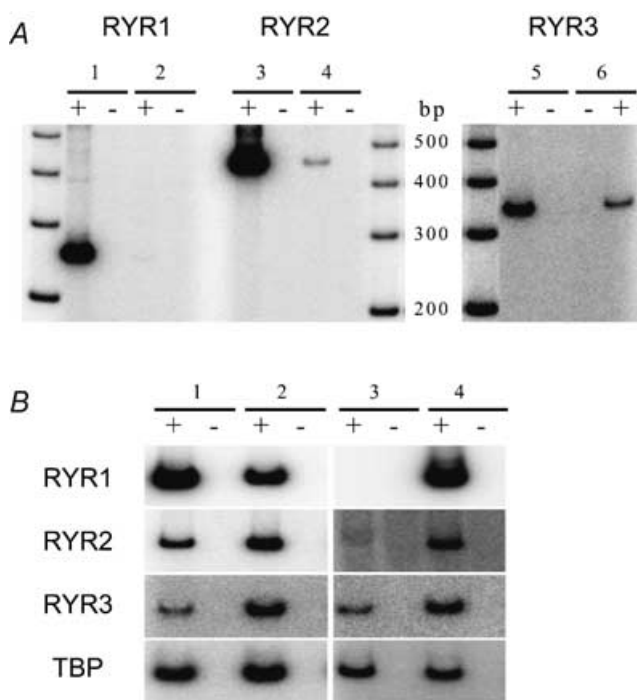


Figure 9. Only RyR3 is weakly expressed in mouse β -cells

A, ryanodine receptor (RyR1, RyR2, and RyR3) mRNAs from islets (lanes 2, 4 and 6) and control tissues (lanes 1, FDB skeletal muscle fibres for RyR1; lanes 3, cardiomyocytes for RyR2; lanes 5, spleen for RyR3) were amplified by RT-PCR (25 cycles for control tissues and 30 cycles for islets). B, ryanodine receptor (RyR1, RyR2, and RyR3) mRNAs and TATA-box binding protein (TBP) mRNAs from fresh islets (lanes 1), overnight cultured islets (lanes 2), FACS-purified β -cells (lanes 3) and non- β -cells (lanes 4) were amplified by RT-PCR (40 cycles for RyR1, 28–34 cycles for RyR2, 28 cycles for RyR3). + and –, addition and omission, respectively, of the reverse transcriptase in RT reaction.

which are largely expressed in β -cells (Lee & Laychock, 2001). This possibility was not easily evaluated because membrane permeant IP₃R blockers, such as caffeine and 2-APB, exerted marked non-specific effects in β -cells, in this and other studies (Islam *et al.* 1995; Missiaen *et al.* 2001). Xestospongine, another putative inhibitor of IP₃Rs (Gafni *et al.* 1997), is not specific (De Smet *et al.* 1999). We therefore microinjected heparin to inhibit IP₃Rs (Nilsson *et al.* 1988; Ehrlich *et al.* 1994). To avoid the possibility that any RyRs might compensate for the inhibition of IP₃Rs, we co-injected ryanodine. The TCP was not altered by the combination of the two drugs, whereas heparin effectively blocked Ca²⁺ mobilization induced by ACh. This indicates that neither IP₃Rs nor RyRs trigger this CICR.

Two other features make the identified CICR atypical. First, it occurs only once during a period of depolarization. Thus, it was never regenerative during prolonged depolarization, and induction of a small CICR by moderate depolarization prevented subsequent stronger depolarization from triggering an additional CICR. Reactivation of the CICR required that [Ca²⁺]_c decreased to basal levels. Second, this CICR does not seem to depend on instantaneous [Ca²⁺]_c as it can occur after repolarization, when [Ca²⁺]_c has started to decrease ([Ca²⁺]_c rebound). Moreover, the time required for 45 mM K⁺ to elicit a CICR is reduced by pre-exposure to a [K⁺] that raises [Ca²⁺]_c and allows refilling of the ER with Ca²⁺ but does not elicit a CICR. The CICR therefore appears to be regulated by the luminal [Ca²⁺] ([Ca²⁺]_{luminal}) and/or a signal dependent on [Ca²⁺]_{luminal} rather than by [Ca²⁺]_c itself.

A CICR mechanism independent from IP₃Rs and RyRs has recently been reported in permeabilized rat hepatocytes (Wissing *et al.* 2002) and A7r5 embryonic rat aorta cells (Kasri *et al.* 2003), but has not been sufficiently characterized to suggest mechanistic similarities with the phenomenon that is described in the present study.

Differences between non-obese mouse β -cells and other models

It has been reported that depolarization of the plasma membrane triggers Ca²⁺ release from the ER in INS-1 cells and in β -cells from *ob/ob* mice (Liu *et al.* 1996; Gamberucci *et al.* 1999). We could indeed detect TG-sensitive Ca²⁺ mobilization in these cells. However, the characteristics of the phenomenon were very different from those of the TCP. These transients were larger and much faster than the TCP, and were regenerative. On the other hand, forskolin which facilitated detection of the [Ca²⁺]_c transients in β -cells from *ob/ob* mice as in other studies (Grapengiesser *et al.* 1991; Fournier *et al.* 1994; Liu *et al.* 1996), failed to activate transients in NMRI and lean C57BL/6J mouse β -cells. Moreover, blockade of voltage-dependent Ca²⁺ channels did not suppress

the [Ca²⁺]_c transients in β -cells from *ob/ob* mice but abrogated the TCP in NMRI β -cells. This is not the first example of differences in [Ca²⁺]_c regulation in β -cells from non-obese and *ob/ob* mice (Ravier *et al.* 2002; Fournier *et al.* 1994).

Physiological significance of this atypical CICR

By using digital image analysis to compare [Ca²⁺]_c changes in individual cells within clusters, we occasionally identified asynchronous [Ca²⁺]_c transients during glucose-induced [Ca²⁺]_c oscillations. Although we cannot exclude the remote possibility that some cells of the clusters transiently desynchronize from the others, we propose that these transients reflect Ca²⁺ mobilization from intracellular stores. When induced by IP₃, such events indeed occur asynchronously in coupled β -cells (Jonkers *et al.* 1999). However, the characteristics of these asynchronous [Ca²⁺]_c transients observed in the presence of glucose (occurrence on top of a slow [Ca²⁺]_c oscillation, exclusively in cells showing a TCP in response to high K⁺) rather suggest that they result from atypical CICR. These mobilizations were seen in a small percentage of cells (12%), and were not induced by the two incretins, GLP-1 and GIP. It is unlikely that rapid transients escaped our image analysis because we acquired 5.4 ratio images in 6.5 s, which is the average duration of the transients detected in β -cells from *ob/ob* mice with our fast photometric system. However, we acknowledge that TCPs similar to those elicited by 25–30 mM K⁺ would be hardly detectable during glucose-induced [Ca²⁺]_c oscillations because of the slowness of their kinetics and/or their small amplitude. Even with this reservation in mind, it is obvious that the Ca²⁺ mobilizations observed here during glucose-induced [Ca²⁺]_c oscillations in non-obese mouse β -cells were much smaller than those reported in some studies using other types of insulin-secreting cells (for review see Islam, 2002). It is therefore difficult to establish their possible physiological function. One theoretical possibility is that the few cells in which this CICR occurs act as pacemakers, the sudden rise in [Ca²⁺]_c causing repolarization by activating Ca²⁺-dependent K⁺ currents and thereby terminating glucose-induced oscillations. This is not the case for two reasons. First, only 30% of the clusters displayed Ca²⁺ mobilizations during glucose-induced [Ca²⁺]_c oscillations in at least one cell. Second, when a cell showed Ca²⁺ mobilization during glucose-induced oscillations, this mobilization was inconsistent (2/3 of the oscillations). It is therefore reasonable to propose that, in non-obese mouse β -cells, glucose-induced [Ca²⁺]_c oscillations mainly result from intermittent Ca²⁺ influx and that Ca²⁺ mobilization plays little role in these changes.

However, this conclusion does not refute our previous proposal that the ER plays an important role during

$[Ca^{2+}]_c$ oscillations induced by glucose (Gilon *et al.* 1999; Arredouani *et al.* 2002b). Indeed, it prolongs them at the end of each period of Ca^{2+} influx by slowly releasing Ca^{2+} that has been taken up during the upstroke phase of $[Ca^{2+}]_c$ oscillations. Moreover, oscillations of $[Ca^{2+}]$ in the ER probably exist and are synchronized with $[Ca^{2+}]_c$ oscillations. They might have a major impact on the control of the oscillations of membrane potential and consequently on $[Ca^{2+}]_c$ oscillations themselves. Indeed, the ER can activate depolarizing currents through the plasma membrane in response to a drop in its Ca^{2+} concentration (Worley *et al.* 1994; Gilon *et al.* 1999).

Conclusion

Our study demonstrates that, in pancreatic β -cells, the ER plays a complex role upon acceleration of Ca^{2+} influx through voltage-dependent Ca^{2+} channels. It rapidly takes up Ca^{2+} during the upstroke phase, via SERCA3, to release it later during the period of Ca^{2+} influx in proportion to the magnitude of the influx. This mobilization is prominent in response to a supraphysiological stimulation with high K^+ , but is operative only to a limited extent during physiological stimulation by glucose. It is mediated by a CICR that is independent from IP_3 Rs and RyRs. No such atypical CICR has previously been described in living cells. Identification of this novel mechanism in β -cells calls for careful examination of its existence in other tissues.

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