# $Ca^{2+}$-induced $Ca^{2+}$ release by activation of inositol 1,4,5-trisphosphate receptors in primary pancreatic β-cells

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**Abstract**

The effect of sarcoendoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) inhibition on the cytoplasmic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) was studied in primary insulin-releasing pancreatic β-cells isolated from mice, rats and human subjects as well as in clonal rat insulinoma INS-1 cells. In $Ca^{2+}$-deficient medium the individual primary β-cells reacted to the SERCA inhibitor cyclopiazonic acid (CPA) with a slow rise of $[Ca^{2+}]_i$ followed by an explosive transient elevation. The $[Ca^{2+}]_i$ transients were preferentially observed at low intracellular concentrations of the $Ca^{2+}$ indicator fura-2 and were unaffected by pre-treatment with 100 μM ryanodine. Whereas 20 mM caffeine had no effect on basal $[Ca^{2+}]_i$ or the slow rise in response to CPA, it completely prevented the CPA-induced $[Ca^{2+}]_i$ transients as well as inositol 1,4,5-trisphosphate-mediated $[Ca^{2+}]_i$ transients in response to carbachol. In striking contrast to the primary β-cells, caffeine readily mobilized intracellular $Ca^{2+}$ in INS-1 cells under identical conditions, and such mobilization was prevented by ryanodine pre-treatment. The results indicate that leakage of $Ca^{2+}$ from the endoplasmic reticulum after SERCA inhibition is feedback-accelerated by $Ca^{2+}$-induced $Ca^{2+}$ release (CICR). In primary pancreatic β-cells this CICR is due to activation of inositol 1,4,5-trisphosphate receptors. CICR by ryanodine receptor activation may be restricted to clonal β-cells.

*Keywords*: $Ca^{2+}$-induced $Ca^{2+}$ release, $IP_3$ receptors, Ryanodine receptors, Insulin secretion, Endoplasmic reticulum, Calcium, Signalling

## 1. Introduction

Insulin secretion is triggered by a rise of the cytoplasmic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in pancreatic β-cells. Glucose, which is the major stimulus for insulin release, achieves this rise of $[Ca^{2+}]_i$ by increased metabolism resulting in closure of ATP/ADP-sensitive $K^+$ channels, membrane depolarization and influx of $Ca^{2+}$ through L-type channels [1]. The primary effects of neurotransmitters and hormones on β-cells often involve formation of inositol 1,4,5-trisphosphate ($IP_3$), which mobilizes $Ca^{2+}$ from the endoplasmic reticulum (ER) [2-4]. Cyclic ADP ribose (cADPr) [5] and nicotinic acid adenine dinucleotide phosphate (NAADP) [6, 7] acting on separate receptors have also been suggested to mediate intracellular $Ca^{2+}$ mobilization in β-cells. Although these pathways for elevation of $[Ca^{2+}]_i$ represent different processes there may be considerable interaction between them. Emptying of the ER may consequently contribute to voltage-dependent influx of $Ca^{2+}$ by activation of a store-operated depolarizing current [8]. Glucose stimulation and the associated depolarization has been proposed to result in increased production of $IP_3$ [9-11], cADPr [5] and NAADP [6]. Another important mechanism is the $Ca^{2+}$-induced $Ca^{2+}$ release (CICR), by which a depolarization-dependent rise of $[Ca^{2+}]_i$ may become amplified by $Ca^{2+}$ release from the ER [12]. Even though RyRs are expressed in β-cells [13-15], the physiological role of cADPr and RyRs remains controversial [16]. Nevertheless, there are several suggestions that RyRs mediate CICR in β-cells [12, 14, 17-19].

Accumulation of $Ca^{2+}$ in the ER is accomplished by the sarcoendoplasmic reticulum $Ca^{2+}$-ATPase (SERCA), which can be selectively inhibited by thapsigargin [20] and cyclopiazonic acid (CPA) [21]. Due to a high basal $Ca^{2+}$ permeability of the ER, SERCA inhibition results in rapid $Ca^{2+}$ depletion, but the nature of this leak is not completely understood [16]. In permeabilized cells clamped at $Ca^{2+}$ concentrations close to the basal

$[Ca^{2+}]_i$ levels SERCA inhibition results in release of $Ca^{2+}$ from the ER, which is unaffected by inhibitors of $IP_3$, ryanodine and NAADP receptors [22, 23]. It was therefore suggested that basal leak occurs through a pathway separate from these receptors [23]. However, in populations of intact cells $IP_3$Rs seem to contribute to loss of $Ca^{2+}$ from the ER after SERCA inhibition, since loading with the receptor antagonist heparin [24] or exposure to another $IP_3$R antagonist caffeine [25, 26] reduced the rate of $Ca^{2+}$ leakage.

The present study was undertaken to clarify the involvement of $IP_3$Rs or RyRs in $Ca^{2+}$ depletion of the ER after SERCA inhibition in β-cells. Studying the effect of the SERCA inhibitor CPA on individual primary β-cells from mice, rats and human subjects, we show that the slow leak of $Ca^{2+}$ from the ER is feedback-accelerated by CICR due to activation of $IP_3$Rs.

## 2. Materials and methods

### 2.1 *Reagents and solutions*

Reagents of analytical grade and deionized water were used. Fura-2 and its acetoxymethyl ester (fura-2/AM) as well as BAPTA acetoxymethyl ester (BAPTA/AM) and ryanodine were from Molecular Probes Inc. (Eugene, OR). Sigma Chemical Co. (St. Louis, MO) provided bovine serum albumin (fraction V), carbachol, EGTA, HEPES and poly-L-lysine. Cyclopiazonic acid (CPA) was from Alexis Corp. (Lausen, Switzerland). Fetal calf serum was bought from Gibco Ltd. (Paisley, Scotland) and collagenase was from Boehringer Mannheim GmbH (Mannheim, Germany). Diazoxide and methoxyverapamil were kindly donated by Schering-Plough Int. (Kenilworth, NJ) and Knoll AG (Ludwigshafen, Germany).

### 2.2 *Preparation of islet cells*

Islets of Langerhans were collagenase-isolated from pieces of pancreas from *ob*/*ob* and NMRI mice as well as Wistar rats. Human islets were obtained from the Nordic Network for

Clinical Islet Transplantation in Uppsala after isolation from cadaver donors (under a protocol approved by the local ethics committee) as previously described [27]. Free cells were prepared by shaking the islets in a $Ca^{2+}$-deficient medium [28]. The cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 $\mu$g/ml streptomycin and 30 $\mu$g/ml gentamicin and allowed to attach to circular 25 mm cover slips (poly-L-lysine coated in the case of rat cells) during 1-3 days culture at 37°C in a humidified atmosphere of 5% $CO_2$. The *ob*/*ob* mouse islets contain more than 90% [29] and rat islets 65-70 % β-cells [30]. The selection of β-cells for analysis was based on their large size and low nuclear/cytoplasmic ratio compared with the cells secreting glucagon, somatostatin [30, 31] and pancreatic polypeptide [32].

2.3 *Culture of INS-1 cells*

Rat insulinoma INS-1 cells (passages 90 - 92) were grown in RPMI 1640 medium containing 11 mM glucose and supplemented with 10% fetal calf serum, 5 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% $CO_2$ at 37°C as previously reported [33]. At 80% confluency, the cells were seeded on circular 25 mm cover slips and used for experiments within 1–3 days.

2.4 *Image analysis of cytoplasmic* $Ca^{2+}$

Loading of cells with the indicator fura-2 was performed during 30 min incubation at 37°C in a HEPES-buffered medium (25 mM; pH 7.4) containing 0.5 mg/ml bovine serum albumin, 138 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 1.28 mM $CaCl_2$, 20 mM glucose, 250 μM diazoxide and 0.2 - 2 $\mu$M fura-2/AM. When testing the effect of ryanodine, 100 μM of this compound was present during loading and throughout the experiment. The cover slips

with attached cells were used as exchangeable bottoms of an open chamber containing 50 $\mu$l medium. The chamber was placed on the stage of an inverted microscope (Eclipse TE2000U, Nikon, Kanagawa, Japan ). The chamber holder and the CFI S Fluor 40x oil immersion objective (Nikon) were maintained at 37°C by custom-built thermostats. The chamber was superfused with close to laminar flow at a rate of 0.3 ml/minute with indicator-free medium supplemented with 50 μM methoxyverapamil. When $Ca^{2+}$-deficient medium was used $CaCl_2$ was omitted and 2 mM EGTA added.

The microscope was equipped with an epifluorescence illuminator (Cairn Research Ltd, Faversham, UK) connected through a 5 mm diameter liquid light guide to an Optoscan monochromator with rapid grating and slit width adjustment (Cairn Research Ltd) and a 150W xenon arc lamp. The monochromator provided excitation light at 340 nm (1.7 nm half bandwidth) and 380 nm (1.4 nm half bandwidth). Emission was measured at 510 nm (40 nm half bandwidth) using a 400 nm dichroic beam splitter and a cooled OrcaER-1394 firewire digital CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan) equipped with a C8600-2 image intensifier (Hamamatsu Photonics). The Metafluor software (Universal Imaging Corp. Downingtown, Pa) controlled the monochromator and the camera, acquiring pairs of 340 and 380 nm images every 2 sec with integration for 60-80 msec at each wavelength and <1 msec for changing wavelength and slits. To minimize bleaching and photo damage, the monochromator slits were closed until the start of the next acquisition cycle. Ratio ($R$) images were calculated after subtraction of background images. $[Ca^{2+}]_i$ values were obtained according to Grynkiewicz et al. [34] using the equation

$$[Ca^{2+}]_i = K_D^{Ca^{2+}} \cdot \frac{F_0}{F_S} \cdot \frac{(R - R_{\min})}{(R_{\max} - R)}$$

$K_D^{Ca^{2+}}$ is 224 nM. $F_0$ and $R_{min}$ are the fura-2 fluorescence at 380 nm and the 340/380 nm fluorescence excitation ratio, respectively, in an "intracellular" $K^+$-rich medium lacking $Ca^{2+}$. $F_S$ and $R_{max}$ are the corresponding data obtained with a saturating concentration of $Ca^{2+}$. Only recordings from isolated individual β-cells were included in the analyses.

### 2.5 *Statistical analysis*

Statistical analyses of the proportion of cells with a certain response were made with Fishers exact test or $\chi^2$ test with Yates' correction. Two-tailed Student's *t*-test was used to compare $[Ca^{2+}]_i$ values. Statistical significance was set at a *P* value of $< 0.05$.

## 3. Results

### 3.1 *SERCA inhibition induces gated release of $Ca^{2+}$*

Studying the effects of SERCA inhibition on $[Ca^{2+}]_i$, we ensured maximal initial filling of the ER by exposing the β-cells to 20 mM glucose, which stimulates $Ca^{2+}$ sequestration in this organelle even when $[Ca^{2+}]_i$ is kept at resting levels [35-37]. Interference from voltage-dependent $Ca^{2+}$ entry was avoided by the presence of hyperpolarizing diazoxide as well as the L-type $Ca^{2+}$ channel blocker methoxyverapamil [35, 38]. To exclude influence of store-operated or any other influx of extracellular $Ca^{2+}$ the exposure to SERCA inhibitor was made during temporary omission of $Ca^{2+}$ and addition of 2 mM EGTA.

In *ob*/*ob* mouse β-cells loaded with 0.5 μM fura-2/AM, SERCA inhibition by 50 μM CPA induced a slow temporary rise of $[Ca^{2+}]_i$ due to leakage from the ER (Fig. 1A-C). Consistent with previous studies [38, 39] there was sustained elevation of $[Ca^{2+}]_i$ by activation of a store-operated $Ca^{2+}$ influx when $Ca^{2+}$ was reintroduced in the continued presence of CPA. In some cells the CPA-induced emptying of the ER was accelerated by pronounced $[Ca^{2+}]_i$ transients. These transients were more common in cells containing less

fura-2 as exemplified in the 3 equally sized β-cells studied in parallel in Fig. 1A-C. The fura-2 contents of the cells in Fig.1B and C were 65 and 58 %, respectively, of that in Fig. 1A as calculated from the $Ca^{2+}$-independent fluorescence [40]. After loading in 0.2 μM fura-2/AM the CPA-induced transients became much more frequent, although the proportion of cells with transients varied in the 25-100% range between different animals. Under such loading conditions β-cells responded with $[Ca^{2+}]_i$ transients during two repeated exposures to CPA provided that the delay was sufficient to replenish the ER (Fig. 1D). The importance of the indicator concentration was apparent from the absence of transients after loading with commonly used concentrations of fura-2/AM (1-2 μM; not shown). This effect was mimicked by increasing the $Ca^{2+}$ buffering capacity of the cytoplasm with BAPTA, a $Ca^{2+}$-chelating agent not interfering with the fura-2 measurements. The transients consequently disappeared when the loading with 0.2 μM fura-2/AM was combined with 4 μM BAPTA/AM (not shown) or when superfusing with 4 μM BAPTA/AM prior to a second exposure to CPA (Fig. 1E).

The onset of the CPA-induced $[Ca^{2+}]_i$ transients varied. In most cells a single transient was preceded by the slower rise of $[Ca^{2+}]_i$ (Figs. 1C-E, 2A, 3, 5, 7). In some cells single (Fig. 2B) or multiple transients (Fig. 2C) appeared to occur immediately after SERCA inhibition. Multiple transients were only observed in 3 experiments on *ob*/*ob* mouse β-cells, but in one of those several individual cells exhibited this response pattern. The gated release of $Ca^{2+}$ from the ER after SERCA inhibition was not restricted to β-cells from *ob*/*ob* mice. As is shown in Fig. 3 CPA induced similar $[Ca^{2+}]_i$ transients preceded by a slower rise also in NMRI mouse, rat and human β-cells.

To establish whether the incidence of CICR was related to the cytoplasmic level of $Ca^{2+}$ we determined whether the appearance of transients was correlated with the basal $[Ca^{2+}]_i$ level in $Ca^{2+}$-deficient medium or with the $[Ca^{2+}]_i$ reached during the slow elevation induced

by CPA. In *ob*/*ob* mouse β-cells, the average basal $[Ca^{2+}]_i$ was significantly lower in cells without CPA-induced transients as compared to those with transients (Fig. 4). Although a higher average $[Ca^{2+}]_i$ was reached after CPA treatment of *ob*/*ob* mouse β-cells responding with transient, this difference did not reach statistical significance. In NMRI mouse and Wistar rat β-cells basal $[Ca^{2+}]_i$ levels were similar to those in *ob*/*ob* mouse β-cells but the number of observations did not allow meaningful analysis of correlation to CPA-induced transients. Basal $[Ca^{2+}]_i$ (25-30 nM) was much lower in the human β-cells but there were no differences between cells responding or not with transients to CPA (Fig. 4). It cannot be excluded that this discrepancy is due to a lower $K_D$ for the $Ca^{2+}$-fura-2 complex in the human β-cells. However, in the human β-cells the CPA-induced slow elevation of $[Ca^{2+}]_i$ preceding the transient was considerably higher than the slow elevation obtained when no transient occurred (Fig. 4).

### 3.2 *CICR is mediated by $IP_3$ receptors in primary β-cell*

A high concentration of caffeine (20 mM), which is commonly used to promote CICR by sensitising RyRs [41] had no effect on basal $[Ca^{2+}]_i$ in any of 79 *ob*/*ob* mouse (Fig. 5A,B), 40 rat (Fig. 5C,D) and 42 human (Fig. 6C) β-cells. The $[Ca^{2+}]_i$ transients induced by SERCA inhibition were instead abolished by caffeine (Fig. 5). This inhibition was reversible, since the transients reappeared when a second CPA challenge was made after caffeine omission (Fig 5B, D). High concentrations of caffeine are known to inhibit $IP_3$-mediated mobilization of $Ca^{2+}$ in different types of cells, including pancreatic β-cells [42, 43]. Consistent with such an action, caffeine was now found to abolish or severely blunt the $Ca^{2+}$ mobilization in response to carbachol in *ob*/*ob* mouse (Fig. 6A), rat (Fig. 6B) and human (Fig. 6C) β-cells. The inhibitory action was apparent not only from suppression of the initial carbachol response but also from the rapid release of intracellular $Ca^{2+}$ when caffeine was omitted. In

contrast to the effect on primary β-cells, caffeine readily mobilized intracellular $Ca^{2+}$ in clonal INS-1 cells (Fig. 7A) and this action was effectively prevented by pre-treatment with 100 μM ryanodine (Fig. 7B). Effective emptying of the ER was apparent from the lack of effect of CPA after ryanodine pre-treatment of the INS-1 cells (Fig. 7B). In support for the idea that CICR induced by SERCA inhibition is mediated by $IP_3Rs$ in primary β-cells ryanodine pre-treatment neither prevented the occurrence of the transients nor the proportion of *ob*/*ob* mouse (Fig. 7C) and human (Fig. 7D) β-cells responding to CPA with gated $Ca^{2+}$ mobilization.

**4. Discussion**

The classical CICR mechanism is usually associated with the RyR, but the $IP_3Rs$ also display this autocatalytic $Ca^{2+}$ release mechanism [44]. The binding of $IP_3$ sensitizes the $IP_3Rs$ to the stimulatory effect of $Ca^{2+}$ [45, 46]. A similar mechanism operates for RyRs where caffeine acts to sensitize them to the stimulatory action of $Ca^{2+}$ [41]. At the concentration presently used, caffeine may even activate RyRs independent of $Ca^{2+}$ [47]. Interestingly, high concentrations of caffeine inhibit agonist-induced production [48] as well as action of $IP_3$ in different cells [42] including pancreatic β-cells [43]. It is therefore possible to use caffeine to discriminate between RyRs and $IP_3Rs$ [49].

In several studies of primary β-cells $Ca^{2+}$ influx across the plasma membrane has been used to trigger CICR [12-14, 18], making it difficult to decide whether $[Ca^{2+}]_i$ transients represent influx or intracellular release. We therefore eliminated any influence of $Ca^{2+}$ influx by using a $Ca^{2+}$-deficient medium containing EGTA in the attempts to evoke CICR by SERCA inhibition or by caffeine. Earlier studies on primary β-cells have revealed that the SERCA inhibitors thapsigargin [39, 50, 51] and CPA [38] effectively empty the ER and activate store-operated influx of $Ca^{2+}$. CPA has major advantages over thapsigargin, being

both water-soluble and reversible. We found that SERCA inhibition resulted in a slow elevation of $[Ca^{2+}]_i$ followed by a pronounced transient indicating that leakage results in a local rise of $[Ca^{2+}]_i$ at the surface of the ER, which then activates CICR. In *ob*/*ob* mouse β-cells, the $[Ca^{2+}]_i$ transients were sometimes observed immediately upon exposure to CPA, perhaps because the basal $[Ca^{2+}]_i$ level is close to the threshold for activation of CICR in rodent β-cells (see below).

The demonstration of $[Ca^{2+}]_i$ transients after SERCA inhibition was facilitated at a low intracellular concentration of the $Ca^{2+}$ indicator fura-2. Transients elicited by CICR may escape detection due to $Ca^{2+}$ buffering by the indicator. The presence of a rapidly diffusible $Ca^{2+}$ indicator can also dissipate intracellular $Ca^{2+}$ gradients [40] preventing the critical concentration to be reached. Moreover, previous studies have demonstrated that the $Ca^{2+}$-free forms of BAPTA-based indicators [52], particularly fura-2 [53], are competitive antagonists of $IP_3$ binding to its receptor. Although the CPA-induced $[Ca^{2+}]_i$ transients were observed in most experiments with mouse, rat and human β-cells, only few transients were found in certain preparations. As expected from a CICR mechanism, the occurrence of the transients depended on the prevailing $[Ca^{2+}]_i$ level. In *ob*/*ob* mouse β-cells, the transients were more clearly related to the basal than to the CPA-induced elevation of $[Ca^{2+}]_i$. However, in the human β-cells, in which the low resting $[Ca^{2+}]_i$ may be further away from the threshold for activation of CICR, the occurrence of the transients instead correlated with the magnitude of the slow response to CPA.

In most studies of CICR in insulin-secreting cells, the phenomenon has been attributed to RyRs [12, 14, 17-19]. Both type 2 RyR mRNA and RyR protein are expressed in primary as well as clonal β-cells [13-15]. Convincing evidence for functional RyRs come only from the clonal β-cells RINm-5F [54], HIT-T15 [14], MIN6 [15] and INS-1 [17, 18, 55] in which caffeine exhibits the characteristic acute effect on $Ca^{2+}$ mobilization. The present

data confirm functional ryanodine receptors in INS-1 cells [17] by showing a $Ca^{2+}$-mobilizing effect of caffeine and that ryanodine pre-treatment abolishes such mobilization. However, these drugs had no corresponding effects on primary β-cells from mice, rats and human subjects under identical conditions. The $[Ca^{2+}]_i$ transients caused by CICR in the primary β-cells were unaffected by ryanodine and blocked by caffeine. In accordance with previous observations [43], caffeine also prevented the $IP_3$-mediated $[Ca^{2+}]_i$ transients in response to carbachol. These data together with the disappearance of the transients at high intracellular concentrations of $Ca^{2+}$ indicator collectively indicate that in primary β-cells the CICR evoked by SERCA inhibition is due to activation of $IP_3$Rs.

Maintenance of mobilizable $Ca^{2+}$ in the ER of β-cells is metabolically expensive requiring exposure to high nutrient concentrations [37, 56]. The reason is probably that $Ca^{2+}$ leakage from the ER is generally a prominent phenomenon [57]. $Ca^{2+}$ uptake into the β-cell ER is due to a high affinity SERCA2 and a low affinity SERCA3 mechanism [58]. Whereas the high affinity mechanism explains active accumulation of $Ca^{2+}$ in the $IP_3$-releasable pool at basal $[Ca^{2+}]_i$ concentrations [37], the low affinity uptake together with the leakage allows the ER to function as a "passive" $Ca^{2+}$ buffer at elevated levels of $[Ca^{2+}]_i$ [58, 59]. A high $Ca^{2+}$ permeability is probably a factor enabling the ER to exert this dual function. In other cells the basal leak of $Ca^{2+}$ is unaffected by inhibitors of $IP_3$, ryanodine and NAADP receptors [22, 23] and probably occurs though separate pathways. However, the established routes for gated release of $Ca^{2+}$ may contribute to a physiological leak, since $IP_3$R antagonists have been found to reduce $Ca^{2+}$ leakage from the ER after SERCA inhibition in intact cells [24-26]. In analogy to previous observations [60, 61] we show that SERCA inhibition not only induces a $Ca^{2+}$ leak from the ER with gradual elevation of $[Ca^{2+}]_i$, but also promotes $IP_3$R-mediated amplification of this release by a positive feedback [62] leading to an explosive pulse of $[Ca^{2+}]_i$. This is the first time when CICR after SERCA inhibition is observed in the absence

of extracellular $Ca^{2+}$ and without stimulating the $IP_3Rs$ by other means than the elevation of $[Ca^{2+}]_i$. Physiological conditions with presence of extracellular $Ca^{2+}$ and a slightly higher basal $[Ca^{2+}]_i$ should be even more favourable for induction of CICR. CICR may therefore be expected to amplify any rise of $Ca^{2+}$ like the voltage-dependent elevation in response to glucose or other depolarizing secretagogues. The present data indicate that in primary β-cells such CICR is due to activation of $IP_3Rs$.

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## Legends to Figures

**Figure 1. SERCA inhibition induced gated release of intracellular $Ca^{2+}$ in mouse β-cells depending on the concentration of $Ca^{2+}$ indicator.**

Pancreatic β-cells from *ob*/*ob*-mice were loaded with 0.5 (A-C) or 0.2 μM fura-2/AM (D-E) in medium containing 20 mM glucose, 1.28 mM $Ca^{2+}$ and 250 μM diazoxide. The cells were then rinsed and superfused with indicator-free medium supplemented with 50 μM methoxyverapamil. As indicated by bars the cells were exposed to $Ca^{2+}$-deficient medium containing 2 mM EGTA, 50 μM CPA, and 4 μM BAPTA/AM. Panels A-C show different responses of 3 individual cells in the same experiment. Panels D and E show individual cells in separate experiments. Note the different time scales in A-C as compared to D-E. The traces in D and E are representative for all of 4 ($P$<0.05) and 8 ($P$<0.001) cells respectively.

**Figure 2. The gated $Ca^{2+}$ release in response to SERCA inhibition shows different patterns.**

Pancreatic β-cells from *ob*/*ob*-mice were loaded with 0.2 μM fura-2/AM in medium containing 20 mM glucose, 1.28 mM $Ca^{2+}$ and 250 μM diazoxide. The cells were then rinsed and superfused with indicator-free medium supplemented with 50 μM methoxyverapamil. As indicated by bars the cells were exposed to $Ca^{2+}$-deficient medium containing 2 mM EGTA and 50 μM CPA. Panel A shows one separately studied and panels B and C two simultaneously studied individual β-cells. The response pattern in A is representative for 88 of 116 ($P$<0.001) β-cells in 12 experiments and those in B and C for 1 and 7 of 9 simultaneously studied β-cells.

**Figure 3. Gated $Ca^{2+}$ release in response to SERCA inhibition is observed in mouse, rat and human β-cells.**

Pancreatic β-cells from *ob*/*ob* mouse (A), NMRI mouse (B), Wistar rat (C) and a human subject (D) were loaded with 0.2 μM fura-2/AM in medium containing 20 mM glucose, 1.28 mM $Ca^{2+}$ and 250 μM diazoxide. The cells were then rinsed and superfused with indicator-free medium supplemented with 50 μM methoxyverapamil. As indicated by bars the cells were exposed to $Ca^{2+}$-deficient medium containing 2 mM EGTA and 50 μM CPA. The response pattern with a $[Ca^{2+}]_i$ transient after exposure to CPA is representative for 88 of 116 ($P$<0.001) *ob*/*ob* mouse β-cells in 12 experiments (A), for 3 of 9 NMRI mouse β-cells in 3 experiments (B), for 22 of 29 ($P$<0.001) Wistar rat β-cells in 8 experiments (C) and for 12 of 19 ($P$<0.001) human β-cells in 3 experiments (D).

**Figure 4. The occurrence of CPA-induced $[Ca^{2+}]_i$ transients in primary β-cells depends on $[Ca^{2+}]_i$.**

Pancreatic β-cells from *ob*/*ob*-mice and human subjects (as indicated) were loaded with 0.2 μM fura-2/AM in medium containing 20 mM glucose, 1.28 mM $Ca^{2+}$, 250 μM diazoxide and in some cases with 100 μM ryanodine. The cells were then rinsed and superfused with indicator-free medium supplemented with 50 μM methoxyverapamil. As shown in Fig. 3 the cells were exposed to $Ca^{2+}$-deficient medium containing 2 mM EGTA and 50 μM CPA. The open bars show the basal $[Ca^{2+}]_i$ levels after introduction of $Ca^{2+}$-deficient medium and the filled bars the maximal levels reached during the subsequent slow elevation of $[Ca^{2+}]_i$ (excluding any transient) in response to CPA. Cells responding to CPA with $[Ca^{2+}]_i$ transients are indicated by +. Values are mean ± s.e.m. for the indicated number of cells. *$P$<0.01, **$P$<0.001. Since ryanodine pretreatment did not prevent the occurrence of the CPA-induced transients we combined the data from cells treated or not with ryanodine.

**Figure 5. Caffeine inhibits the gated but not the slow release of $Ca^{2+}$ after SERCA inhibition.**

Pancreatic β-cells from *ob*/*ob*-mouse (A, B) or Wistar rat (C, D) were loaded with 0.2 μM fura-2/AM in medium containing 20 mM glucose, 1.28 mM $Ca^{2+}$ and 250 μM diazoxide. The cells were then rinsed and superfused with indicator-free medium supplemented with 50 μM methoxyverapamil. As indicated by bars the cells were exposed to $Ca^{2+}$-deficient medium containing 2 mM EGTA, 20 mM caffeine (Caff) and 50 μM CPA. The response pattern in A is representative for all of 19 ($P$<0.001) *ob*/*ob* mouse β-cells in 3 experiments, that in B for all of 7 ($P$<0.001) *ob*/*ob* mouse β-cells in 2 experiments, that in C for all of 10 ($P$<0.001) Wistar rat β-cells in 4 experiments and that in D for all of 2 Wistar rat β-cells in one experiment.

**Figure 6. Caffeine inhibits carbachol-stimulated intracellular release of $Ca^{2+}$.**

Pancreatic β-cells from *ob*/*ob*-mouse (A) or Wistar rat (B) and a human subject (C) were loaded with 0.2 μM fura-2/AM in medium containing 20 mM glucose, 1.28 mM $Ca^{2+}$ and 250 μM diazoxide. The cells were then rinsed and superfused with indicator-free medium supplemented with 50 μM methoxyverapamil. As indicated by bars the cells were exposed to $Ca^{2+}$-deficient medium containing 2 mM EGTA, 20 mM caffeine (Caff), and 100 μM carbachol (Carb). Caffeine had a complete or partial inhibitory effect on the carbachol response all out of 15 ($P$<0.001) *ob*/*ob* mouse β-cells in 3 experiments (A), all out of 8 ($P$<0.001) Wistar rat β-cells in 3 experiments (B) and all out of 24 ($P$<0.001) human β-cells in 4 experiments.

**Figure 7. Caffeine and ryanodine empties the ER in clonal INS-1 β-cells but not in primary β-cells.**

Pancreatic β-cells from clonal INS-1 β-cells (A, B) and primary *ob*/*ob*-mouse (C) or human (D) β-cells were loaded with 0.2 μM fura-2/AM alone (A) or together with 100 μM ryanodine (B-D) in medium containing 20 mM glucose, 1.28 mM $Ca^{2+}$ and 250 μM diazoxide. The cells were then rinsed and superfused with indicator-free medium supplemented with 50 μM methoxyverapamil. As indicated by bars the cells were exposed to $Ca^{2+}$-deficient medium containing 2 mM EGTA, 20 mM caffeine (Caff) and 50 μM CPA. The $Ca^{2+}$ mobilizing effect of caffeine is representative for 152 of 190 ($P$<0.001) INS-1 cells (A). The blocking action of ryanodine on this $Ca^{2+}$ mobilization as well as that in response to CPA was representative for all out of 45 ($P$<0.001) and 38 ($P$<0.001) INS-1 cells, respectively (B). The maintenance of a $[Ca^{2+}]_i$ transient in response to CPA after ryanodine treatment is representative for 42 of 48 ($P$<0.001) *ob*/*ob* mouse β-cells in 5 experiments (C), and 28 of 68 ($P$<0.001) human β-cells (D).

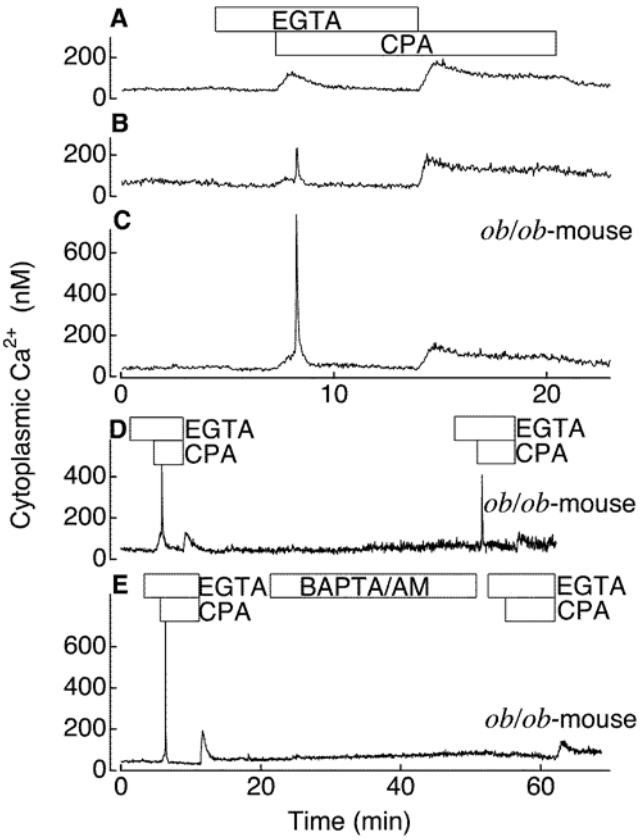

A
EGTA
CPA
200
0
B
200
0
C
ob/ob-mouse
600
400
200
0
0
10
20
Cytoplasmic Ca2+ (nM)
D
EGTA
CPA
EGTA
CPA
ob/ob-mouse
400
200
0
E
EGTA
CPA
BAPTA/AM
EGTA
CPA
ob/ob-mouse
600
400
200
0
0
20
40
60
Time (min)

**Fig. 1**

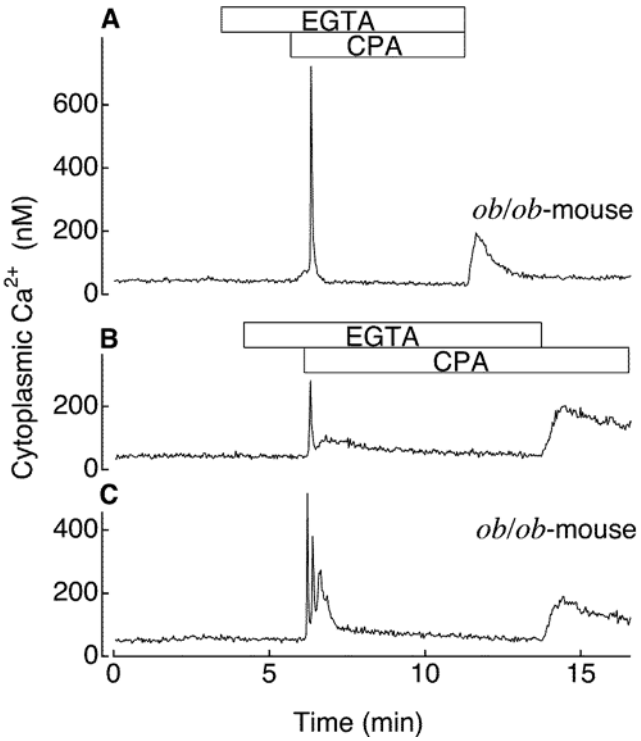

A
EGTA
CPA
600
400
200
0
ob/ob-mouse
B
EGTA
CPA
200
0
C
400
200
0
ob/ob-mouse
0
5
10
15
Time (min)
Cytoplasmic Ca2+ (nM)

**Fig. 2**

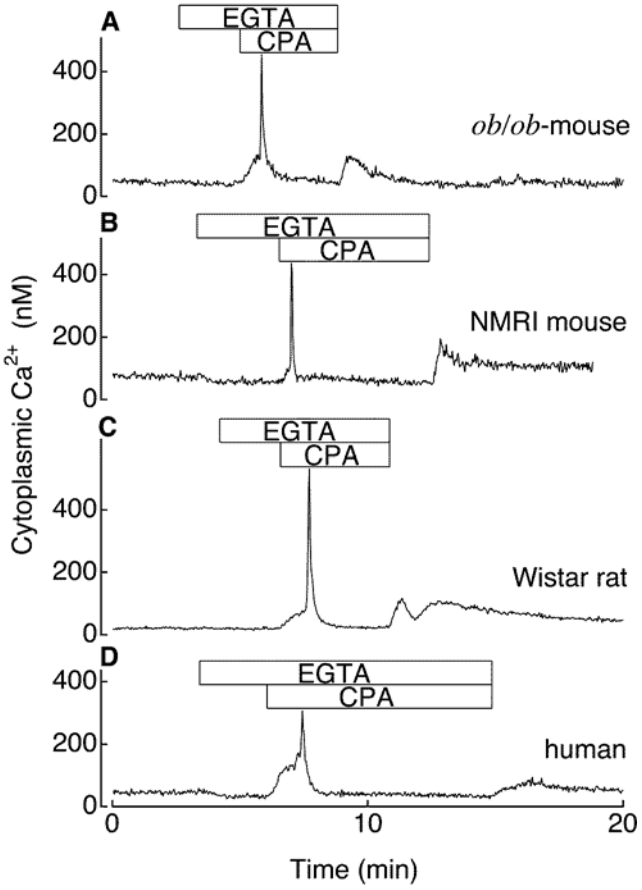

A
EGTA
CPA
400
200
0
ob/ob-mouse
B
EGTA
CPA
400
200
0
NMRI mouse
C
EGTA
CPA
400
200
0
Wistar rat
D
EGTA
CPA
400
200
0
human
Cytoplasmic Ca2+ (nM)
0
10
20
Time (min)

**Fig. 3**

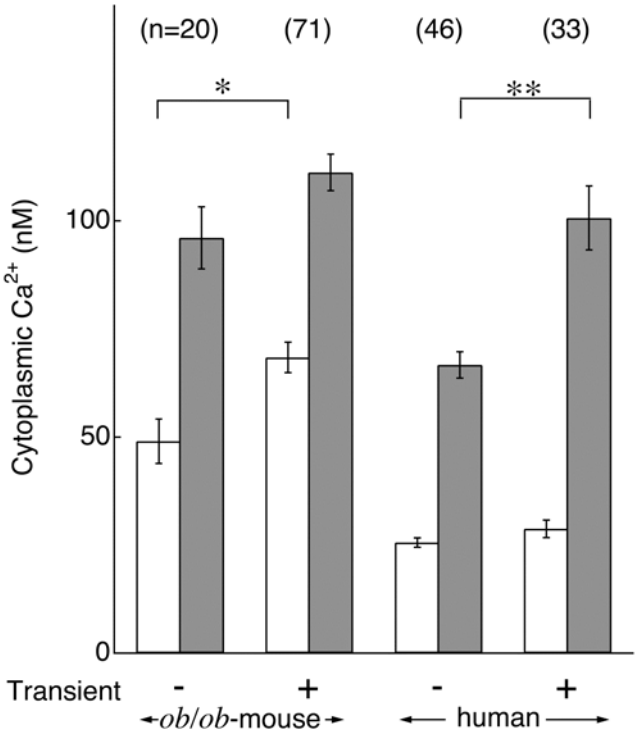

(n=20)
(71)
(46)
(33)
*
**
Cytoplasmic Ca2+ (nM)
100
50
0
Transient
-
+
-
+
ob/ob-mouse
human

**Fig. 4**

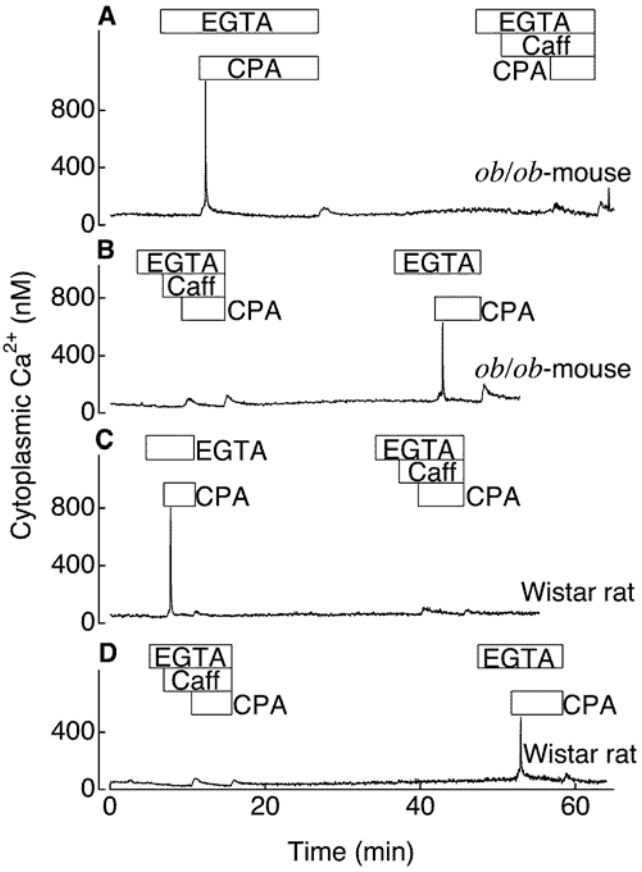
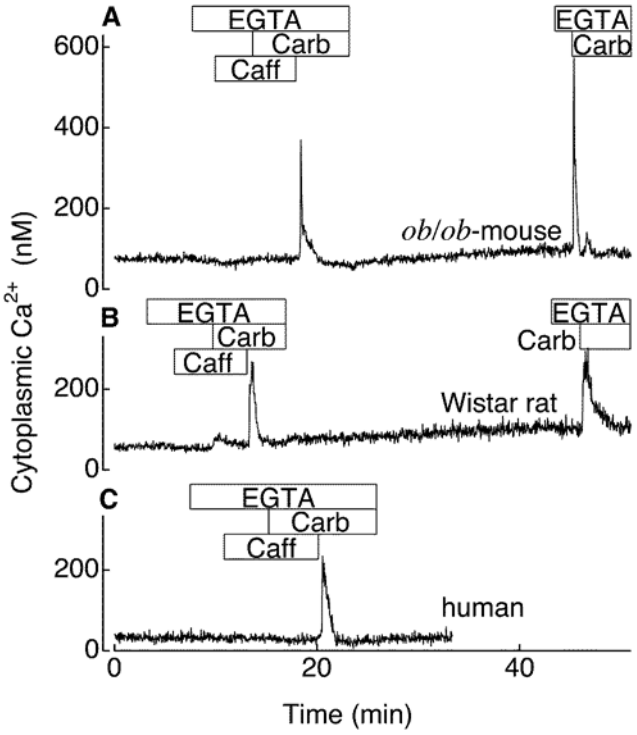

A
EGTA
CPA
EGTA
Caff
CPA
800
400
0
ob/ob-mouse
B
EGTA
Caff
CPA
EGTA
CPA
800
400
0
ob/ob-mouse
C
EGTA
CPA
EGTA
Caff
CPA
800
400
0
Wistar rat
D
EGTA
Caff
CPA
EGTA
CPA
400
0
Wistar rat
0
20
40
60
Time (min)
Cytoplasmic Ca2+ (nM)

**Fig. 5**


A
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Carb
Caff
EGTA
Carb
600
400
200
0
ob/ob-mouse
B
EGTA
Carb
Caff
EGTA
Carb
200
0
Wistar rat
C
EGTA
Carb
Caff
200
0
human
0
20
40
Time (min)
Cytoplasmic Ca2+ (nM)

**Fig. 6**

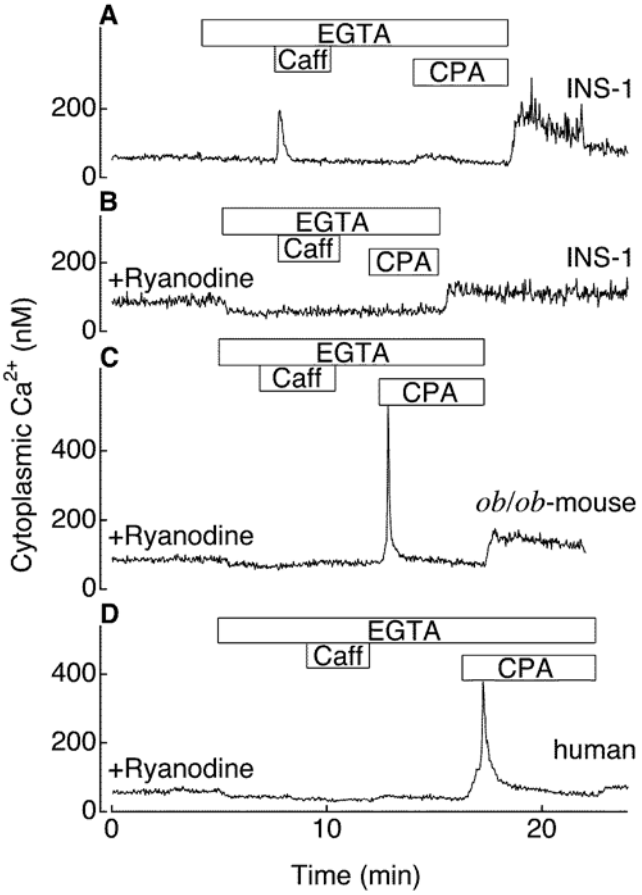
A
EGTA
Caff
CPA
INS-1
200
0
B
EGTA
Caff
CPA
INS-1
200
+Ryanodine
0
C
EGTA
Caff
CPA
400
200
ob/ob-mouse
+Ryanodine
0
D
EGTA
Caff
CPA
400
200
human
+Ryanodine
0
0
10
20
Time (min)
Cytoplasmic Ca2+ (nM)

**Fig. 7**

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