

## Emptying of intracellular $\text{Ca}^{2+}$ stores stimulates $\text{Ca}^{2+}$ entry in mouse pancreatic $\beta$ -cells by both direct and indirect mechanisms

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1. In non-excitabile cells, the depletion of intracellular  $\text{Ca}^{2+}$  stores triggers  $\text{Ca}^{2+}$  influx by a process called capacitative  $\text{Ca}^{2+}$  entry. In the present study, we have investigated how the emptying of these stores by thapsigargin ( $1\ \mu\text{M}$ ) influences  $\text{Ca}^{2+}$  influx in electrically excitable pancreatic  $\beta$ -cells. The cytoplasmic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) was monitored in clusters of mouse  $\beta$ -cells or in whole islets loaded with fura-2.
2. The membrane was first held hyperpolarized by diazoxide, an opener of ATP-sensitive  $\text{K}^+$  ( $\text{K}_{\text{ATP}}$ ) channels, in the presence of  $4.8\ \text{mM}\ \text{K}^+$ . Alternating between  $\text{Ca}^{2+}$ -free medium and medium containing  $2.5\ \text{mM}\ \text{Ca}^{2+}$  caused a minor rise in  $[\text{Ca}^{2+}]_i$  ( $\sim 14\ \text{nM}$ ) in clusters of  $\beta$ -cells. A larger rise ( $\sim 65\ \text{nM}$ ), resistant to the blockade of voltage-dependent  $\text{Ca}^{2+}$  channels by D600, occurred when extracellular  $\text{Ca}^{2+}$  was readmitted after emptying intracellular  $\text{Ca}^{2+}$  stores with thapsigargin or acetylcholine. Thus there exists a small capacitative  $\text{Ca}^{2+}$  entry in  $\beta$ -cells.
3. When the membrane potential was clamped at depolarized levels with  $10$ ,  $20$  or  $45\ \text{mM}\ \text{K}^+$  in the presence of diazoxide,  $[\text{Ca}^{2+}]_i$  increased to different plateau levels ranging between  $100$  and  $900\ \text{nM}$ . Thapsigargin consistently caused a further transient rise in  $[\text{Ca}^{2+}]_i$ , but had little (at  $10\ \text{mM}\ \text{K}^+$ ) or no effect on the plateau level. This confirms that the capacitative  $\text{Ca}^{2+}$  entry is small.
4. In clusters of cells whose membrane potential was not clamped with diazoxide,  $15\ \text{mM}$  glucose (in  $4.8\ \text{mM}\ \text{K}^+$ ) induced  $[\text{Ca}^{2+}]_i$  oscillations by promoting  $\text{Ca}^{2+}$  influx through voltage-dependent  $\text{Ca}^{2+}$  channels. The application of thapsigargin accelerated these oscillations and increased their amplitude, sometimes causing a sustained elevation of  $[\text{Ca}^{2+}]_i$ . Similar results were obtained from whole islets perfused with a medium containing  $\geq 6\ \text{mM}$  glucose. The effect of thapsigargin was always much larger than expected from the capacitative  $\text{Ca}^{2+}$  entry, probably because of a potentiation of  $\text{Ca}^{2+}$  influx through voltage-dependent  $\text{Ca}^{2+}$  channels.
5. This potentiating effect of thapsigargin did not result from an acceleration of cell metabolism since the drug did not affect glucose-induced changes in NAD(P)H fluorescence. It is also unlikely to involve the inhibition of  $\text{K}_{\text{ATP}}$  channels because thapsigargin steadily elevated  $[\text{Ca}^{2+}]_i$  in cells in which  $[\text{Ca}^{2+}]_i$  oscillations persisted in the presence of a maximally effective concentration of tolbutamide.
6. In conclusion, the emptying of intracellular  $\text{Ca}^{2+}$  stores in  $\beta$ -cells induces a small capacitative  $\text{Ca}^{2+}$  entry and activates a depolarizing current which potentiates glucose-induced  $\text{Ca}^{2+}$  influx through voltage-dependent  $\text{Ca}^{2+}$  channels.

$\text{Ca}^{2+}$  plays a major role in stimulus–response coupling. In electrically non-excitabile cells, the activation of receptors linked to phospholipid hydrolysis evokes a biphasic increase in the concentration of cytoplasmic free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ). The first phase is due to the stimulation by inositol 1,4,5-tris-

phosphate ( $\text{IP}_3$ ) of  $\text{Ca}^{2+}$  release from intracellular  $\text{Ca}^{2+}$  stores. The second phase results from an entry of  $\text{Ca}^{2+}$  across the plasma membrane (Berridge, 1993). It is now generally accepted that the emptying of intracellular  $\text{Ca}^{2+}$  stores triggers  $\text{Ca}^{2+}$  influx by a process termed capacitative  $\text{Ca}^{2+}$

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entry (Putney, 1986; Putney & Bird, 1993). In mast cells, this influx involves specific  $\text{Ca}^{2+}$  channels, and produces a small current termed  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  (CRAC) current or  $I_{\text{CRAC}}$  (Hoth & Penner, 1992, 1993). Other types of channels with distinct ion selectivity ( $\text{Ca}^{2+}$ ,  $\text{K}^{+}$  or  $\text{Na}^{+}$ ) and conductances are activated by intracellular  $\text{Ca}^{2+}$  store depletion in different cell types (Parekh, Terlau & Stühmer, 1993; Fasolato, Innocenti & Pozzan, 1994; Clapham, 1996; Friel, 1996; Hoth, 1996). They constitute a family of channels, which have been termed store-operated channels (SOC), and include the CRAC channel originally described in mast cells (Clapham, 1996; Friel, 1996; Hoth, 1996). In electrically excitable cells,  $\text{Ca}^{2+}$  enters through voltage-dependent  $\text{Ca}^{2+}$  channels activated by the depolarization of the plasma membrane. However, it is still unclear whether intracellular store depletion also induces an entry of  $\text{Ca}^{2+}$  that co-exists with the voltage-mediated  $\text{Ca}^{2+}$  influx.

The insulin-secreting pancreatic  $\beta$ -cell is electrically excitable. Its stimulation by glucose requires the metabolism of the sugar. The acceleration of metabolism increases the ATP/ADP ratio which closes ATP-sensitive  $\text{K}^{+}$  channels ( $\text{K}_{\text{ATP}}$  channels) in the plasma membrane. This closure causes a decrease in  $\text{K}^{+}$  conductance leading to membrane depolarization with the subsequent opening of voltage-dependent  $\text{Ca}^{2+}$  channels.  $\text{Ca}^{2+}$  influx through these channels then increases, leading to a rise in  $[\text{Ca}^{2+}]_{\text{i}}$  and the stimulation of insulin secretion (Henquin, 1994). Although the membrane potential of  $\beta$ -cells is mainly determined by the  $\text{K}^{+}$  permeability of the plasma membrane, it is also influenced by a number of other permeabilities (Ashcroft & Rorsman, 1989). A recent study has suggested that the emptying of intracellular  $\text{Ca}^{2+}$  stores may influence the membrane potential of  $\beta$ -cells by activating a  $\text{Na}^{+}$ -dependent conductance (Worley, McIntyre, Spencer & Dukes, 1994a; Worley, McIntyre, Spencer, Mertz, Roe & Dukes, 1994b). A small capacitative  $\text{Ca}^{2+}$  entry has also been described in RINm5F cells (Bode & Göke, 1994). However, neither the existence nor the contribution of a capacitative  $\text{Ca}^{2+}$  entry to the overall  $[\text{Ca}^{2+}]_{\text{i}}$  increase induced by various agents has been evaluated in normal  $\beta$ -cells.

In the present study we used thapsigargin, a potent and selective inhibitor of the sarco-endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA pump) (Thastrup, Cullen, Drobak, Hanley & Dawson, 1990), to assess the influence of intracellular  $\text{Ca}^{2+}$  store emptying on  $\text{Ca}^{2+}$  influx in mouse pancreatic  $\beta$ -cells. To distinguish between membrane potential-dependent and -independent effects we used diazoxide, which opens  $\text{K}_{\text{ATP}}$  channels and permits clamping of the membrane potential at stable levels determined by the concentration of extracellular  $\text{K}^{+}$  (Gembal, Gilon & Henquin, 1992). We also evaluated the requirement of intracellular  $\text{Ca}^{2+}$  stores for glucose-induced  $[\text{Ca}^{2+}]_{\text{i}}$  oscillations.

## METHODS

### Solutions

Different types of solutions were used which had a pH of 7.4 at 37 °C. They were all supplemented with 1 mg ml<sup>-1</sup> bovine serum albumin (BSA; fraction V; Boehringer-Mannheim). The solution used for islet isolation and for most experiments was a bicarbonate-buffered medium which contained (mM): 120 NaCl, 4.8 KCl, 2.5  $\text{CaCl}_2$ , 1.2  $\text{MgCl}_2$ , and 24  $\text{NaHCO}_3$ , and which was gassed with 94%  $\text{O}_2$ -6%  $\text{CO}_2$ . When the concentration of KCl was increased, that of NaCl was decreased accordingly.  $\text{Ca}^{2+}$ -free solutions were prepared by substituting  $\text{MgCl}_2$  for  $\text{CaCl}_2$ , and except for the experiments shown in Fig. 2, they all contained 50  $\mu\text{M}$  EGTA.

### Preparation of islets and cells

All experiments were performed with tissue from fed female NMRI mice (25–30 g) killed by decapitation. Pancreatic islets were isolated after collagenase digestion of the pancreas. To obtain dispersed cells, islets were first rinsed with the usual bicarbonate-buffered medium containing 10 mM glucose, but without  $\text{CaCl}_2$  and with 0.1 mM EGTA. They were then incubated at 37 °C for 2–3 min in the same medium supplemented with trypsin (0.1 mg ml<sup>-1</sup>; Merck A.G., Darmstadt, Germany), with gentle pipetting through a siliconized glass pipette until the islets disappeared. After 1 min, the trypsin digestion was stopped and the cells were rinsed twice with cold RPMI 1640 medium (Gibco BRL) containing 10% (v/v) heat-inactivated fetal calf serum (FCS; Gibco BRL). Cells were allowed to attach to 22 mm circular coverslips and cultured for 1–3 days. Intact islets were maintained in culture for 1 or 2 days. The culture medium was RPMI 1640 medium containing 10 mM glucose and supplemented with 10% FCS, 100 i.u. ml<sup>-1</sup> penicillin and 100  $\mu\text{g}$  ml<sup>-1</sup> streptomycin.

### Measurements of $[\text{Ca}^{2+}]_{\text{i}}$

Cultured cells or islets were loaded with fura-2 for 40 min at 37 °C in a bicarbonate-buffered solution containing 10 mM glucose and 1  $\mu\text{M}$  fura-2 acetoxymethyl ester (Molecular Probes). In control experiments, the cells were loaded with fura-2 during incubation at 22 °C to minimize dye trapping into organelles. The results were not different from those obtained routinely after loading at 37 °C, which suggests that fura-2 compartmentalization is unlikely to exert an important influence on  $[\text{Ca}^{2+}]_{\text{i}}$  measurements in intact cells. Loaded cells or islets were then transferred into a temperature-controlled perfusion chamber, the base of which was made from a glass coverslip.  $[\text{Ca}^{2+}]_{\text{i}}$  was directly measured in cells attached to the coverslip, or in islets held in place close to the coverslip by gentle suction with a glass micropipette. Perfusion solutions were kept at 37 °C in a water bath and the temperature controller ensured a temperature of 37 °C close to the tissue. The flow rate of perfusion was 1.3 ml min<sup>-1</sup>. The tissue was excited successively at 340 and 380 nm, and the fluorescence emitted at 510 nm was captured and analysed by a photomultiplier-based system (Photon Technologies International Ltd, Princeton, NJ, USA). In some experiments, the emitted fluorescence was captured by a CCD camera (Photonic Science Ltd, Tunbridge Wells, UK), and the images were analysed by the MagiCal system (Applied Imaging, Sunderland, UK). In both cases,  $[\text{Ca}^{2+}]_{\text{i}}$  was calculated by comparing the ratio of the signals successively acquired at 340 and 380 nm with a calibration curve based on the equation of Grynkiewicz, Poenie & Tsien (1985). The technique has previously been described in detail (Gilon & Henquin, 1992; Gilon, Obie, Bian, Bird & Putney, 1995b).

### Measurements of reduced pyridine nucleotides

Measurements of endogenous reduced pyridine nucleotides, referred to as NAD(P)H, were performed as previously described (Panten, Christians, Kriegstein, Poser & Hasselblatt, 1973; Gilon & Henquin, 1992). In brief, after 45 min of pre-incubation in bicarbonate buffer, cultured islets or cells were transferred into the same experimental set-up as for the  $[\text{Ca}^{2+}]_i$  measurements. Reduced pyridine nucleotides were excited at 360 nm, and the emitted fluorescence was filtered at 470 nm. The changes in NAD(P)H fluorescence were expressed as percentages of control values by dividing the intensity of the fluorescence recorded at a given time by that recorded during the last minute preceding stimulation.

### Drugs

Acetylcholine chloride, thapsigargin and tolbutamide were obtained from Sigma. Diazoxide was from Schering-Plough Avondale (Rathdrum, Ireland), and D600 was a gift from Knoll (Ludwigshafen, Germany).

### Presentation of results

The experiments are illustrated either as means  $\pm$  S.E.M. or by recordings representative of results obtained from the indicated number of cells or islets. Several cells or islets from the same culture were tested with the same protocol, but each protocol was repeated with at least three different cultures. The statistical significance of observed differences was assessed either by Student's paired or unpaired *t* tests as appropriate.

## RESULTS

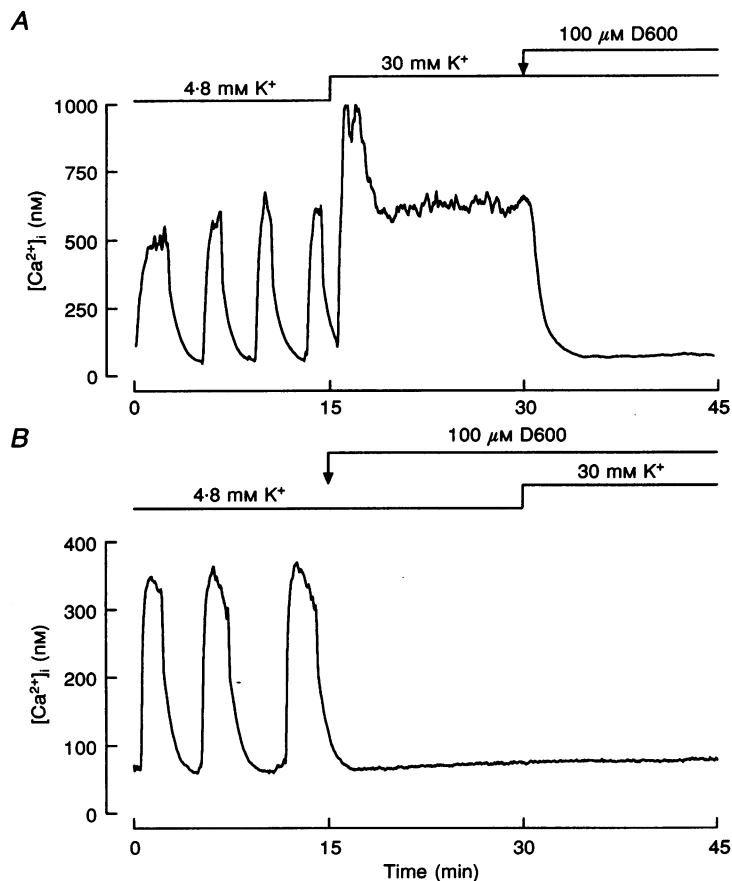
### Effects of control agents on $[\text{Ca}^{2+}]_i$ in cell clusters

As reported by others (Grapengiesser, Gylfe & Hellman, 1988; Pralong, Bartley & Wollheim, 1990; Wang & McDaniel, 1990; Herchuelz, Pochet, Pastiels & Van Praet, 1991), glucose (15 mM) induced highly variable  $[\text{Ca}^{2+}]_i$  responses in single islet cells. Responsive cells displayed a sustained increase or oscillations in  $[\text{Ca}^{2+}]_i$ , whereas other cells did not respond at all (data not shown). In contrast, glucose triggered a consistent response in clusters of five to eight islet cells, which consisted of large amplitude oscillations in  $[\text{Ca}^{2+}]_i$  with a frequency of  $0.27 \pm 0.03 \text{ min}^{-1}$  ( $n = 82$ ; Fig. 1). Clusters of cells were therefore used in subsequent experiments.

When the concentration of  $\text{K}^+$  in the perfusion medium was increased from 4.8 to 30 mM to depolarize the membrane, a biphasic increase in  $[\text{Ca}^{2+}]_i$  occurred with an initial peak averaging  $1318 \pm 165 \text{ nM}$  and a plateau averaging  $588 \pm 50 \text{ nM}$  after 10 min ( $n = 6$ ). Both this  $\text{K}^+$ -induced increase in  $[\text{Ca}^{2+}]_i$  (Fig. 1A) and the glucose-induced  $[\text{Ca}^{2+}]_i$  oscillations (Fig. 1B) were abolished by D600. That 100  $\mu\text{M}$  D600 completely blocked voltage-dependent  $\text{Ca}^{2+}$  channels is shown by the absence of any rise in  $[\text{Ca}^{2+}]_i$  when the concentration of  $\text{K}^+$  in the medium was increased from 4.8 to 30 mM in its presence (Fig. 1B).

**Figure 1.** D600 blockade of depolarization-induced  $[\text{Ca}^{2+}]_i$  changes in mouse pancreatic  $\beta$ -cells perfused with a medium containing 15 mM glucose

The  $\text{K}^+$  concentration of the perfusion medium was increased from 4.8 to 30 mM, and 100  $\mu\text{M}$  D600 was added as indicated. The traces are representative of results obtained in 6 (A) and 4 (B) clusters of cells.

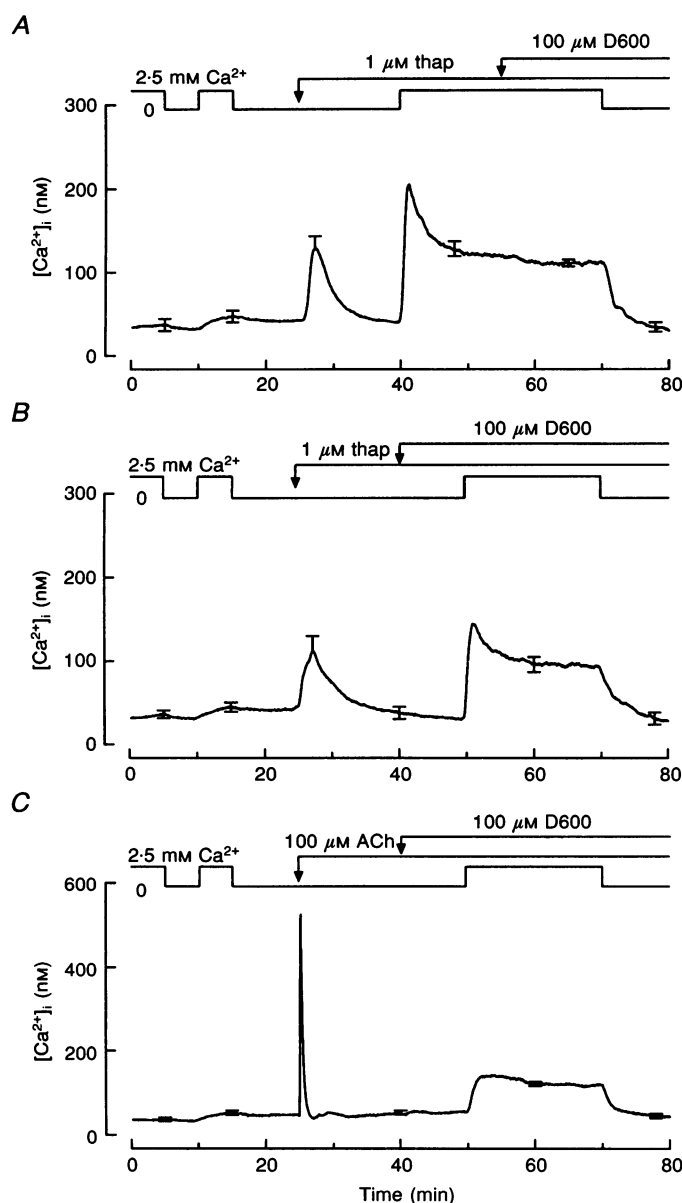


At a concentration of  $1\text{ }\mu\text{M}$ , thapsigargin empties intracellular  $\text{Ca}^{2+}$  stores in  $\beta$ -cells. This has previously been demonstrated by its ability to suppress completely ACh-induced  $\text{Ca}^{2+}$  mobilization in clusters of islet cells (Miura, Gilon & Henquin, 1996; see also Fig. 5) and in whole islets (Gilon, Nenquin & Henquin, 1995a).

#### Effects of thapsigargin on $[\text{Ca}^{2+}]_i$ in hyperpolarized cell clusters

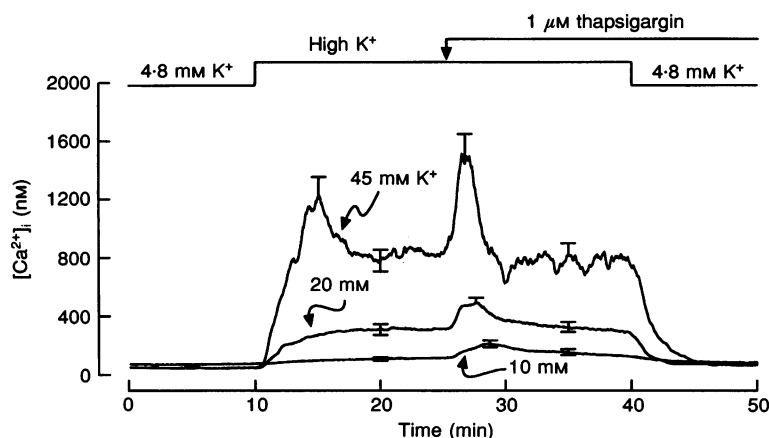
These experiments were carried out in the presence of  $4.8\text{ mM}$  KCl and  $250\text{ }\mu\text{M}$  diazoxide which, by fully opening  $\text{K}_{\text{ATP}}$  channels, holds the membrane potential at the resting level. Under these conditions,  $[\text{Ca}^{2+}]_i$  was low and stable, averaging  $47 \pm 4\text{ nM}$  ( $n = 22$ ). Switching between  $\text{Ca}^{2+}$ -containing and  $\text{Ca}^{2+}$ -free media induced small fluctuations in  $[\text{Ca}^{2+}]_i$  with an amplitude ranging between 6 and 26 nM and averaging  $14 \pm 1\text{ nM}$  ( $n = 22$ ; Fig. 2A–C). The addition of  $1\text{ }\mu\text{M}$  thapsigargin to the  $\text{Ca}^{2+}$ -free medium induced a

transient increase in  $[\text{Ca}^{2+}]_i$ , reflecting the emptying of intracellular  $\text{Ca}^{2+}$  stores. Subsequent readmission of  $2.5\text{ mM}$  extracellular  $\text{Ca}^{2+}$  produced a biphasic rise in  $[\text{Ca}^{2+}]_i$  that was much larger than that before thapsigargin application (Fig. 2A). At the plateau, i.e. 15 min after extracellular  $\text{Ca}^{2+}$  readmission,  $[\text{Ca}^{2+}]_i$  reached  $120 \pm 7\text{ nM}$  ( $n = 7$ ), which corresponds to an elevation by  $79 \pm 6\text{ nM}$  above the values measured before  $\text{Ca}^{2+}$  readmission. The subsequent addition of  $100\text{ }\mu\text{M}$  D600 induced a marginal decrease in  $[\text{Ca}^{2+}]_i$  suggesting that a minor fraction of  $\text{Ca}^{2+}$  could have entered the cells through voltage-dependent  $\text{Ca}^{2+}$  channels (Fig. 2A). Therefore, D600 was also applied immediately after the emptying of  $\text{Ca}^{2+}$  stores by thapsigargin in a  $\text{Ca}^{2+}$ -free medium (Fig. 2B). Readmission of  $2.5\text{ mM}$   $\text{Ca}^{2+}$  in the presence of D600 induced a biphasic rise in  $[\text{Ca}^{2+}]_i$  that averaged  $64 \pm 3\text{ nM}$  above basal values at the plateau ( $n = 10$ ) and was completely reversible upon return to a  $\text{Ca}^{2+}$ -free medium (Fig. 2B). The effects of  $\text{Ca}^{2+}$  store



**Figure 2. Thapsigargin- and ACh-induced capacitative  $\text{Ca}^{2+}$  entry in mouse pancreatic  $\beta$ -cells**

All solutions contained 15 mM glucose and  $250\text{ }\mu\text{M}$  diazoxide. The  $\text{Ca}^{2+}$  concentration of the perfusion medium was changed between 2.5 and 0 mM as indicated. Thapsigargin (thap,  $1\text{ }\mu\text{M}$ ), ACh ( $100\text{ }\mu\text{M}$ ) and D600 ( $100\text{ }\mu\text{M}$ ) were added at times shown on top of each panel. Note the different scale in panel C. The traces are means  $\pm$  S.E.M. of results obtained in 7 (A), 10 (B) and 5 (C) clusters of cells.



**Figure 3. Effect of thapsigargin on  $\text{K}^+$ -induced  $[\text{Ca}^{2+}]_i$  changes in mouse pancreatic  $\beta$ -cells**

All solutions contained 15 mM glucose, 250  $\mu\text{M}$  diazoxide and 2.5 mM  $\text{Ca}^{2+}$ . At 10 min, the  $\text{K}^+$  concentration of the medium was increased from 4.8 mM to 10, 20 or 45 mM. Thapsigargin was then added at the time shown on top of the figure. The traces are means  $\pm$  s.e.m. of results obtained in 4–5 clusters of cells.

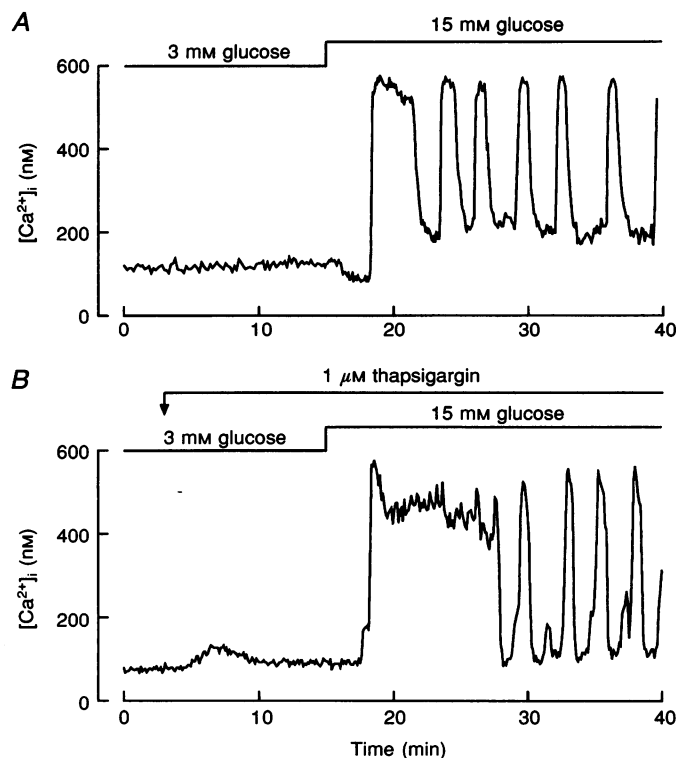
depletion by a physiological agent, ACh, were also tested. The addition of 100  $\mu\text{M}$  ACh to  $\text{Ca}^{2+}$ -free medium triggered a large but transient peak of  $[\text{Ca}^{2+}]_i$  (Fig. 2C). When 2.5 mM  $\text{Ca}^{2+}$  was then readmitted after D600,  $[\text{Ca}^{2+}]_i$  increased by  $65 \pm 5$  nM ( $n = 5$ ) above basal values, as it did after thapsigargin. The small rise in  $[\text{Ca}^{2+}]_i$  induced by the emptying of intracellular  $\text{Ca}^{2+}$  stores and resistant to D600 corresponds to a capacitative  $\text{Ca}^{2+}$  entry. It is important to emphasize that its amplitude is much smaller ( $\sim 8$ -fold) than the increase in  $[\text{Ca}^{2+}]_i$  evoked by depolarization with high  $\text{K}^+$  (compare Figs 1 and 2).

#### Effects of thapsigargin on $[\text{Ca}^{2+}]_i$ in stably depolarized cell clusters

Islet cells were then depolarized to various degrees by perfusion with a medium containing diazoxide and 10, 20 or 45 mM  $\text{K}^+$ . These depolarizations resulted in elevations of  $[\text{Ca}^{2+}]_i$  ranging between 100 and 900 nM at the plateau (Fig. 3). The addition of thapsigargin caused a transient rise in  $[\text{Ca}^{2+}]_i$ , the amplitude of which increased with the concentration of  $\text{K}^+$ . In contrast, the plateau of  $[\text{Ca}^{2+}]_i$  that followed was slightly increased by thapsigargin in the presence of 10 mM  $\text{K}^+$  (by  $36 \pm 13$  nM after 10 min,  $n = 5$ ), but was not affected by the drug in 20 or 45 mM  $\text{K}^+$ .

**Figure 4. Effect of thapsigargin on glucose-induced  $[\text{Ca}^{2+}]_i$  oscillations in mouse pancreatic  $\beta$ -cells**

The glucose concentration of the medium was increased from 3 to 15 mM at 15 min. In the experiment shown in panel B, thapsigargin was added as indicated on top of the figure. The traces are representative of results obtained in 28 (A) and 25 (B) clusters of cells.



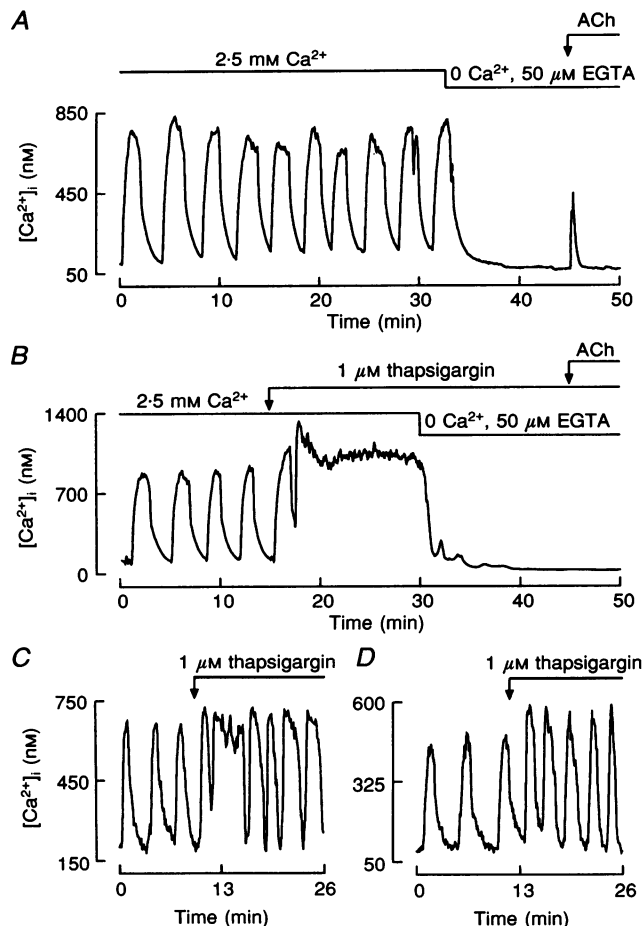
### Effects of thapsigargin on $[Ca^{2+}]_i$ in clusters whose membrane potential was not clamped

A further series of experiments was carried out with islet cell clusters perfused with a medium devoid of diazoxide and containing a normal (4.8 mM) concentration of  $K^+$ , thus under conditions where the membrane potential was not clamped. In the presence of 3 mM glucose,  $[Ca^{2+}]_i$  was low and stable. Increasing the concentration of glucose to 15 mM first induced a drop in  $[Ca^{2+}]_i$  that was followed by a biphasic rise characterized by a long first phase and large amplitude oscillations in  $[Ca^{2+}]_i$  (Fig. 4A). After addition of thapsigargin to medium containing 3 mM glucose,  $[Ca^{2+}]_i$  increased and then declined to stabilize at a level  $12.6 \pm 2.5$  nM ( $n = 25$ ) above the values measured before the application of the drug ( $P < 0.01$  by Student's paired *t* test). A subsequent increase in the glucose concentration from 3 to 15 mM no longer lowered  $[Ca^{2+}]_i$  but caused a long initial rise followed by  $[Ca^{2+}]_i$  oscillations in most cells (Fig. 4B).

The influence of thapsigargin on glucose-induced  $[Ca^{2+}]_i$  changes was also studied in experiments in which the drug was added to a medium already containing 15 mM glucose. As shown in Fig. 5C and D, thapsigargin accelerated the frequency of  $[Ca^{2+}]_i$  oscillations and sometimes augmented their amplitude. In some clusters, it also transformed the oscillations into a sustained increase in  $[Ca^{2+}]_i$  (Fig. 5B). The effect of thapsigargin was quantified by integrating  $[Ca^{2+}]_i$

before (last 10 min with glucose alone) and after (5–15 min) addition of the drug. Control  $[Ca^{2+}]_i$  averaged  $317 \pm 22$  nM and increased to  $501 \pm 56$  nM ( $n = 14$ ) in the presence of thapsigargin ( $P < 0.01$ , Student's paired *t* test). In control cells that were not challenged with thapsigargin,  $[Ca^{2+}]_i$  remained stable with time ( $350 \pm 31$  versus  $359 \pm 29$  nM,  $n = 9$ ; Fig. 5A). The increase in  $[Ca^{2+}]_i$  which thapsigargin provoked under these conditions was thus much larger (~3-fold) than that resulting from the capacitative  $Ca^{2+}$  entry.

To evaluate whether  $K_{ATP}$  channels are involved in the effects of thapsigargin, the drug was also tested in the presence of tolbutamide, which closes these channels. The addition of 250  $\mu$ M tolbutamide to medium containing 15 mM glucose induced longer  $[Ca^{2+}]_i$  oscillations in eight out of twelve cell clusters (Fig. 6A) and transformed these oscillations into a sustained increase in the other four clusters (Fig. 6B). No further major change in  $[Ca^{2+}]_i$  occurred when the tolbutamide concentration was raised to 500  $\mu$ M, which shows that  $K_{ATP}$  channels were fully inhibited under these conditions. However, thapsigargin was still effective. When  $[Ca^{2+}]_i$  was already steadily increased, thapsigargin produced a further transient rise (Fig. 6B), as it did when the cells were depolarized with high  $K^+$ . When  $[Ca^{2+}]_i$  was still oscillating in the presence of tolbutamide, thapsigargin caused a transient peak followed by a sustained elevation of  $[Ca^{2+}]_i$  (Fig. 6A).



**Figure 5.** Effect of thapsigargin on glucose-induced  $[Ca^{2+}]_i$  oscillations in mouse pancreatic  $\beta$ -cells

The glucose concentration of the medium was 15 mM throughout, and 1  $\mu$ M thapsigargin was added during the periods of time shown on top of each panel. In the experiments illustrated by panels A and B, the  $Ca^{2+}$  concentration of the medium was decreased from 2.5 to 0 mM, and 100  $\mu$ M ACh was added as indicated. In the experiments illustrated by panels C and D, the  $Ca^{2+}$  concentration was kept at 2.5 mM throughout. The traces are representative of results obtained in 9–14 clusters of cells.

When  $\text{K}_{\text{ATP}}$  channels were opened by diazoxide, thapsigargin induced a transient rise followed by a small plateau of  $[\text{Ca}^{2+}]_{\text{i}}$  corresponding to  $\text{Ca}^{2+}$  mobilization and capacitative entry, respectively. In contrast, the reversal of the diazoxide effect by tolbutamide resulted in a large, sustained elevation of  $[\text{Ca}^{2+}]_{\text{i}}$  (Fig. 6C). That the effects of thapsigargin do not involve blockade of  $\text{K}_{\text{ATP}}$  channels is also supported by the observation that the drug does not inhibit  $^{86}\text{Rb}^{+}$  efflux from islet cells, an indirect measure of  $\text{K}_{\text{ATP}}$  channel activity (not shown), and does not affect the  $\beta$ -cell membrane potential in the presence of low glucose (Worley *et al.* 1994b).

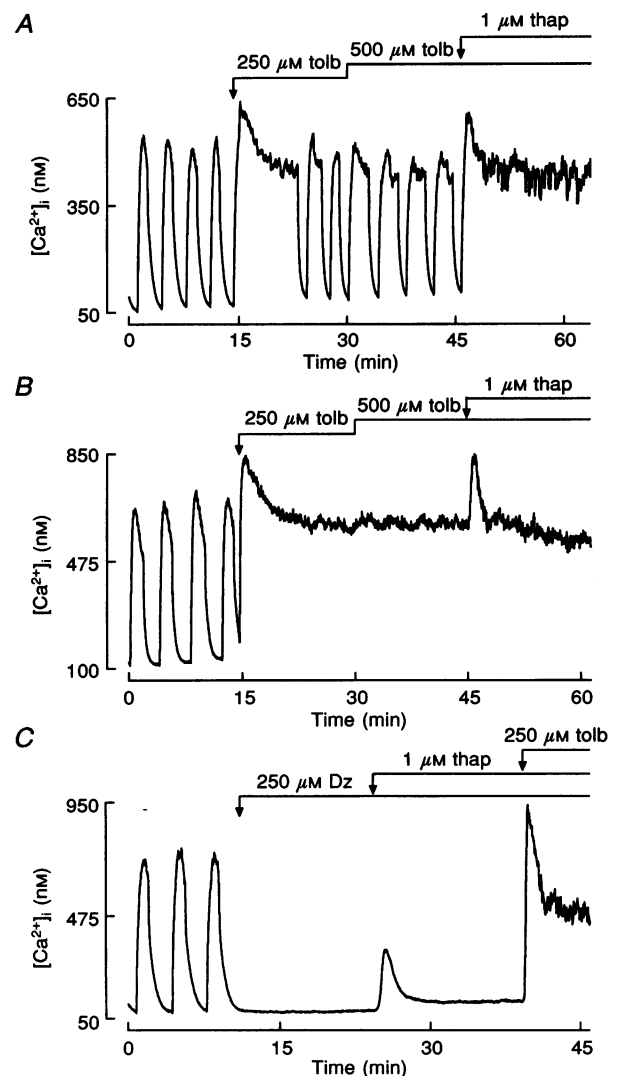
#### Effects of thapsigargin on $[\text{Ca}^{2+}]_{\text{i}}$ in whole islets

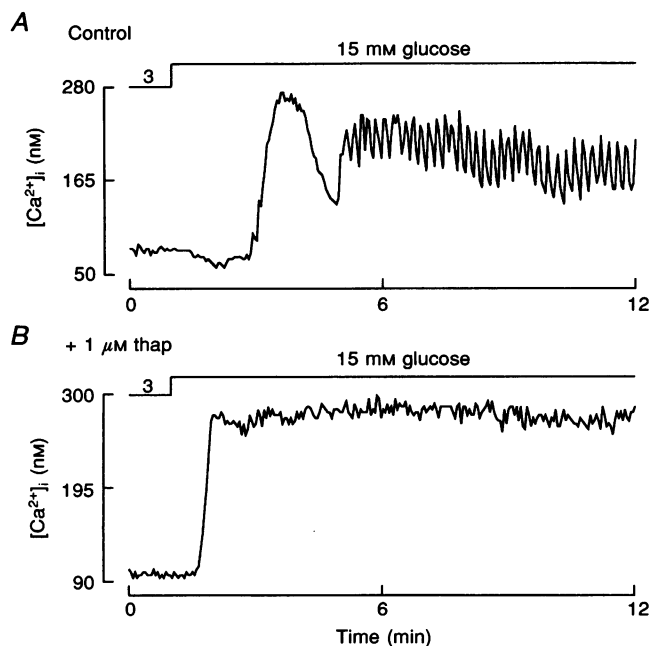
In control islets, increasing the glucose concentration from 3 to 15 mM induced a transient decrease in  $[\text{Ca}^{2+}]_{\text{i}}$  followed by a biphasic increase that was characterized by a long first phase and a second phase consisting of oscillations (Fig. 7A). After treatment of the islets with thapsigargin, the initial transient drop in  $[\text{Ca}^{2+}]_{\text{i}}$  disappeared, the first rise occurred sooner, and the oscillations of the second phase were replaced by a sustained elevation of  $[\text{Ca}^{2+}]_{\text{i}}$  (Fig. 7B).

Switching from a glucose-free medium to a medium containing 6 mM glucose induced a transient decrease in  $[\text{Ca}^{2+}]_{\text{i}}$  sometimes followed by a small and transient elevation in  $[\text{Ca}^{2+}]_{\text{i}}$  in control islets. When the glucose concentration was then raised to 10 mM,  $[\text{Ca}^{2+}]_{\text{i}}$  increased rapidly and started to oscillate (Fig. 8A). In thapsigargin-treated islets, 6 mM glucose no longer lowered  $[\text{Ca}^{2+}]_{\text{i}}$  but induced oscillations in twelve out of sixteen islets. These oscillations were always much slower than those elicited by 10 mM glucose in control islets (compare Fig. 8A with B and C). Subsequent stimulation by 10 mM glucose produced a further increase in  $[\text{Ca}^{2+}]_{\text{i}}$  characterized by slow and large amplitude oscillations in twelve out of sixteen islets and by a sustained rise in the remaining islets. In three out of sixteen islets, the slow oscillations co-existed with some rapid oscillations during stimulation by 10 mM glucose (Fig. 8B). The rise in  $[\text{Ca}^{2+}]_{\text{i}}$  produced by glucose was quantified by integrating  $[\text{Ca}^{2+}]_{\text{i}}$  during the last 6 min of stimulation with 6 and 10 mM glucose (from 7 to 13 min and from 18 to 24 min, respectively; Fig. 8). During stimulation with 6 mM glucose,  $[\text{Ca}^{2+}]_{\text{i}}$  averaged  $92 \pm 5.1$  nM in control islets ( $n = 15$ ) and  $150 \pm 13$  nM in thapsigargin-treated islets ( $n = 16$ ,  $P < 0.01$ ). In the presence of 10 mM glucose,

**Figure 6.** Comparison of the effects of tolbutamide, diazoxide and thapsigargin on  $[\text{Ca}^{2+}]_{\text{i}}$  in mouse pancreatic  $\beta$ -cells stimulated with glucose

The glucose concentration of the medium was 15 mM throughout, and tolbutamide (tolb), diazoxide (Dz) and thapsigargin (thap) were added during the periods of time shown on top of each panel. The traces are representative of results obtained in 8 (A), 4 (B) and 4 (C) clusters of cells.



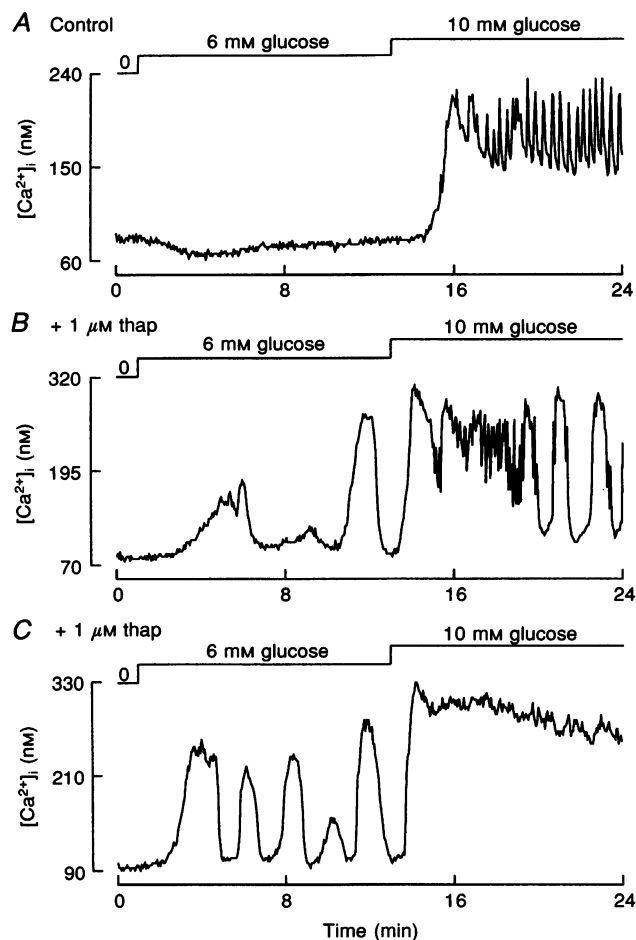


**Figure 7. Effect of thapsigargin on glucose-induced  $[Ca^{2+}]_i$  changes in mouse pancreatic islets**

The glucose concentration of the medium was increased from 3 to 15 mM when indicated. *A* shows the control response. In the experiment shown in panel *B*, both the medium used for the loading with fura-2 and the perfusion medium were supplemented with 1  $\mu$ M thapsigargin (thap). The traces are representative of results obtained in 12 (*A*) and 17 (*B*) islets.

average  $[Ca^{2+}]_i$  was  $169 \pm 9$  and  $237 \pm 12$  nM ( $P < 0.01$ ) in control and thapsigargin-treated islets, respectively. Other control experiments using diazoxide showed that the  $[Ca^{2+}]_i$  rise produced by glucose occurs entirely through voltage-dependent  $Ca^{2+}$  channels even after thapsigargin treatment of the islets (not shown).

To verify that the thapsigargin-induced increase in  $[Ca^{2+}]_i$  did not result from an acceleration of glucose metabolism, the fluorescence of reduced pyridine nucleotides (NAD(P)H) was monitored. Raising the glucose concentration from 3 to 15 mM induced a rapid increase in the NAD(P)H fluorescence, the amplitude and the kinetics of which were similar in



**Figure 8. Effect of thapsigargin on glucose-induced  $[Ca^{2+}]_i$  changes in mouse pancreatic islets**

The glucose concentration of the medium was increased from 0 to 6 and 10 mM as indicated. *A* shows the control response. In the experiments shown in panels *B* and *C*, both the medium used for the loading with fura-2 and the perfusion medium were supplemented with 1  $\mu$ M thapsigargin. The traces are representative of results obtained in 15 (*A*) and 16 (*B* and *C*) islets.

control and thapsigargin-treated islets (Fig. 9). Thapsigargin was also without acute effect as indicated by the lack of change in autofluorescence of clusters of islet cells upon addition of the drug (not shown). Experiments in which thapsigargin was not found to modify glucose oxidation by whole islets (Aizawa *et al.* 1995) also led to the conclusion that the drug does not affect glucose metabolism in  $\beta$ -cells.

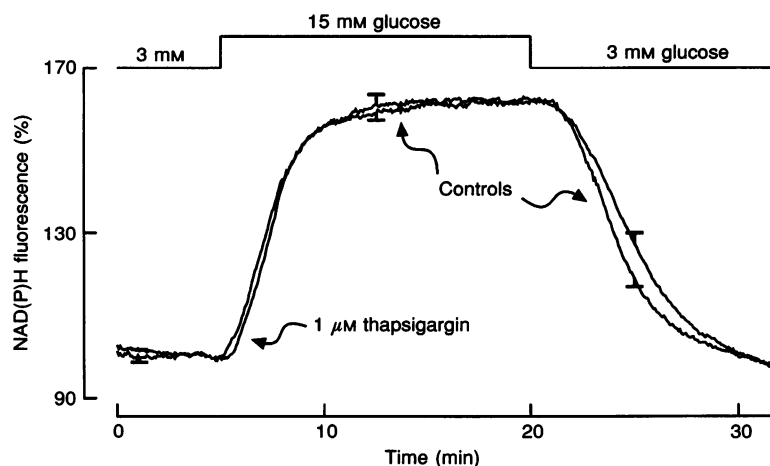
## DISCUSSION

The present study shows that emptying of intracellular  $\text{Ca}^{2+}$  stores by a SERCA pump inhibitor, thapsigargin, stimulates membrane potential-dependent and -independent modalities of  $\text{Ca}^{2+}$  influx in the electrically excitable pancreatic  $\beta$ -cells.

That thapsigargin effectively empties intracellular  $\text{Ca}^{2+}$  stores in  $\beta$ -cells has previously been established by its ability to suppress completely ACh-induced mobilization of  $\text{Ca}^{2+}$  without affecting  $\text{IP}_3$  levels (Gilon *et al.* 1995a; Hamakawa & Yada 1995; Liu, Grapengiesser, Gylfe & Hellman, 1995). The ability of ACh to mimic the effect of thapsigargin on  $\text{Ca}^{2+}$  influx under hyperpolarizing conditions strongly supports the conclusion that the increased  $\text{Ca}^{2+}$  influx is secondary to  $\text{Ca}^{2+}$  store depletion. Unfortunately, the effects of thapsigargin and ACh could not be compared under other conditions (i.e. in stably depolarized cells or in cells whose membrane potential was not clamped) for two reasons. First, in contrast to thapsigargin, ACh also causes a large increase in  $\text{Na}^+$  conductance (Miura *et al.* 1996) that stimulates  $\text{Ca}^{2+}$  influx through voltage-dependent  $\text{Ca}^{2+}$  channels (Henquin, Garcia, Bozem, Hermans & Nenquin, 1988). Second, ACh also exerts an inhibitory effect on voltage-dependent  $\text{Ca}^{2+}$  channels (Gilon *et al.* 1995a).

Identification of a capacitative  $\text{Ca}^{2+}$  entry in excitable cells requires suppression of the large  $\text{Ca}^{2+}$  influx that normally occurs through voltage-dependent channels. This was achieved not only by blocking these channels with a high concentration of D600, but also by hyperpolarizing the membrane with diazoxide (Gembal *et al.* 1992). Under these conditions, emptying of intracellular  $\text{Ca}^{2+}$  stores by thapsigargin was followed by a rise in  $[\text{Ca}^{2+}]_i$  that was dependent on  $\text{Ca}^{2+}$  influx and can thus be attributed to a capacitative  $\text{Ca}^{2+}$  influx. The magnitude of this rise was small compared with that produced by depolarization of the membrane with high  $\text{K}^+$ . Moreover, when voltage-dependent  $\text{Ca}^{2+}$  channels were not blocked and the membrane potential was clamped at different levels with 10–45 mM  $\text{K}^+$ , the additional sustained increase in  $[\text{Ca}^{2+}]_i$  produced by thapsigargin was inversely related to the rise in  $[\text{Ca}^{2+}]_i$  already produced by  $\text{K}^+$  (and significant only in 10 mM  $\text{K}^+$ ). This may be explained by the fact that the driving force for  $\text{Ca}^{2+}$  entry diminishes as  $[\text{Ca}^{2+}]_i$  increases and as the membrane depolarizes towards the equilibrium potential for  $\text{Ca}^{2+}$  ions as already demonstrated in other cell types (Fischer, Illek, Negulescu, Clauss & Machen, 1992; Girard & Clapham, 1993). On the other hand, the initial transient increase in  $[\text{Ca}^{2+}]_i$  induced by thapsigargin augmented the preceding  $[\text{Ca}^{2+}]_i$ . This suggests that the  $\text{Ca}^{2+}$  content of the store is influenced by the concentration of  $\text{Ca}^{2+}$  within the cytosol. In summary, when the  $\beta$ -cell membrane potential is clamped, emptying of intracellular  $\text{Ca}^{2+}$  stores can induce a capacitative  $\text{Ca}^{2+}$  influx that increases  $[\text{Ca}^{2+}]_i$  above basal values, but insignificantly adds to the  $[\text{Ca}^{2+}]_i$  rise brought about by activation of voltage-dependent  $\text{Ca}^{2+}$  channels.

Under physiological conditions the membrane potential of  $\beta$ -cells is not clamped. It changes with the glucose concentration



**Figure 9.** Lack of effect of thapsigargin on glucose-induced changes in NAD(P)H fluorescence in mouse pancreatic islets

When indicated, the concentration of glucose was increased from 3 to 15 mM. In one set of experiments, both the medium used for the pre-incubation of the islets and the perfusion medium were supplemented with 1  $\mu\text{M}$  thapsigargin. NAD(P)H fluorescence is expressed as a percentage of the signal measured during the last minute preceding the change in glucose concentration. The traces correspond to the mean responses  $\pm$  s.e.m. in 15 (Controls) and 18 (thapsigargin) islets.

and, when the latter is between 7 and 20 mM, even displays slow waves of depolarization which cause an intermittent  $\text{Ca}^{2+}$  influx and oscillations in  $[\text{Ca}^{2+}]_i$  (Santos, Rosario, Nadal, Garcia-Sancho, Soria & Valdeolmillos, 1991; Gilon & Henquin, 1992). In a medium containing a non-stimulatory concentration of glucose (3 mM), thapsigargin induced a small increase in  $[\text{Ca}^{2+}]_i$ , which probably reflects the activation of the capacitative  $\text{Ca}^{2+}$  entry. It may seem surprising that this increase was smaller than that produced by thapsigargin in the presence of 15 mM glucose and diazoxide. The difference can, however, be explained by the fact that intracellular  $\text{Ca}^{2+}$  stores are already partially depleted in 3 mM glucose. It is well known that high glucose fills intracellular  $\text{Ca}^{2+}$  stores (Gylfe, 1988; Yada, Kakei & Tanaka, 1992; Roe, Mertz, Lancaster, Worley & Dukes, 1994). The initial drop in  $[\text{Ca}^{2+}]_i$  that follows a rise in glucose concentration and is prevented by thapsigargin reflects this sequestration of  $\text{Ca}^{2+}$  in the endoplasmic reticulum.

Larger effects of thapsigargin on  $[\text{Ca}^{2+}]_i$  were observed when the ambient glucose concentration was 6 mM or higher. They are unlikely to result simply from a  $\text{Ca}^{2+}$  influx through SOC. Thus, the effect of thapsigargin in 6, 10 or 15 mM glucose was clearly larger than that observed when the drug was added to cells whose membrane was stably held at a hyperpolarized or depolarized potential. Specifically, thapsigargin did not significantly increase  $[\text{Ca}^{2+}]_i$  in cell clusters stimulated with 20 mM  $\text{K}^+$  in the presence of diazoxide, but did so in clusters stimulated with 15 mM glucose (without diazoxide) although the integrated  $[\text{Ca}^{2+}]_i$  were similar. Our interpretation is that intracellular store depletion by thapsigargin also activates a small depolarizing current. In low glucose or in high glucose with diazoxide, this current might not affect the membrane potential because of the large repolarizing current flowing through  $\text{K}_{\text{ATP}}$  channels. When these channels are largely closed by glucose ( $\geq 6$  mM in absence of diazoxide) the current activated by store depletion may facilitate depolarization and  $\text{Ca}^{2+}$  influx through voltage-dependent  $\text{Ca}^{2+}$  channels whose threshold potential is almost reached in 6 mM glucose. This may also explain the dual effect of thapsigargin in the presence of tolbutamide. Thapsigargin had no sustained influence in cells in which  $[\text{Ca}^{2+}]_i$  was steadily elevated by tolbutamide presumably through continuous depolarization, whereas it raised  $[\text{Ca}^{2+}]_i$  to a plateau in cells in which  $[\text{Ca}^{2+}]_i$  oscillations persisted in the presence of tolbutamide.

Different types of current ( $\text{Ca}^{2+}$ ,  $\text{Na}^+$  and  $\text{K}^+$ ) activated by intracellular  $\text{Ca}^{2+}$  store depletion have been identified in various cell types (Parekh *et al.* 1993; Fasolato *et al.* 1994; Clapham, 1996; Friel, 1996; Hoth, 1996). Recently, thapsigargin has been reported to activate a non-selective cationic current in  $\beta$ -cells (Worley *et al.* 1994a). Our previous observation that thapsigargin does not increase  $[\text{Na}^+]_i$  in

$\beta$ -cells (Miura *et al.* 1996) suggests that the depolarizing current is not carried by  $\text{Na}^+$  unless it is too small to produce a detectable change in  $[\text{Na}^+]_i$ . It is possible that it is the capacitative  $\text{Ca}^{2+}$  entry itself that underlies the depolarizing current. However, the nature of these SOC in pancreatic  $\beta$ -cells is still unknown and their identification would require another approach based on patch-clamp techniques.

This study also shows that  $[\text{Ca}^{2+}]_i$  oscillations persisted after thapsigargin treatment in clusters of islet cells stimulated with 15 mM glucose. This was also the case in islets perfused with 6 or 10 mM glucose. Since intracellular  $\text{Ca}^{2+}$  stores were completely emptied by thapsigargin, our observations indicate that, in contrast to a previous suggestion (Ämmälä *et al.* 1991), periodic mobilization of intracellular  $\text{Ca}^{2+}$  is not indispensable for glucose to induce  $[\text{Ca}^{2+}]_i$  oscillations. Similar conclusions were reached in a study using  $\text{Ba}^{2+}$ , which is not sequestered in or released from the  $\text{IP}_3$ -sensitive stores, as a substitute for  $\text{Ca}^{2+}$  (Liu *et al.* 1995). However, the characteristics of the  $[\text{Ca}^{2+}]_i$  oscillations that we recorded were usually affected by thapsigargin. This raises the possibility that  $\text{Ca}^{2+}$  stores at least subtly influence the oscillations.

In conclusion, the emptying of intracellular  $\text{Ca}^{2+}$  stores stimulates both membrane potential-independent and -dependent  $\text{Ca}^{2+}$  influx in pancreatic  $\beta$ -cells. The membrane potential-independent mechanism corresponds to the capacitative  $\text{Ca}^{2+}$  entry that exists in non-excitabile cells, but the  $[\text{Ca}^{2+}]_i$  rise that it directly causes is rather small. The membrane potential-dependent mechanism indirectly increases  $[\text{Ca}^{2+}]_i$  by generating a depolarizing current that potentiates  $\text{Ca}^{2+}$  influx through voltage-dependent  $\text{Ca}^{2+}$  channels provided these are already activated by glucose-induced depolarization. Agents that modulate the  $\text{Ca}^{2+}$  content of intracellular stores will thus have an effect on  $\text{Ca}^{2+}$  influx, which greatly depends on the membrane potential. In excitable cells, this membrane potential-dependent mechanism permits a small depolarizing current triggered by store depletion to activate a much larger  $\text{Ca}^{2+}$  influx than in non-excitabile cells.

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