Store-operated influx of Ca^{2+} in pancreatic β -cells exhibits graded dependence on the filling of the endoplasmic reticulum

Oleg Dyachok^{1,2} and Erik Gylfe^{1,*}

¹Department of Medical Cell Biology, Uppsala University, Biomedicum, Box 571, SE-751 23 Uppsala, Sweden

²Department of Biophysics, National T. Shevchenko University of Kiev, Kiev, Ukraine

*Author for correspondence (e-mail: erik.gylfe@medcellbiol.uu.se)

Accepted 5 March 2001

Journal of Cell Science 114, 2179–2186 (2001) © The Company of Biologists Ltd

SUMMARY

The store-operated pathway for Ca^{2+} entry was studied in individual mouse pancreatic β -cells by measuring the cytoplasmic concentrations of Ca^{2+} ($[\text{Ca}^{2+}]_i$) and Mn^{2+} ($[\text{Mn}^{2+}]_i$) with the fluorescent indicator fura-2. Influx through the store-operated pathway was initially shut off by pre-exposure to 20 mM glucose, which maximally stimulates intracellular Ca^{2+} sequestration. To avoid interference with voltage-dependent Ca^{2+} entry the cells were hyperpolarized with diazoxide and the channel blocker methoxyverapamil was present. Activation of the store-operated pathway in response to Ca^{2+} depletion of the endoplasmic reticulum was estimated from the sustained elevation of $[\text{Ca}^{2+}]_i$ or from the rate of increase in $[\text{Mn}^{2+}]_i$ due to influx of these extracellular ions. Increasing concentrations of the inositol 1,4,5-

trisphosphate-generating agonist carbachol or the sarco(endo)plasmic reticulum Ca^{2+} -ATPase inhibitor cyclopiazonic acid (CPA) cause gradual activation of the store-operated pathway. In addition, the carbachol- and CPA-induced influx of Mn^{2+} depended on store filling in a graded manner. The store-operated influx of $\text{Ca}^{2+}/\text{Mn}^{2+}$ was inhibited by Gd^{3+} and 2-aminoethoxydiphenyl borate but neither of these agents discriminated between store-operated and voltage-dependent entry. The finely tuned regulation of the store-operated mechanisms in the β -cell has direct implications for the control of membrane potential and insulin secretion.

Key words: Pancreatic β -cell, Store-operated, Calcium channels, Insulin secretion

INTRODUCTION

A rise of the cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is the key trigger of insulin secretion from pancreatic β -cells exposed to glucose and other nutrient stimuli (Wollheim and Sharp, 1981; Hellman and Gylfe, 1986). The signal transduction involves metabolism of the stimulus causing a rise of the ATP/ADP ratio and subsequent closure of the ATP-sensitive K^+ (K_{ATP}) channels, resulting in depolarization and influx of Ca^{2+} through voltage-dependent channels (Ashcroft and Rorsman, 1989). However, there is increasing evidence that intracellular sequestration and release of Ca^{2+} are also important in the regulation of insulin secretion. Glucose is consequently a potent stimulus for Ca^{2+} accumulation in the endoplasmic reticulum (ER) of pancreatic (Hellman et al., 1986; Gylfe, 1991; Tengholm et al., 1999) as well as clonal β -cells (Gylfe and Hellman, 1986; Maechler et al., 1999). Moreover, depolarization during glucose stimulation triggers formation of inositol 1,4,5 trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) (Roe et al., 1993; Liu et al., 1996), which seems to be the major messenger for Ca^{2+} mobilization from the β -cell ER (Tengholm et al., 1998). Apart from affecting $[\text{Ca}^{2+}]_i$ directly, the intracellular release has indirect actions resulting from changes in membrane potential. The $\text{Ins}(1,4,5)\text{P}_3$ -induced rise of $[\text{Ca}^{2+}]_i$ can activate a hyperpolarizing K^+ current shutting

off the voltage-dependent entry of the cation (Ämmälä et al., 1991; Liu et al., 1998; Dryselius et al., 1999). The associated emptying of the ER has the opposite effect, activating a depolarizing store-operated current carried by Ca^{2+} or Na^+ (Worley et al., 1994; Bertram et al., 1995; Liu and Gylfe, 1997; Gilon et al., 1999).

The role of the store-operated current in the physiology of the glucose-stimulated β -cell ultimately depends on how it is regulated by store filling. In some types of cells Ca^{2+} influx is activated in an all-or-none fashion after almost complete emptying of the intracellular Ca^{2+} stores (Fierro and Parekh, 2000; Fierro et al., 2000), whereas in others there is gradual activation with increasing depletion of the stores (Hofer et al., 1998; Sedova et al., 2000). The present study provides the first evidence that the store-operated entry of Ca^{2+} into the β -cell exhibits a graded dependence on Ca^{2+} filling of the ER. Small variations in the ER Ca^{2+} concentration may consequently contribute to the regulation of the membrane potential and $[\text{Ca}^{2+}]_i$ determining insulin release.

MATERIALS AND METHODS

Chemicals

Reagents of analytical grade and deionized water were used. Fura-2

and its acetoxymethyl ester were from Molecular Probes Inc. (Eugene, OR). EGTA and carbachol were provided by Sigma (St Louis, MO), 2-aminoethoxydiphenyl borate (2-APB) by Aldrich (Gillingham, UK), cyclopiazonic acid (CPA) by Calbiochem (La Jolla, CA). Collagenase and HEPES were bought from Boehringer Mannheim GmbH (Mannheim, Germany). Schering (Kenilworth, NJ) and Knoll AG (Ludwigshafen, Germany) kindly donated diazoxide and methoxyverapamil, respectively. *Staphylococcus aureus* α -toxin was a product of BioSys Inova (Stockholm, Sweden).

Preparation of pancreatic islets and β -cells

Islets of Langerhans were isolated by collagenase digestion from the pancreas of adult ob/ob mice from a local colony (Hellman, 1965). These islets consist of more than 90% β -cells, which respond normally to glucose and other regulators of insulin release (Hahn et al., 1974). Free cells were prepared by shaking the islets in a Ca^{2+} -deficient medium (Lemmark, 1974). The cells were suspended in RPMI 1640 medium containing 11 mM glucose and supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 30 $\mu\text{g}/\text{ml}$ gentamicin and allowed to attach to circular 25 mm coverslips during culture for 4–7 days in an atmosphere of 5% CO_2 in humidified air. Further experimental handling of cells was performed with a medium containing 25 mM HEPES (pH 7.40), 1 mg/ml bovine serum albumin, 137 mM Na^+ , 5.9 mM K^+ , 1.2 mM Mg^{2+} , and <1 nM, 1.28 or 10 mM Ca^{2+} with Cl^- as the sole anion. The lowest Ca^{2+} concentration was obtained by including 2 mM EGTA in a Ca^{2+} -deficient medium. When testing the effects of Gd^{3+} , bovine serum albumin and EGTA were omitted.

Measurements of cytoplasmic Ca^{2+} and Mn^{2+}

In most experiments, loading of the cells with the fluorescent indicator fura-2 was performed in the presence of 1.28 mM Ca^{2+} during a 40 minute incubation at 37°C in a medium supplemented with 1 μM fura-2 acetoxymethyl ester, 400 μM diazoxide and 20 mM glucose. However, when testing the effect of K^+ depolarization, fura-2 loading was made in medium lacking diazoxide and containing 3 mM glucose. With these procedures $90 \pm 0.5\%$ ($n=4$) of the fura-2 is cytoplasmic as judged from the release of indicator in response to plasma membrane permeabilization using a previously described technique (Tengholm et al., 2000) with 1250 hemolytic units/ml α -toxin. Calculations of $[\text{Ca}^{2+}]_i$ and $[\text{Mn}^{2+}]_i$ (see below) were compensated for this compartmentalization of fura-2. The coverslips with attached cells were used as exchangeable bottoms of an open chamber containing 50 μl medium. The chamber was placed on the stage of an inverted microscope (Nikon Diaphot) within a climate box maintained at 37°C by an air stream incubator, and the cells were superfused at a rate of 0.3 ml/minute with similar indicator-free medium. When studying store-operated Ca^{2+} influx this medium was supplemented with 50 μM methoxyverapamil.

The microscope was equipped with an epifluorescence illuminator and a $100\times$ UV fluorite objective. A filter changer of a time-sharing multichannel spectrophotofluorometer (Chance et al., 1975) provided excitation light flashes of 1 millisecond duration every 10 milliseconds at 340 and 380 nm, and the emission was measured at 510 nm with a photomultiplier. A computer recorded the electronically separated fluorescence signals at the two wavelengths.

$[\text{Ca}^{2+}]_i$ values were obtained according to a previously described method (Grynkiewicz et al., 1985) using Equation 1:

$$[\text{Ca}^{2+}]_i = K_D^{\text{Ca}^{2+}} \cdot \frac{F_0}{F_S} \cdot \frac{(R - R_{\min})}{(R_{\max} - R)} \quad (1)$$

$K_D^{\text{Ca}^{2+}}$ 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 calibration solution lacking Ca^{2+} . F_S and $R_{\max}$ are the corresponding data obtained with saturating concentrations of Ca^{2+} .

Fig. 1. Estimation of store-operated Mn^{2+} influx in an individual β -cell. The pancreatic β -cell was loaded with fura-2 in hyperpolarizing medium containing 400 μM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+} . The same medium lacking indicator but containing 50 μM methoxyverapamil was present at the beginning of the experiment. The medium was then supplemented with 200 μM Mn^{2+} (upper bar) and 30 μM carbachol (Carb; lower bars). The cytoplasmic Mn^{2+} concentration (A) is shown above the calculated Ca^{2+} -independent fluorescence of fura-2 compensated for fading and loss of indicator (B); the calculated Ca^{2+} -independent fluorescence of fura-2 without such compensation (C) and $[\text{Ca}^{2+}]_i$ (D), which is not reliable after the introduction of Mn^{2+} (shaded area). The broken lines indicate the rate of change in Mn^{2+} concentration (A) and Ca^{2+} -independent fluorescence of fura-2 (B,C) before addition of Mn^{2+} (0), immediately after addition of Mn^{2+} (1) and after subsequent addition of 30 μM carbachol (2).

Variations in the influx through the store-operated pathway were estimated more directly by a Mn^{2+} quench approach. However, instead of measuring only the reduction in fura-2 fluorescence in β -cells exposed to this cation (Liu and Gylfe, 1997) we introduced a novel approach linearizing the data by calculating the cytoplasmic Mn^{2+} concentration ($[\text{Mn}^{2+}]_i$). Because Mn^{2+} quenches the fluorescence of fura-2, irrespective of excitation wavelength, a single wavelength technique is used. To make such measurements independent of changes in $[\text{Ca}^{2+}]_i$ the isosbestic wavelength of fura-2 is utilized. However, instead of measuring the fluorescence excited at the isosbestic wavelength, a Ca^{2+} insensitive 'isosbestic' fluorescence signal was calculated as $F_i = F_{340} + \alpha \cdot F_{380}$. In this equation, α is the isocoefficient that scales the negative F_{380} response to compensate exactly for the positive F_{340} response (fluorescence excited at 340 and 380 nm, respectively) when $[\text{Ca}^{2+}]_i$ is increased (Zhou and Neher, 1993). The effectiveness of this procedure is illustrated in Fig. 1, in which panel C shows lack of effect of carbachol on the calculated

'isosbestic' fluorescence despite a pronounced carbachol-induced $[\text{Ca}^{2+}]_i$ response (panel D). Owing to photobleaching and loss of indicator from the cells there is a slow gradual decrease of the Ca^{2+} -independent fluorescence even in the absence of Mn^{2+} (Fig. 1C, broken line 0). After compensating for this decrease (Fig. 1B), $[\text{Mn}^{2+}]_i$ can be calculated in analogy to the method previously described (Gryniewicz et al., 1985) using Equation 2:

$$[\text{Mn}^{2+}]_i = K_D^{\text{Mn}^{2+}} \cdot \frac{(F_{\text{max}} - F)}{(F - F_{\text{min}})} \quad (2)$$

$K_D^{\text{Mn}^{2+}}$ is 2.8 nM (Kwan and Putney, 1990). F_{max} is the unquenched (Fig. 1B, line 0) and F_{min} the maximally quenched fura-2 fluorescence in the presence of Mn^{2+} , which was set to 1% of F_{max} (Kwan and Putney, 1990). Fig. 1A illustrates the slow rise of $[\text{Mn}^{2+}]_i$ upon introduction of the ion (broken line 1) and acceleration of this effect after stimulation with carbachol (broken line 2). Although the apparent $K_D^{\text{Mn}^{2+}}$ may be expected to change slightly with the Ca^{2+} concentration, we found no evidence for such interference because $[\text{Ca}^{2+}]_i$ peaks occurred without fluctuations in the Mn^{2+} signal.

Presentation of data and statistical analysis

Results are presented as means $\pm$ s.e.m. Differences were statistically evaluated by the two-tailed Student's *t* test. The dose-response data (Fig. 2B; Fig. 4B) were fitted to a sigmoidal equation (logistic function) using the Marquart-Levenberg algorithm (SigmaPlot, SPSS Inc. Chicago, IL). The linear curve fits (Fig. 1; Fig. 3; Fig. 5) and all illustrations were made with the Igor Pro software (Wavemetrics Inc., Lake Oswego, OR).

RESULTS

Omission of extracellular Ca^{2+} (reduction from 1.28 to <1 nM) resulted in a modest slow lowering of $[\text{Ca}^{2+}]_i$ in the hyperpolarized β -cells exposed to 20 mM glucose (Fig. 2A). Subsequent introduction of 10 mM Ca^{2+} caused a rapid but modest increase of $[\text{Ca}^{2+}]_i$ above the baseline. This effect was considered to be due to leakage of Ca^{2+} through pathways other than the store-operated and voltage-dependent Ca^{2+} channels, which are inhibited by glucose exposure (Liu and Gylfe, 1997) and hyperpolarization, respectively. After return to 1.28 mM Ca^{2+} for a few minutes, Ca^{2+} was again omitted and carbachol added to mobilize Ca^{2+} from the ER. When Ca^{2+} was subsequently increased to 10 mM in the continued presence of carbachol, there was a marked increase in $[\text{Ca}^{2+}]_i$ owing to contribution of the store-operated Ca^{2+} channels. Carbachol was then omitted and Ca^{2+} lowered to 1.28 mM. Similar cycles were then repeated with increasing concentrations of carbachol (Fig. 2A). When 0.3–1 μM carbachol was introduced in the absence of Ca^{2+} there were no detectable changes in $[\text{Ca}^{2+}]_i$ and sequentially adding higher concentrations of the drug resulted in small temporary elevations. The effect of 10 mM Ca^{2+} depended on the prevailing carbachol concentration in a graded fashion, the maximal increase in $[\text{Ca}^{2+}]_i$ being quite pronounced. Fig. 2B shows the dose-response relationship for carbachol-induced elevation of $[\text{Ca}^{2+}]_i$ in the presence of 10 mM Ca^{2+} . Half-maximal and maximal effects were reached at 2.48 ± 0.31 and 30 μM carbachol, respectively, whereas 100 μM gave a slightly smaller response.

After exposure to Mn^{2+} in the absence of agonist there was a linear rise in $[\text{Mn}^{2+}]_i$ in the hyperpolarized and glucose-

Fig. 2. Effect of carbachol concentration on elevation of $[\text{Ca}^{2+}]_i$ due to store-operated influx of Ca^{2+} in individual β -cells. Pancreatic β -cells were loaded with fura-2 in hyperpolarizing medium containing 400 μM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+} . The same medium lacking indicator but containing 50 μM methoxyverapamil was present at the beginning of the representative experiment shown in A. The Ca^{2+} concentration was then changed between 1.28 (gray), 0 (white; Ca^{2+} -free+2 mM EGTA) and 10 mM (black), as indicated by the upper bars; 0.3–100 μM carbachol was introduced as shown by the lower bars. B shows the dose-response relationship for carbachol-induced elevation of $[\text{Ca}^{2+}]_i$ in the presence of 10 mM Ca^{2+} . A single observation is shown at 0.3 μM carbachol and means $\pm$ s.e.m. for six observations at the other concentrations. The solid line shows a fit of the 25 individual data points in the 0.3–30 μM carbachol range to a logistic function ($r=0.977$; $P<0.0001$); the broken line shows that the effect decreases at 100 μM carbachol.

exposed β -cells. This rise can be expected to represent entry of the ion through pathways other than the voltage-dependent and store-operated Ca^{2+} channels (Fig. 3A). Subsequent addition of carbachol dose-dependently accelerated the rate of $[\text{Mn}^{2+}]_i$ increase, owing to activation of the store-operated pathway. In a series of three experiments, the acceleration obtained with 3.6 μM carbachol was $29.9 \pm 9.5\%$ of the maximal activation obtained with 30 μM of the drug.

During maximal SERCA inhibition the ER is rapidly depleted owing to leakage of Ca^{2+} (Liu and Gylfe, 1997; Tengholm et al., 1999). We then used increasing concentrations of the SERCA inhibitor CPA to gradually deplete the ER in individual β -cells. The protocol shown in Fig. 4A is similar to

Fig. 3. Store-operated influx of Mn^{2+} in response to carbachol in an individual β -cell. Pancreatic β -cells were loaded with fura-2 in hyperpolarizing medium containing 400 μM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+} . The same medium lacking indicator but containing 50 μM methoxyverapamil was present at the beginning of the experiment. The medium was then supplemented with 200 μM Mn^{2+} (upper bar) and 3.6 or 30 μM carbachol (Car; lower bars). The cytoplasmic Mn^{2+} concentration (A) is shown above $[\text{Ca}^{2+}]_i$ (B), which is not reliable after the introduction of Mn^{2+} (shaded area). The broken lines indicate the rate of change in Mn^{2+} concentration immediately after addition of Mn^{2+} (1) and after subsequent addition of 3.6 (2) and 30 μM carbachol (3). The results are representative of three independent experiments.

that used for carbachol in Fig. 2A except that extracellular Ca^{2+} was varied only between <1 nM and 1.28 mM. Like carbachol, CPA caused some increase of $[\text{Ca}^{2+}]_i$ in Ca^{2+} -deficient medium and a dose-dependent, more pronounced rise in the presence of extracellular cation. The latter effect was half-maximal and maximal at 1.94 ± 0.23 and 10 μM CPA, respectively, whereas 30 μM gave a slightly smaller response (Fig. 3B). Using influx of Mn^{2+} as measure of the store-operated pathway, 2 μM CPA accelerated the influx by $23.6 \pm 2.7\%$ ($n=5$) of the maximal activation obtained with 20 μM of the drug (Fig. 5A).

Gd^{3+} at a concentration of 1 μM has been found to inhibit the store-operated Ca^{2+} entry in a smooth muscle cell line without affecting vasopressin-stimulated influx of the ion (Broad et al., 1999). We now find that 1 μM Gd^{3+} does not interfere with mobilization of ER Ca^{2+} in response to 100 μM carbachol (Fig. 6A) or 50 μM CPA (Fig. 6B) in hyperpolarized β -cells exposed to Ca^{2+} -deficient medium. In accordance with

Fig. 4. Effect of CPA concentration on elevation of $[\text{Ca}^{2+}]_i$ due to store-operated influx of Ca^{2+} in individual β -cells. Pancreatic β -cells were loaded with fura-2 in hyperpolarizing medium containing 400 μM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+} . The same medium lacking indicator but containing 50 μM methoxyverapamil was present at the beginning of the representative experiment shown in A. The Ca^{2+} concentration was then changed between 1.28 (gray) and 0 (white; Ca^{2+} -free+2 mM EGTA), as indicated by the upper bars, and 0.3–30 μM CPA was introduced as shown by the lower bars. B shows the dose-response relationship for CPA-induced elevation of $[\text{Ca}^{2+}]_i$ in the presence of 1.28 mM Ca^{2+} . Means $\pm$ s.e.m. for 4–5 observations are shown. The solid line shows a fit of the 19 individual data points in the 0.3–10 μM CPA range to a logistic function ($r=0.984$; $P<0.0001$) and the broken line that the effect decreases at 30 μM CPA.

an inhibitory effect on store-operated Ca^{2+} influx, subsequent restoration of a physiological Ca^{2+} concentration (1.28 mM) in the continued presence of carbachol or CPA resulted in elevation of $[\text{Ca}^{2+}]_i$ only when Gd^{3+} was absent. When the store-operated influx in response to carbachol was monitored with Mn^{2+} , it was completely abolished by Gd^{3+} , which even reduced the basal Mn^{2+} influx (Fig. 7). In addition, Gd^{3+} was an effective blocker of the voltage-dependent rise of $[\text{Ca}^{2+}]_i$ in response to K^+ depolarization (Fig. 6C). Other experiments indicated that the effect of Gd^{3+} is not reversible and that 0.1–5 μM of this ion fails to discriminate between the store-operated and voltage-dependent entry of Ca^{2+} (data not shown).

Studies with the cell-permeable $\text{Ins}(1,4,5)\text{P}_3$ receptor inhibitor 2-APB have indicated that $\text{Ins}(1,4,5)\text{P}_3$ receptors are

Fig. 5. Store-operated influx of Mn^{2+} in response to CPA in an individual β -cell. Pancreatic β -cells were loaded with fura-2 in hyperpolarizing medium containing 400 μM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+} . The same medium lacking indicator but containing 50 μM methoxyverapamil was present at the beginning of the experiment. The medium was then supplemented with 400 μM Mn^{2+} (upper bar) and 2 or 20 μM CPA (lower bars) as indicated. The cytoplasmic Mn^{2+} concentration (A) is shown above $[\text{Ca}^{2+}]_i$ (B), which is not reliable after the introduction of Mn^{2+} (shaded area). The broken lines indicate the rate of change in Mn^{2+} concentration immediately after addition of Mn^{2+} (1) and after subsequent addition of 2 (2) and 20 μM CPA (3). The results are representative of five independent experiments.

important for activation and maintenance of store-operated Ca^{2+} entry by a mechanism other than merely emptying the ER (Maruyama et al., 1997; Ma et al., 2000; van Rossum et al., 2000). Using an experimental approach similar to that in Fig. 6A,B, we found that 2-APB diminishes not only intracellular Ca^{2+} mobilization and store-operated influx of the ion in response to the $\text{Ins}(1,4,5)\text{P}_3$ -elevating agonist carbachol (Fig. 8A), but also the store-operated influx induced by SERCA inhibition with CPA (Fig. 8B). The small increase in basal $[\text{Ca}^{2+}]_i$ upon introduction of 2-APB (Fig. 8A,B,D) might be caused by the slight SERCA inhibition (Maruyama et al., 1997). Fig. 9 indicates that 2-APB blocks the store-operated influx of Mn^{2+} in response to carbachol as well as the basal influx. Because $[\text{Mn}^{2+}]_i$ even seems to decrease in the presence of 2-APB, it is not excluded that extrusion of Mn^{2+} dominates under these conditions. Although the presently used concentration of 2-APB has been reported to block store-operated Ca^{2+} influx without effect on L-type Ca^{2+} channels

Fig. 6. Effect of Gd^{3+} on elevation of $[\text{Ca}^{2+}]_i$ due to store-operated and voltage-dependent influx of Ca^{2+} in individual β -cells. Pancreatic β -cells were loaded with fura-2 in hyperpolarizing medium containing 400 μM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+} (A,B), or in medium containing 3 mM glucose and 1.28 mM Ca^{2+} (C). The same medium lacking indicator but containing 50 μM methoxyverapamil was present at the beginning of the experiments shown in A and B, whereas there were no further additive in C. One μM Gd^{3+} was present as indicated by the upper bars. Ca^{2+} was omitted during the periods shown by the middle bars. The presence of 100 μM carbachol (Carb), 50 μM CPA and 31 mM K^+ are shown by the lower bars. The results are representative of seven (A) or five (B,C) independent experiments.

(Maruyama et al., 1997), we find a marked inhibitory action on the voltage-dependent rise of $[\text{Ca}^{2+}]_i$ obtained with K^+ depolarization (Fig. 8C,D). This effect is evidently not readily reversible, as there was only a modest temporary rise in $[\text{Ca}^{2+}]_i$ when omitting 2-APB during K^+ depolarization (Fig. 8C), and the initial response to K^+ depolarization was not fully restored even 8 minutes after 2-APB omission (Fig. 8D).

DISCUSSION

The presence of a store-operated or capacitative pathway was first suggested by Putney (Putney, 1990; Putney, 1986) and has since proved to be the most important mechanism for Ca^{2+} entry into non-excitable cells. In the excitable pancreatic β -cell, this mechanism seems to have only modest direct effects on $[\text{Ca}^{2+}]_i$ (Liu and Gylfe, 1997) but, by modulating the

Fig. 7. Gd^{3+} inhibits store-operated influx of Mn^{2+} in response to carbachol in an individual β -cell. Pancreatic β -cells were loaded with fura-2 in hyperpolarizing medium containing 400 μM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+} . The same medium lacking indicator but containing 50 μM methoxyverapamil was present at the beginning of the experiment. The medium was then supplemented with 200 μM Mn^{2+} (upper bar), 100 μM carbachol (Carb; middle bar) and 1 μM Gd^{3+} (lower bar). The cytoplasmic Mn^{2+} concentration (A) is shown above $[Ca^{2+}]_i$ (B), which is not reliable after the introduction of Mn^{2+} (shaded area). The broken lines indicate the rate of change in Mn^{2+} concentration immediately after addition of Mn^{2+} (1), after subsequent addition of 100 μM carbachol (2) and 1 μM Gd^{3+} (3). The results are representative of nine independent experiments.

membrane potential, store-operated fluxes of Ca^{2+} and Na^{+} may be significant for the more pronounced Ca^{2+} influx through the voltage-dependent channels (Worley et al., 1994; Bertram et al., 1995; Liu and Gylfe, 1997; Gilon et al., 1999). The molecular events coupling Ca^{2+} emptying of the ER to activation of Ca^{2+} influx have not yet been unequivocally identified (Putney, 1999). More than one mechanism may be involved, explaining why the Ca^{2+} influx is activated in an all-or-none fashion after almost complete emptying of the intracellular Ca^{2+} stores in some types of cells (Fierro and Parekh, 2000; Fierro et al., 2000), whereas there is gradual activation with increasing depletion of the stores in others (Hofer et al., 1998; Sedova et al., 2000). To date, it is not known how the store-operated Ca^{2+} entry depends on store filling in the pancreatic β -cell, although such knowledge is a

Fig. 8. Effect of 2-APB on elevation of $[Ca^{2+}]_i$ due to store-operated and voltage-dependent influx of Ca^{2+} in individual β -cells. Pancreatic β -cells were loaded with fura-2 in hyperpolarizing medium containing 400 μM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+} (A,B) or in medium containing 3 mM glucose and 1.28 mM Ca^{2+} (C,D). The same medium lacking indicator but containing 50 μM methoxyverapamil was present at the beginning of the experiments shown in A and B, whereas there was no further additive in C and D. 100 μM 2-APB was present as indicated by the upper bars. Ca^{2+} was omitted and 2 mM EGTA added during the periods shown by the middle bars. The presence of 100 μM carbachol (Carb), 50 μM CPA and 31 mM K^{+} are shown by the lower bars. The results are representative of five (A), six (B), four (C) or three (D) independent experiments.

prerequisite for current models attributing important functions to the store-operated pathway in the regulation of insulin release (Worley et al., 1994; Bertram et al., 1995; Liu and Gylfe, 1997; Gilon et al., 1999).

To selectively study the store-operated pathway in individual β -cells without interference from voltage-dependent Ca^{2+} entry we employed a previously developed technique (Gylfe, 1991; Liu and Gylfe, 1997). In this approach the β -cells are hyperpolarized with diazoxide, which activates the K_{ATP} channels (Trube et al., 1986). As an extra precaution, the medium was supplemented with methoxyverapamil, a voltage-dependent Ca^{2+} channel blocker lacking effects on the store-operated entry (Gylfe, 1991; Liu and Gylfe, 1997). Maximal filling of the $Ins(1,4,5)P_3$ -sensitive store of ER Ca^{2+} was ascertained by pre-exposure to 20 mM glucose (Gylfe, 1988; Gylfe, 1991; Tengholm et al., 1999), which was present throughout the experiments. In every cell we found that

Fig. 9. 2-APB inhibits store-operated influx of Mn^{2+} in response to carbachol in an individual β -cell. Pancreatic β -cells were loaded with fura-2 in hyperpolarizing medium containing 400 μM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+} . The same medium lacking indicator but containing 50 μM methoxyverapamil was present at the beginning of the experiment. The medium was then supplemented with 200 μM Mn^{2+} (upper bar), 100 μM carbachol (Carb; middle bar) and 100 μM 2-APB (lower bar). The cytoplasmic Mn^{2+} concentration (A) is shown above $[\text{Ca}^{2+}]_i$ (B), which is not reliable after the introduction of Mn^{2+} (shaded area). The broken lines indicate the rate of change in Mn^{2+} concentration immediately after addition of Mn^{2+} (1), after subsequent addition of 100 μM carbachol (2) and 100 μM 2-APB (3). The results are representative of ten independent experiments.

increasing concentrations of the Ca^{2+} -mobilizing carbachol cause gradual elevation of $[\text{Ca}^{2+}]_i$ depending on store-operated influx. In most experiments, one observation point was on the steepest part of the dose-response curve, contrary to what can be expected if the entry is regulated in an all-or-none fashion. Similar results were obtained with increasing concentrations of the SERCA inhibitor CPA, which empties the ER via a leakage pathway after inhibition of Ca^{2+} uptake. Unlike a previous study (Liu and Gylfe, 1997), we did not attempt to correlate the effects of carbachol and CPA on mobilization of intracellular Ca^{2+} with the magnitude of the store-operated influx. Such an approach requires separate experiments at each concentration to ascertain that the ER is completely filled when introducing the test substance.

Mn^{2+} quenching of the fura-2 fluorescence is a potent technique for more direct studies of fluxes through the voltage-

dependent (Dryselius et al., 1999) and store-operated (Liu and Gylfe, 1997) pathways in the β -cell. Because quenching exhibits a non-linear dependence on Mn^{2+} concentration, we introduced a novel approach calculating actual $[\text{Mn}^{2+}]_i$ levels from the quenching curve. In all situations studied, $[\text{Mn}^{2+}]_i$ increased linearly throughout the observation periods, although the rate varied depending on stimulation. The rate of increase can therefore be taken as a measure of influx with little interference from outward transport. Using this approach we found that 3.6 μM carbachol and 2 μM CPA, concentrations slightly higher than those giving half-maximal elevation of $[\text{Ca}^{2+}]_i$, induced only 30 and 24% activation of the store-operated influx, respectively. Consequently, there is no linear relationship between influx rate and elevation of $[\text{Ca}^{2+}]_i$. An explanation may be that, at high agonist concentrations, fura-2 in the submembrane space becomes saturated with Ca^{2+} resulting in underestimation of $[\text{Ca}^{2+}]_i$ and a left shift of the dose-response relationships.

Individual pancreatic β -cells respond to glucose with slow $[\text{Ca}^{2+}]_i$ oscillations with a frequency of 0.2–0.5/minute (Grapengiesser et al., 1988). Similar oscillations are observed in pancreatic islets but, within the islets, the β -cell response is dominated by about tenfold faster oscillations (Valdeolmillos et al., 1989; Bergsten et al., 1994; Gilon et al., 1994). It was previously shown that the fast oscillations depend on cAMP and that they can be transformed into slow oscillations by SERCA inhibition (Liu et al., 1998). Modeling the generation of the fast oscillatory pattern it has been suggested that release of Ca^{2+} from the ER causes a hyperpolarizing current, which shuts off the voltage-dependent entry of Ca^{2+} (Ämmälä et al., 1991; Liu et al., 1998; Dryselius et al., 1999). However, the associated emptying of the ER has been suggested to generate the fast oscillations by activating a depolarizing store-operated current (Worley et al., 1994; Bertram et al., 1995; Gilon et al., 1999). To discriminate between these seemingly inconsistent alternatives it would be valuable to have an inhibitor of the store-operated pathway, which does not affect mobilization of ER Ca^{2+} or voltage-dependent entry of the ion. Testing suggested inhibitors we found that Gd^{3+} and 2-APB lack the required Ca^{2+} channel specificity. The usefulness of 2-APB is limited because this $\text{Ins}(1,4,5)\text{P}_3$ receptor inhibitor will interfere with Ca^{2+} mobilization from the ER.

Taken together, this study provides the first evidence that the store-operated entry of Ca^{2+} into the β -cell exhibits a graded dependence on Ca^{2+} filling of the ER. Small variations in the ER Ca^{2+} concentration may consequently contribute to the regulation of the membrane potential and $[\text{Ca}^{2+}]_i$ determining insulin release.

This work was supported by grants from the Swedish Medical Research Council (12X-6240), the Swedish Foundation for Strategic Research, the Swedish Foundation for International Cooperation in Research and Higher Education, the Wenner-Gren Center Foundation, the Swedish Diabetes Association, Novo-Nordisk Foundation, Family Ernfors foundation, Åke Wiberg's Foundation and the Swedish Society for Medical Research.

REFERENCES

- Ämmälä, C., Larsson, O., Berggren, P. O., Bokvist, K., Juntti-Berggren, L., Kindmark, H. and Rorsman, P. (1991). Inositol trisphosphate-

- dependent periodic activation of a Ca^{2+} -activated K^+ conductance in glucose-stimulated pancreatic β -cells. *Nature* **353**, 849-852.
- Ashcroft, F. M. and Rorsman, P.** (1989). Electrophysiology of the pancreatic β -cell. *Progress in Biophysics and Molecular Biology* **54**, 87-143.
- Bergsten, P., Grapengiesser, E., Gylfe, E., Tengholm, A. and Hellman, B.** (1994). Synchronous oscillations of cytoplasmic Ca^{2+} and insulin release in glucose-stimulated pancreatic islets. *J. Biol. Chem.* **269**, 8749-8753.
- Bertram, R., Smolen, P., Sherman, A., Mears, D., Atwater, I., Martin, F. and Soria, B.** (1995). A role for calcium release-activated current (CRAC) in cholinergic modulation of electrical activity in pancreatic β -cells. *Biophys. J.* **68**, 2323-2332.
- Broad, L. M., Cannon, T. R. and Taylor, C. W.** (1999). A non-capacitative pathway activated by arachidonic acid is the major Ca^{2+} entry mechanism in rat A7r5 smooth muscle cells stimulated with low concentrations of vasopressin. *J. Physiol. (London)* **517**, 121-134.
- Chance, B., Legallais, V., Sorge, J. and Graham, N.** (1975). A versatile time-sharing multichannel spectrophotometer, reflectometer, and fluorometer. *Anal. Biochem.* **66**, 498-514.
- Dryselius, S., Grapengiesser, E., Hellman, B. and Gylfe, E.** (1999). Voltage-dependent entry and generation of slow Ca^{2+} oscillations in glucose-stimulated pancreatic β -cells. *Am. J. Physiol.* **276**, E512-E518.
- Fierro, L., Lund, P. E. and Parekh, A. B.** (2000). Comparison of the activation of the Ca^{2+} release-activated Ca^{2+} current I_{CRAC} to InsP_3 in Jurkat T-lymphocytes, pulmonary artery endothelia and RBL-1 cells. *Pflügers Archiv. Eur. J. Physiol.* **440**, 580-587.
- Fierro, L. and Parekh, A. B.** (2000). Substantial depletion of the intracellular Ca^{2+} stores is required for macroscopic activation of the Ca^{2+} release-activated Ca^{2+} current in rat basophilic leukaemia cells. *J. Physiol. (London)* **522**, 247-257.
- Gilon, P., Arredouani, A., Gailly, P., Gromada, J. and Henquin, J. C.** (1999). Uptake and release of Ca^{2+} by the endoplasmic reticulum contribute to the oscillations of the cytosolic Ca^{2+} concentration triggered by Ca^{2+} influx in the electrically excitable pancreatic B-cell. *J. Biol. Chem.* **274**, 20197-20205.
- Gilon, P., Jonas, J. C. and Henquin, J. C.** (1994). Culture duration and conditions affect the oscillations of cytoplasmic calcium concentration induced by glucose in mouse pancreatic islets. *Diabetologia* **37**, 1007-1014.
- Grapengiesser, E., Gylfe, E. and Hellman, B.** (1988). Glucose-induced oscillations of cytoplasmic Ca^{2+} in the pancreatic β -cell. *Biochem. Biophys. Res. Commun.* **151**, 1299-1304.
- Gryniewicz, G., Poenie, M. and Tsien, R. Y.** (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440-3450.
- Gylfe, E.** (1988). Nutrient secretagogues induce bimodal early changes in cytoplasmic calcium of insulin-releasing *ob/ob* mouse β -cells. *J. Biol. Chem.* **263**, 13750-13754.
- Gylfe, E.** (1991). Carbachol induces sustained glucose-dependent oscillations of cytoplasmic Ca^{2+} in hyperpolarized pancreatic β -cells. *Pflügers Archiv. Eur. J. Physiol.* **419**, 639-643.
- Gylfe, E. and Hellman, B.** (1986). Glucose-stimulated sequestration of Ca^{2+} in clonal insulin-releasing cells. Evidence for an opposing effect of muscarinic receptor activation. *Biochem. J.* **233**, 865-870.
- Hahn, H. J., Hellman, B., Lernmark, Å., Sehlin, J. and Täljedal, I. B.** (1974). The pancreatic β -cell recognition of insulin secretagogues. Influence of neuraminidase treatment on the release of insulin and the islet content of insulin, sialic acid, and cyclic adenosine 3':5'-monophosphate. *J. Biol. Chem.* **249**, 5275-5284.
- Hellman, B.** (1965). Studies in obese-hyperglycemic mice. *Ann. New York Acad. Sci.* **131**, 541-558.
- Hellman, B. and Gylfe, E.** (1986). Calcium and the control of insulin secretion. In *Calcium and Cell Function*. Vol. VI (ed. W.Y. Cheung), pp. 253-326. Orlando: Academic Press.
- Hellman, B., Gylfe, E. and Wesslén, N.** (1986). Inositol 1,4,5-trisphosphate mobilizes glucose-incorporated calcium from pancreatic islets. *Biochem. Int.* **13**, 383-389.
- Hofer, A. M., Fasolato, C. and Pozzan, T.** (1998). Capacitative Ca^{2+} entry is closely linked to the filling state of internal Ca^{2+} stores: A study using simultaneous measurements of I_{CRAC} and intraluminal $[\text{Ca}^{2+}]$. *J. Cell Biol.* **140**, 325-334.
- Kwan, C. Y. and Putney, J. W. J.** (1990). Uptake and intracellular sequestration of divalent cations in resting and methacholine-stimulated mouse lacrimal acinar cells. Dissociation by Sr^{2+} and Ba^{2+} of agonist-stimulated divalent cation entry from the refilling of the agonist-sensitive intracellular pool. *J. Biol. Chem.* **265**, 678-684.
- Lernmark, Å.** (1974). The preparation of, and studies on, free cell suspensions from mouse pancreatic islets. *Diabetologia* **10**, 431-438.
- Liu, Y. J., Grapengiesser, E., Gylfe, E. and Hellman, B.** (1996). Crosstalk between the cAMP and inositol trisphosphate signalling pathways in pancreatic β -cells. *Arch. Biochem. Biophys.* **334**, 295-302.
- Liu, Y. J. and Gylfe, E.** (1997). Store-operated Ca^{2+} entry in insulin-releasing pancreatic β -cells. *Cell Calcium* **22**, 277-286.
- Liu, Y. J., Tengholm, A., Grapengiesser, E., Hellman, B. and Gylfe, E.** (1998). Origin of slow and fast oscillations of Ca^{2+} in mouse pancreatic islets. *J. Physiol. (London)* **508**, 471-481.
- Ma, H. T., Patterson, R. L., van Rossum, D. B., Birnbaumer, L., Mikoshiba, K. and Gill, D. L.** (2000). Requirement of the inositol trisphosphate receptor for activation of store-operated Ca^{2+} channels. *Science* **287**, 1647-1651.
- Maechler, P., Kennedy, E. D., Sebö, E., Valeva, A., Pozzan, T. and Wollheim, C. B.** (1999). Secretagogues modulate the calcium concentration in the endoplasmic reticulum of insulin-secreting cells. *J. Biol. Chem.* **274**, 12583-12592.
- Maruyama, T., Kanaji, T., Nakade, S., Kanno, T. and Mikoshiba, K.** (1997). 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release. *J. Biochem.* **122**, 498-505.
- Putney, J. W. J.** (1986). A model for receptor-regulated calcium entry. *Cell Calcium* **7**, 1-12.
- Putney, J. W. J.** (1990). Capacitative calcium entry revisited. *Cell Calcium* **11**, 611-624.
- Putney, J. W. J.** (1999). 'Kissin' cousins': intimate plasma membrane-ER interactions underlie capacitative calcium entry. *Cell* **99**, 5-8.
- Roe, M. W., Lancaster, M. E., Mertz, R. J., Worley, J. F. I. and Dukes, I. D.** (1993). Voltage-dependent intracellular calcium release from mouse islets stimulated by glucose. *J. Biol. Chem.* **268**, 9953-9956.
- Sedova, M., Klishin, A., Hüser, J. and Blatter, L. A.** (2000). Capacitative Ca^{2+} entry is graded with degree of intracellular Ca^{2+} store depletion in bovine vascular endothelial cells. *J. Physiol. (London)* **523**, 549-559.
- Tengholm, A., Hagman, C., Gylfe, E. and Hellman, B.** (1998). *In situ* characterization of non mitochondrial Ca^{2+} stores in individual pancreatic β -cells. *Diabetes* **47**, 1224-1230.
- Tengholm, A., Hellman, B. and Gylfe, E.** (1999). Glucose regulation of free Ca^{2+} in the endoplasmic reticulum of mouse pancreatic beta cells. *J. Biol. Chem.* **274**, 36883-36890.
- Tengholm, A., Hellman, B. and Gylfe, E.** (2000). Mobilisation of Ca^{2+} stores in individual pancreatic β -cells permeabilised or not with digitonin or α -toxin. *Cell Calcium* **27**, 43-51.
- Trube, G., Rorsman, P. and Ohno-Shosaku, T.** (1986). Opposite effects of tolbutamide and diazoxide on the ATP-dependent K^+ channel in mouse pancreatic β -cells. *Pflügers Archiv. Eur. J. Physiol.* **407**, 493-499.
- Valdeolmillos, M., Santos, R. M., Contreras, D., Soria, B. and Rosario, L. M.** (1989). Glucose-induced oscillations of intracellular Ca^{2+} concentration resembling bursting electrical activity in single mouse islets of Langerhans. *FEBS Lett.* **259**, 19-23.
- van Rossum, D. B., Patterson, R. L. and Gill, D. L.** (2000). Ca^{2+} entry mediated by store depletion, S-nitrosylation, and TRP3 channels. Comparison of coupling and function. *J. Biol. Chem.* **275**, 28562-28568.
- Wollheim, C. B. and Sharp, G. W. G.** (1981). Regulation of insulin release by calcium. *Physiol. Rev.* **61**, 914-973.
- Worley, J. F. I., McIntyre, M. S., Spencer, B., Mertz, R. J., Roe, M. W. and Dukes, I. D.** (1994). Endoplasmic reticulum calcium store regulates membrane potential in mouse islet β -cells. *J. Biol. Chem.* **269**, 14359-14362.
- Zhou, Z. and Neher, E.** (1993). Mobile and immobile calcium buffers in bovine adrenal chromaffin cells. *J. Physiol. (London)* **469**, 245-273.