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

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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+} ($[Ca^{2+}]_i$) and Mn^{2+} ($[Mn^{2+}]_i$) 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 $[Ca^{2+}]_i$ or from the rate of increase in $[Mn^{2+}]_i$ due to influx of these extracellular ions. Increasing concentrations of the inositol 1,4,5-trisphosphate-generating agonist carbachol or the sarco(endo)plasmatic 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 Ca^{2+}/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 ($[Ca^{2+}]_i$) 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 ($Ins(1,4,5)P_3$) (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 $[Ca^{2+}]_i$ directly, the intracellular release has indirect actions resulting from changes in membrane potential. The $Ins(1,4,5)P_3$-induced rise of $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 (Lernmark, 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 μg/ml streptomycin and 30 μg/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±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 $[Ca^{2+}]_i$ and $[Mn^{2+}]_i$ (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× 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.

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

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

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

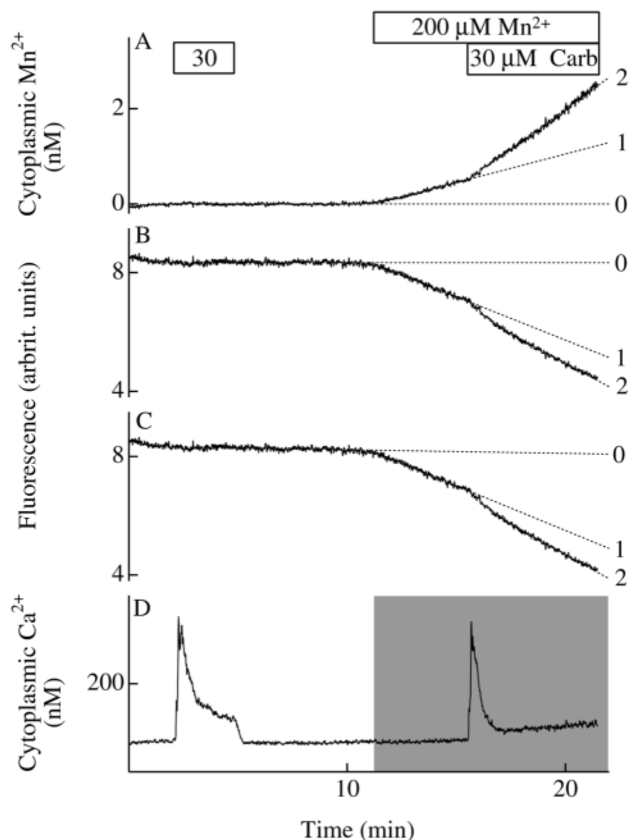

Fig. 1. Estimation of store-operated Mn^{2+} influx in an individual β-cell. The pancreatic β-cell was loaded with fura-2 in hyperpolarizing medium containing 400 μM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+}. The same medium lacking indicator but containing 50 μM methoxyverapamil was present at the 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 $[Ca^{2+}]_i$ (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 ($[Mn^{2+}]_i$). 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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), $[Mn^{2+}]_i$ can be calculated in analogy to the method previously described (Grynkiewicz et al., 1985) using Equation 2:

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

$K_D^{Mn^{2+}}$ 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 $[Mn^{2+}]_i$ upon introduction of the ion (broken line 1) and acceleration of this effect after stimulation with carbachol (broken line 2). Although the apparent $K_D^{Mn^{2+}}$ may be expected to change slightly with the Ca^{2+} concentration, we found no evidence for such interference because $[Ca^{2+}]_i$ peaks occurred without fluctuations in the Mn^{2+} signal.

Presentation of data and statistical analysis

Results are presented as means±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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ being quite pronounced. Fig. 2B shows the dose-response relationship for carbachol-induced elevation of $[Ca^{2+}]_i$ 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 $[Mn^{2+}]_i$ in the hyperpolarized and glucose-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 $[Mn^{2+}]_i$ 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±9.5% of the maximal activation obtained with 30 μM of the drug.

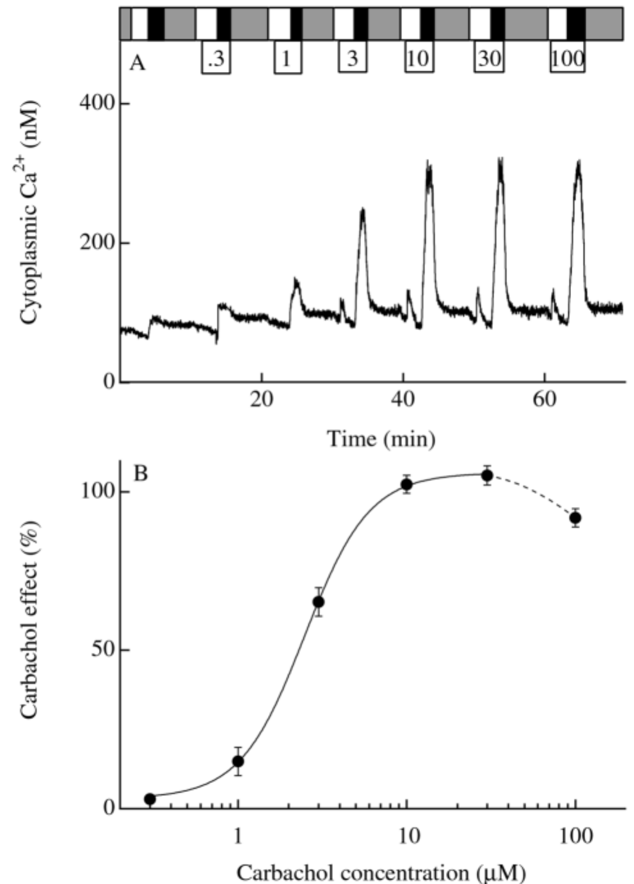

Fig. 2. Effect of carbachol concentration on elevation of $[Ca^{2+}]_i$ due to store-operated influx of Ca^{2+} in individual β-cells. Pancreatic β-cells were loaded with fura-2 in hyperpolarizing medium containing 400 μM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+}. The same medium lacking indicator 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 $[Ca^{2+}]_i$ in the presence of 10 mM Ca^{2+}. A singe observation is shown at 0.3 μM carbachol and means±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.

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

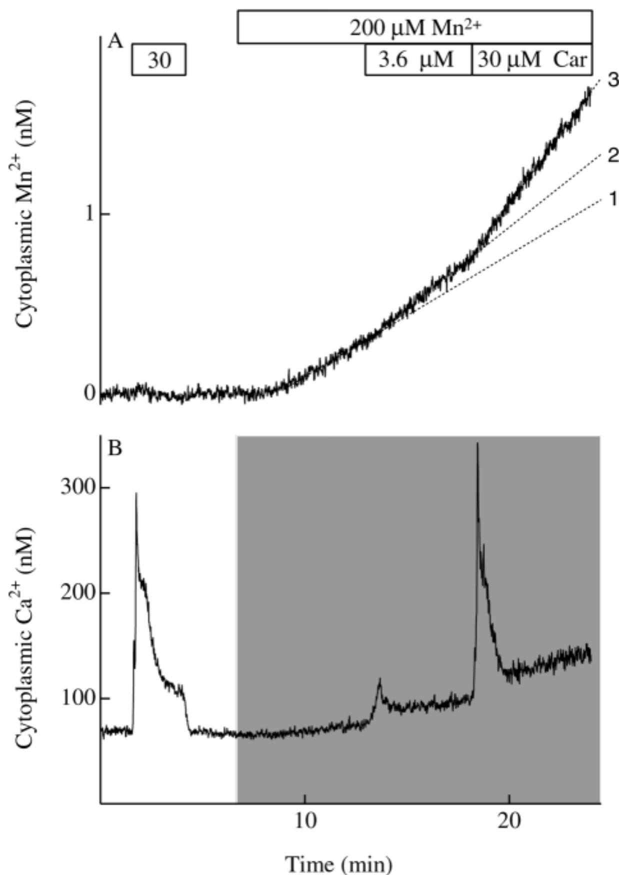

Fig. 3. Store-operated influx of Mn^{2+} in response to carbachol in an individual β-cell. Pancreatic β-cells were loaded with fura-2 in hyperpolarizing medium containing 400 µM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+}. The same medium lacking indicator but containing 50 µM methoxyverapamil was present 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 $[Ca^{2+}]_i$ (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.

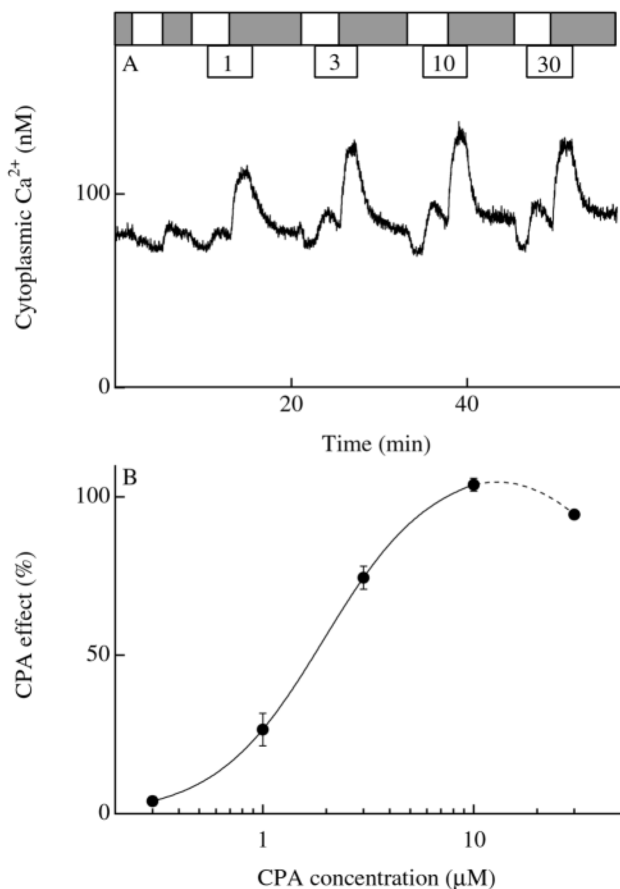

Fig. 4. Effect of CPA concentration on elevation of $[Ca^{2+}]_i$ due to store-operated influx of Ca^{2+} in individual β-cells. Pancreatic β-cells were loaded with fura-2 in hyperpolarizing medium containing 400 µM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+}. The same medium lacking indicator but containing 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 $[Ca^{2+}]_i$ in the presence of 1.28 mM Ca^{2+}. Means±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.

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 $[Ca^{2+}]_i$ 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±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 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $Ins(1,4,5)P_3$ receptor inhibitor 2-APB have indicated that $Ins(1,4,5)P_3$ receptors are

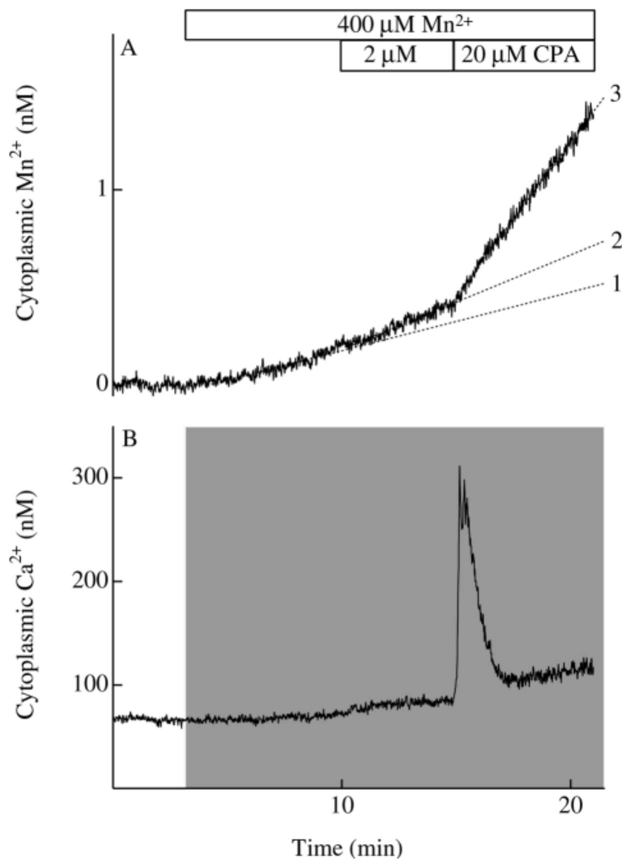

Fig. 5. Store-operated influx of Mn^{2+} in response to CPA in an individual β-cell. Pancreatic β-cells were loaded with fura-2 in hyperpolarizing medium containing 400 μM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+}. The same medium lacking indicator but containing 50 μM methoxyverapamil was present 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 $[Ca^{2+}]_i$ (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.

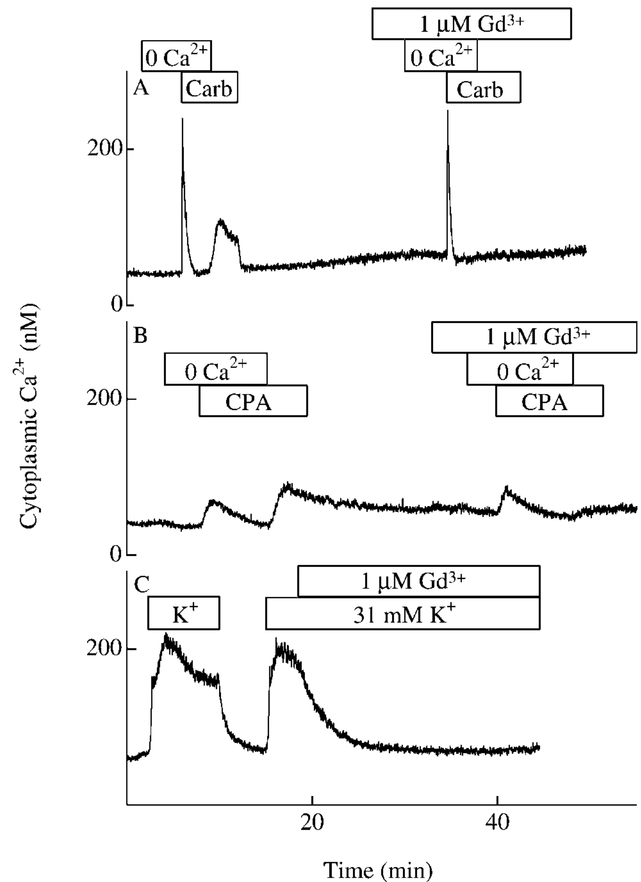

Fig. 6. Effect of Gd^{3+} on elevation of $[Ca^{2+}]_i$ due to store-operated and voltage-dependent influx of Ca^{2+} in individual β-cells. Pancreatic β-cells were loaded with fura-2 in hyperpolarizing medium containing 400 μM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+} (A,B), or in medium containing 3 mM glucose and 1.28 mM Ca^{2+} (C). 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.

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 Ins(1,4,5)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 $[Ca^{2+}]_i$ 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 $[Mn^{2+}]_i$ 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 (Maruyama et al., 1997), we find a marked inhibitory action on the voltage-dependent rise of $[Ca^{2+}]_i$ obtained with K^+ depolarization (Fig. 8C,D). This effect is evidently not readily reversible, as there was only a modest temporary rise in $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ (Liu and Gylfe, 1997) but, by modulating the

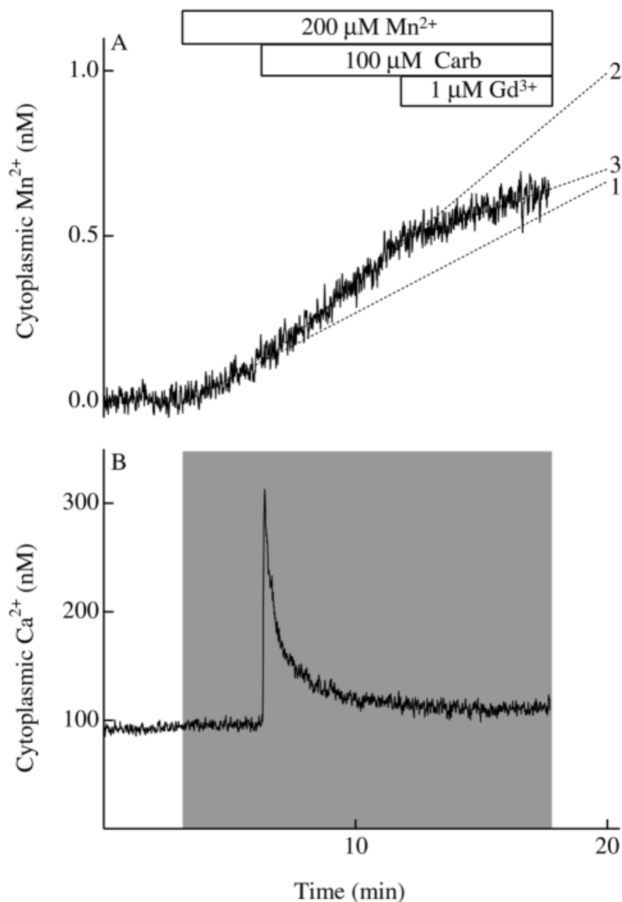

Fig. 7. Gd^{3+} inhibits store-operated influx of Mn^{2+} in response to carbachol in an individual β-cell. Pancreatic β-cells were loaded with fura-2 in hyperpolarizing medium containing 400 μM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+}. The same medium lacking indicator but containing 50 μM methoxyverapamil 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.

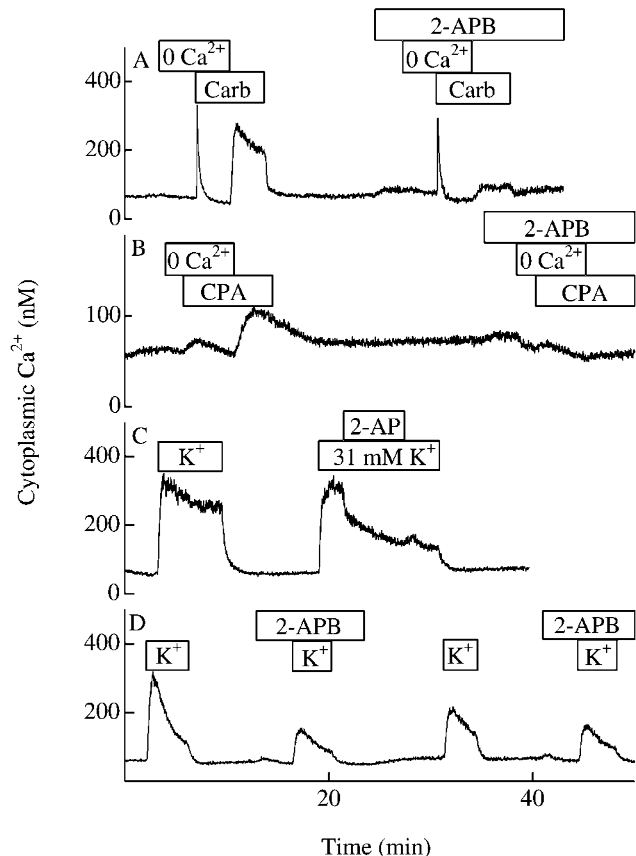

Fig. 8. Effect of 2-APB on elevation of $[Ca^{2+}]_i$ due to store-operated and voltage-dependent influx of Ca^{2+} in individual β-cells. Pancreatic β-cells were loaded with fura-2 in hyperpolarizing medium containing 400 μM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+} (A,B) or in medium containing 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.

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 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

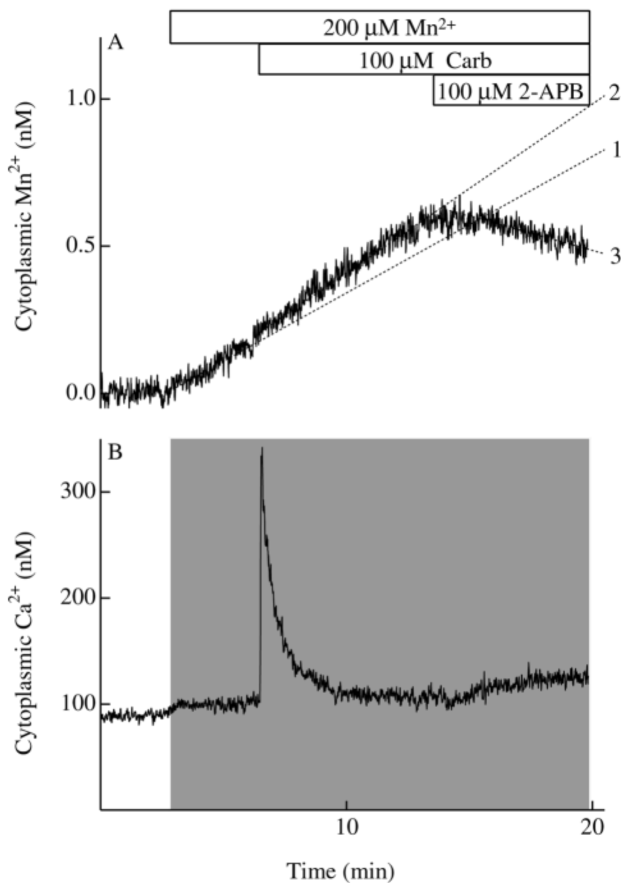

Fig. 9. 2-APB inhibits store-operated influx of Mn^{2+} in response to carbachol in an individual β-cell. Pancreatic β-cells were loaded with fura-2 in hyperpolarizing medium containing 400 µM diazoxide, 20 mM glucose and 1.28 mM Ca^{2+}. The same medium lacking indicator but containing 50 µM methoxyverapamil 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 $[Ca^{2+}]_i$ (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 $[Ca^{2+}]_i$ 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 $[Mn^{2+}]_i$ levels from the quenching curve. In all situations studied, $[Mn^{2+}]_i$ 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 $[Ca^{2+}]_i$, induced only 30 and 24% activation of the store-operated influx, respectively. Consequently, there is no linear relationship between influx rate and elevation of $[Ca^{2+}]_i$. An explanation may be that, at high agonist concentrations, fura-2 in the submembrane space becomes saturated with Ca^{2+} resulting in underestimation of $[Ca^{2+}]_i$ and a left shift of the dose-response relationships.

Individual pancreatic β-cells respond to glucose with slow $[Ca^{2+}]_i$ 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 Ins(1,4,5)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 $[Ca^{2+}]_i$ determining insulin release.

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