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Secretagogues Modulate the Calcium Concentration in the Endoplasmic Reticulum of Insulin-secreting Cells

STUDIES IN AEQUORIN-EXPRESSING INTACT AND PERMEABILIZED INS-1 CELLS*

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The precise regulation of the Ca^{2+} concentration in the endoplasmic reticulum ($[Ca^{2+}]_{er}$) is important for protein processing and signal transduction. In the pancreatic β-cell, dysregulation of $[Ca^{2+}]_{er}$ may cause impaired insulin secretion. The Ca^{2+}-sensitive photoprotein aequorin mutated to lower its Ca^{2+} affinity was stably expressed in the endoplasmic reticulum (ER) of rat insulinoma INS-1 cells. The steady state $[Ca^{2+}]_{er}$ was 267 ± 9 μM. Both the Ca^{2+}-ATPase inhibitor cyclopiazonic acid and 4-chloro-*m*-cresol, an activator of ryanodine receptors, caused an almost complete emptying of ER Ca^{2+}. The inositol 1,4,5-trisphosphate generating agonists, carbachol, and ATP, reduced $[Ca^{2+}]_{er}$ by 20–25%. Insulin secretagogues that raise cytosolic $[Ca^{2+}]$ by membrane depolarization increased $[Ca^{2+}]_{er}$ in the potency order $K^+ \gg$ glucose > leucine, paralleling their actions in the cytosolic compartment. Glucose, which augmented $[Ca^{2+}]_{er}$ by about 25%, potentiated the Ca^{2+}-mobilizing effect of carbachol, explaining the corresponding observation in cytosolic $[Ca^{2+}]$. The filling of ER Ca^{2+} by glucose is not directly mediated by ATP production as shown by the continuous monitoring of cytosolic ATP in luciferase expressing cells. Both glucose and K^+ increase $[Ca^{2+}]_{er}$, but only the former generated whereas the latter consumed ATP. Nonetheless, drastic lowering of cellular ATP with a mitochondrial uncoupler resulted in a marked decrease in $[Ca^{2+}]_{er}$, emphasizing the requirement for mitochondrially derived ATP above a critical threshold concentration. Using α-toxin permeabilized cells in the presence of ATP, glucose 6-phosphate did not change $[Ca^{2+}]_{er}$, invalidating the hypothesis that glucose acts through this metabolite. Therefore, insulin secretagogues that primarily stimulate Ca^{2+} influx, elevate $[Ca^{2+}]_{er}$ to ensure β-cell homeostasis.

Increases in cytosolic calcium concentration ($[Ca^{2+}]_c$)[1] accompany diverse known cellular responses including hormone secretion. Ca^{2+} originates from two main sources, one of which involves the entry of Ca^{2+} from the extracellular space into the cell via Ca^{2+} channels in the plasma membrane. The other that has generated much interest in the last decade revolves around the endoplasmic reticulum (ER), which plays a pivotal role in the regulation of $[Ca^{2+}]_c$ (1, 2).

One of the better understood pathways concerning Ca^{2+} release and uptake by the ER involves the application of hormones or neurotransmitters that bind to cell surface receptors activating the phospholipase C-mediated hydrolysis of polyphosphatidylinositol lipids on the inner surface of the plasma membrane (3, 4). This leads to the production of inositol 1,4,5-trisphosphate ($InsP_3$), the second messenger directly responsible for the release of Ca^{2+} from the ER that occurs via the stimulation of $InsP_3$ receptors situated in the ER membrane (1). Another mechanism by which Ca^{2+} is released from the ER implicates the ryanodine receptor family. This process is activated by several putative second messengers including Ca^{2+} itself (5). The filled state and the reuptake of mobilized Ca^{2+} by the ER is mediated by a Ca^{2+}-ATPase of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) type, which pumps Ca^{2+} back into the ER against the ionic gradient (6).

The Ca^{2+} concentration within the ER ($[Ca^{2+}]_{er}$) has been variously estimated to be anywhere from the low micromolar to the high millimolar range depending upon the cell type and the method of detection (7–16). The more widely used fluorescent Ca^{2+} probes such as fura2 fail to segregate specifically into intracellular organelles, and those that do localize to the ER compartment are rapidly saturated, indicating that there is a relatively high resting $[Ca^{2+}]_{er}$ (17). Other groups have reported the use of low affinity indicators to measure $[Ca^{2+}]_{er}$ including mag-fura2 and mag-indo1 (18–20). Again, however, due to calibration irregularities, it is difficult to measure the free lumenal $[Ca^{2+}]_{er}$ with certainty. This approach is best suited for permeabilized cells or patch-clamped cells from which the cytosolic indicator can be dialyzed (21) but has also been used successfully in intact cells (20).

By exploiting the recombinant aequorin photoprotein technology it has become possible to target aequorin directly to the ER, which overcomes this problem and allows selective Ca^{2+} measurements to be made. This targeting is achieved by incorporating part of the immunoglobulin Igγ2b heavy chain gene

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[1] The abbreviations used are: $[Ca^{2+}]_c$, cytosolic calcium concentration; $[Ca^{2+}]_{er}$, endoplasmic reticulum calcium concentration; CPA, cyclopiazonic acid; ER, endoplasmic reticulum; erAEQmut, mutated ER aequorin; FCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; $InsP_3$, inositol 1,4,5-trisphosphate; KRBH, Krebs-Ringer bicarbonate-Hepes buffer; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase.

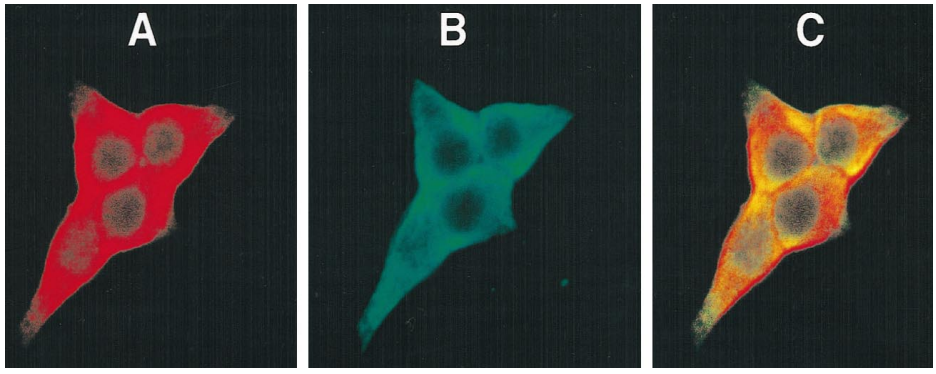

FIG. 1. **Co-localization of ER-targeted aequorin with the ER lumenal protein, calreticulin, by immunofluorescence confocal microscopy**. In *A* the aequorin immunofluorescence was detected with a primary antibody directed against the hemagglutinin epitope incorporated into the aequorin construct and visualized in the rhodamine fluorescence channel (red). In *B* the immunofluorescence from the ER lumenal protein calreticulin was detected in the fluorescein isothiocyanate fluorescence channel (*green*). *C* shows in *orange-yellow* the regions of virtual co-localization of the two antigens.

upstream of the aequorin cDNA as described by Montero *et al.* (10). Furthermore, this targeting is specific to the ER due to the interaction between the immunoglobulin heavy chain domain CH1 and the ER lumenal protein, BiP. In B lymphocytes and plasma cells this interaction will be dissociated by the arrival of the immunoglobulin light chain, but in cells that do not synthesize immunoglobulin this retention can be expected to remain unperturbed. To allow measurements in a high $[Ca^{2+}]$ compartment, a mutated ER-targeted aequorin was constructed that introduced a point mutation into the protein. This $Asp^{119} \rightarrow Ala$ mutation, previously shown to reduce the Ca^{2+} affinity of the photoprotein (22), effectively shifts the detectable $[Ca^{2+}]$ from the 0.1 to 10 μM range to the submillimolar range anticipated in the ER. Furthermore, Montero *et al.* (12) and Barrero *et al.* (14) have introduced the use of an altered prosthetic group to facilitate the use of Ca^{2+} rather than the Ca^{2+} surrogate, Sr^{2+}, in such experiments. Coelenterazine n displays lower oxidation rates than the wild type following Ca^{2+} binding to apoaequorin.

It has been reported previously that the $[Ca^{2+}]_{er}$ plays a pivotal role in the functioning of the pancreatic β-cell. As in other cell types, $InsP_3$-generating receptor agonists mobilize Ca^{2+} from the ER (23). The pancreatic β-cells also express the ryanodine receptor, as low levels of mRNA for the type 2 receptor were reported recently (24). Miura *et al.* (25) have demonstrated a small capacitative entry into murine β-cells facilitated by the emptying of intracellular Ca^{2+} stores. Moreover, studies on the proteolytic processing and intracellular transport of proinsulin and prohormone convertases in isolated rat pancreatic islets suggest that the Ca^{2+} required for these events originates from the ER (26). It has been proposed that the nutrient secretagogue, glucose, elicits Ca^{2+} sequestration by the ER in experiments using $^{45}Ca^{2+}$ (Refs. 27 and references therein). In addition, the muscarinic receptor agonist, carbachol, and its second messenger $InsP_3$, caused greater Ca^{2+} mobilization in broken cells (27) and a higher $[Ca^{2+}]_c$ rise in β-cells pretreated with high glucose concentrations compared with low glucose concentrations (28, 29). However, direct measurements of fluctuations of $[Ca^{2+}]_{er}$ during the stimulation of insulin secretion are not yet available.

We have used an INS-1 cell line stably expressing the ER-targeted aequorin to study directly the effects of agents that stimulate Ca^{2+} influx such as glucose, leucine, another nutrient secretagogue, and depolarizing concentrations of KCl. These effects were compared with those of the $InsP_3$-producing agonists carbachol and ATP. The INS-1 cell line used throughout this study is morphologically similar to the native pancreatic β-cell sharing enzymatic and secretory profiles with the primary cells (30–32). We show here that INS-1 cells stably expressing high levels of mutated ($Asp^{119} \rightarrow Ala$) aequorin targeted to the ER retain glucose-induced insulin secretion and provide information on the regulation of $[Ca^{2+}]_{er}$ in this secretory cell system.

EXPERIMENTAL PROCEDURES

Cell Culture—INS-1 cells were cultured in RPMI 1640 medium including 10% fetal calf serum and additions as described previously (30, 32). Stable clones were made according to Kennedy *et al.* (32). Briefly, 10 μg of mutated ER aequorin (erAEQmut)/pcDNA1 (see Introduction and Ref. 10) and 2 μg pSV2neo were transfected into INS-1 cells set on a 10-cm Petri dish. Selection of clones stably expressing erAEQmut was made after several days with 0.4 μg/ml G418. The highest expresser, ER#18, was maintained in medium containing 0.25 μg/ml G418. For the luciferase-expressing cell line, the transfection was done in two separate steps using the Ca^{2+}-phosphate-DNA co-precipitation method as outlined in Maechler *et al.* (33). Measurement of the resultant luminescence was made as described previously (33).

Aequorin Measurements—Cells were seeded at a density of 4×10^5 cells/ml on 13-mm polyornithine-coated coverslips and kept for 2–3 days prior to the experiments. Before the measurement of divalent cation in the ER could be made, the ER store had to be depleted of Ca^{2+} as described previously (10, 14). This was done by a series of incubations as follows: (i) cells were washed twice briefly in Ca^{2+}-free KRBH (135 mM NaCl, 3.6 mM KCl, 2 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 2.8 mM glucose, and 10 mM Hepes, pH 7.4); (ii) cells were then incubated for 5 min in Ca^{2+}-free KRBH containing 3 mM EGTA; (iii) following this, the ER stores were emptied for 5 min in Ca^{2+}-free KRBH containing 3 mM EGTA and 10 μM cyclopiazonic acid (CPA; Sigma) which, like thapsigargin, acts as an inhibitor of the Ca^{2+}-ATPase; (iv) finally, the erAEQmut was reconstituted with 5 μM coelenterazine n (Molecular Probes, Amsterdam, The Netherlands) in Ca^{2+}-free KRBH containing 0.1 mM EGTA and 10 μM CPA for approximately 60 min. Coelenterazine is the prosthetic group for apoaequorin (34), and the coelenterazine n synthetic analog displays significantly reduced Ca^{2+} sensitivity (12).

For the permeabilized cell experiments, cells were plated on extracellular matrix-coated coverslips as described by Maechler *et al.* (33). The ER Ca^{2+} stores were emptied as above, and the erAEQmut was reconstituted with coelenterazine n. Following this procedure, cells were permeabilized with *Staphylococcus* α-toxin (1 μg/4–5 $\times 10^5$ cells) for 8 min. As in our previous studies (33, 35) the cells were perifused with an intracellular type buffer adjusted to approximately 100 nM free calcium (140 mM KCl, 5 mM NaCl, 7 mM $MgSO_4$, 20 mM Hepes, pH 7.0, 10 mM ATP, 10.2 mM EGTA, 1.65 mM $CaCl_2$), 500 nM free calcium (*idem* except 6.67 mM $CaCl_2$), and 1.3 μM free calcium (*idem* except 10.0 mM $CaCl_2$).

For both intact and permeabilized cells, the coverslips were placed in a thermostatted chamber at 37 °C 5 mm from the photomultiplier apparatus (model EMI 9789, Thorn EMI Electron Tubes Ltd., Middlesex, UK) and perifused constantly at a rate of 1 ml/min in the appropriate buffer. In the case of intact cells the perifusion conditions were changed sequentially as follows. First, cells were exposed to continued 0.1 mM EGTA and 10 μM CPA. After 100 s, the CPA was

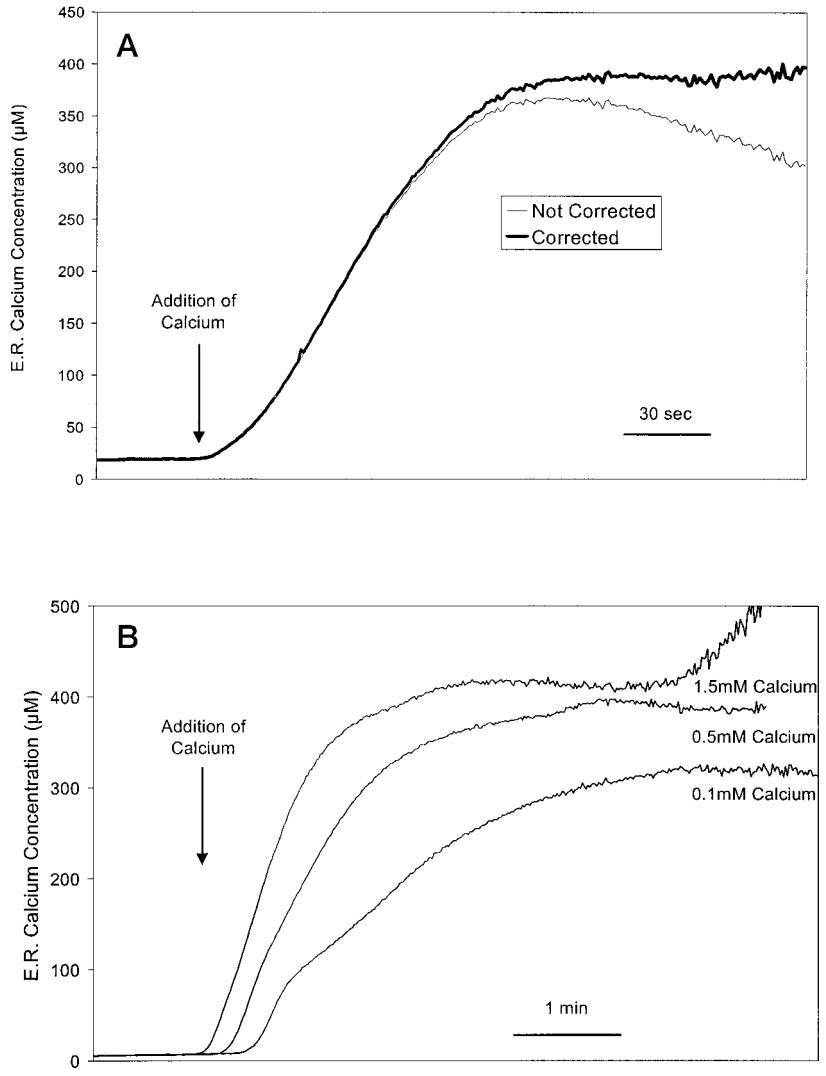

FIG. 2. **Calibration and dependence on extracellular Ca^{2+} of the steady state $[Ca^{2+}]_{er}$ in INS-1 cells.** *A* shows the effect of removing the last 3% of the data points collected (*thick line*) compared with no correction (*thin line*). *B* shows the dependence of the corrected steady state $[Ca^{2+}]_{er}$ on the concentration of extracellular Ca^{2+}. The *traces* are representative of three independent experiments.

removed, and the perifusion proceeded in the absence of CPA for a further 5 min to ensure complete removal of the Ca^{2+}-ATPase inhibitor. Calcium was then added to the cells in a stepwise manner commencing in 0.1 mM Ca^{2+} for 60 s before increasing the concentration to 1.5 mM. This pre-exposure to low calcium concentrations was found to slow the consumption of aequorin (possibly because it prevents an overshoot in $[Ca^{2+}]_c$ during Ca^{2+} repletion) and thereby to facilitate a prolongation of the steady state in $[Ca^{2+}]_{er}$. Data were collected every second with a photon counting board (EMI 660) and calibrated according to Montero *et al.* (11, 12). It has previously been reported that 1–5% of erAEQmut protein is missorted, which biases the calibrated $[Ca^{2+}]_{er}$ (11). Therefore, 2–4% of the total photon emission at the end of each trace was subtracted from the cumulative total before calibration.

Immunofluorescence Studies—ER#18 cells were plated at a density of 50×10^3/ml on polyornithine-coated glass. For immunofluorescence studies, cells were fixed in 4% paraformaldehyde and permeabilized as described previously (32). The 12CA5 antibody that recognizes the hemagglutinin tag (10, 32) incorporated in the erAEQmut construct was kindly donated by Dr. S. Arkinstall (Ares Serono, Geneva, Switzerland). The antibody raised against the ER lumenal protein, calreticulin, was kindly donated by Dr. M. Michalek (Toronto, Canada). All other antibodies were from Pierce. Immunofluorescence was observed using a Zeiss laserscan 460 confocal microscope.

Insulin Secretion Measurements—Cells were seeded at a density of 4×10^5 in 24-well plates and allowed to settle for 2–3 days. Cellular Ca^{2+} stores were then emptied according to the above protocol where necessary. All cells were then preincubated for 30 min in KRBH as detailed above and subsequently stimulated for 15 min. Rat insulin in buffers and in acid-ethanol extracts of cells was measured by radioimmunoassay using rat insulin as standard (30) and an anti-insulin antibody from Linco (St. Louis, MO).

Statistical Analyses—Values are given as the means ± S.E., and the significance of differences was assessed by Student's *t* test for unpaired data except for rises in $[Ca^{2+}]_{er}$ in Fig. 4, where the paired test was applied.

RESULTS

Using an insulin-secreting INS-1 cell line that stably expresses aequorin targeted to the ER, we have studied the effects of several agents on the filling and the release of Ca^{2+} from this organelle. Thirty-three G418 resistant INS-1 cell clones were kept and of these twenty-seven expressed aequorin, nine of which displayed high expression levels. The clone ER#18, which contained the highest level of aequorin, had a total photon emission of 4.5×10^6 counts/coverslip and was used throughout the study. This gives an approximate concentration of 4.4 ng of aequorin/10^6 cells or 1 μl of cell volume. Taking the ER as 10% of the cell volume this results in a concentration of approximately 2 μM in this organelle, a level not expected to affect $[Ca^{2+}]_{er}$ by buffering the ion. Indeed, transient transfection protocols that yield variable expression levels in HeLa cells result in negligible variations of steady state $[Ca^{2+}]_{er}$ (36).

Fig. 1*A* shows the immunolocalization of the hemagglutinin epitope-tagged aequorin expressed in the ER using an anti-hemagglutinin antibody. The pattern of staining is identical to that observed when the cells were exposed to anti-calreticulin antibody (Fig. 1*B*). This protein is known to be located in the lumen of the ER (37). Fig. 1*C* reveals the co-localization of the two proteins. Using this technique, no obvious missorting of the

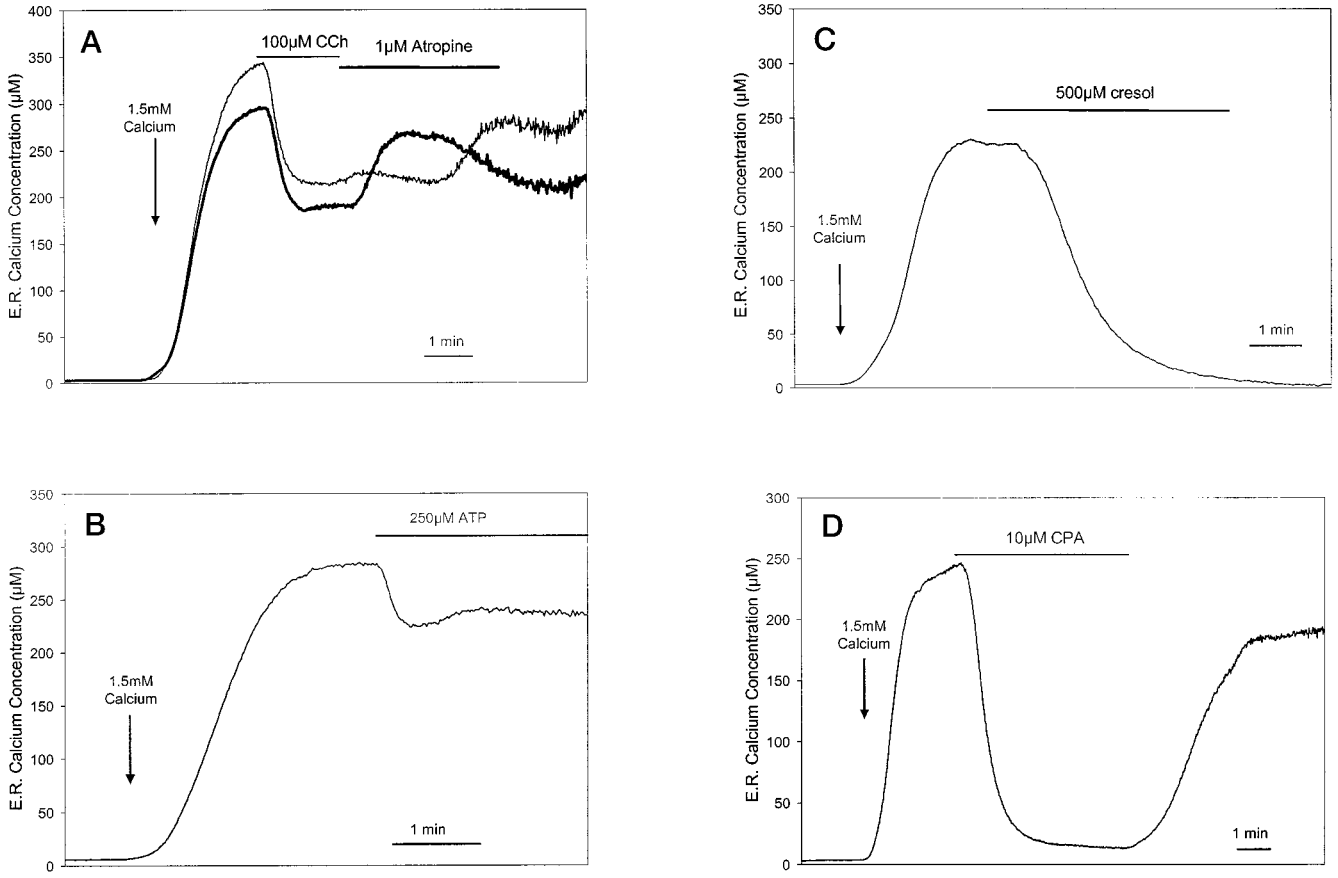

FIG. 3. **Effects of Ca^{2+}-mobilizing receptor agonists and of the SERCA inhibitor CPA on $[Ca^{2+}]_{er}$ in INS-1 cells.** *A*, time course of emptying and refilling of the ER Ca^{2+} store with carbachol (*CCh*, 100 μM) alone or following displacement with the muscarinic receptor antagonist, atropine (1 μM). *B*, $[Ca^{2+}]_{er}$ after the addition of 250 μM ATP. *C*, effect of 4-chloro-*m*-cresol (*cresol*, 500 μM) on $[Ca^{2+}]_{er}$. *D*, emptying of the ER Ca^{2+} stores following the addition of the SERCA inhibitor, CPA (10 μM). The *traces* are representative of at least three independent experiments.

ER targeted aequorin to other cellular compartments could be detected.

The calibrated $[Ca^{2+}]_{er}$ values shown in Fig. 2*A* indicate, however, that a minor degree of missorting does occur. In particular upon readdition of Ca^{2+} to depleted cells, the apparent kinetics of Ca^{2+} concentration in the ER lumen (Fig. 2*A*, *thin line*) was biphasic, the rapid uptake being followed by a slow decrease that eventually reached levels similar to those of depleted cells. This kinetic behavior was observed previously by Montero *et al.* (11) and was shown to be due to a small (1–5%) fraction of aequorin missorted into a low Ca^{2+} compartment. A close inspection of the kinetics of aequorin consumption in fact revealed that, as in the case of Montero *et al.* (11), the decrease in the calibrated $[Ca^{2+}]_{er}$ occurred when over 80% of total aequorin content had been consumed (data not shown). A simple, practical solution to avoid the calibration artifact due to missorted aequorin is the elimination from the final algorithm of 2–4% of the total light emission. With this simple trick, the calibrated kinetics of Ca^{2+} refilling (Fig. 2*A*, *thick line*) are as expected, *i.e.* rapid uptake followed by a prolonged steady state plateau. It is worth stressing that the plateau level is almost identical to the peak of $[Ca^{2+}]_{er}$ measured without the correction and that no significant elevation in steady state $[Ca^{2+}]_{er}$ is generated if the fraction of missorted aequorin is varied between 2–5% of total (data not shown and see Ref. 11). The plateau phase of $[Ca^{2+}]_{er}$ occurs at around 300 μM (267 ± 9 μM; n = 20 independent experiments) and can last for several minutes, particularly if the temperature at which the experiment is performed is lowered to 22 °C (data not shown). The kinetics of the filling of the ER as well as the level of the observed steady state is influenced by the extracellular $[Ca^{2+}]$ as illustrated in Fig. 2*B*.

The localization of the aequorin to $InsP_3$-sensitive stores shown in Fig. 1 is further substantiated by the results obtained with receptor agonists known to activate phospholipase C and to raise $[Ca^{2+}]_c$ in this cell system (32, 38). Carbachol (100 μM) elicits Ca^{2+} mobilization from the ER as evidenced by a marked decrease in the $[Ca^{2+}]_{er}$ (Fig. 3*A*, *thin line*). This effect is mediated by the activation of muscarinic receptors because the addition of atropine not only prevented the carbachol effect (data not shown) but also caused a more rapid refilling of the stores than occurs after simple removal of the agonist (Fig. 3*A*, *thick line*). The addition of extracellular ATP that activates P_{2Y} receptors in insulin-secreting cells (39) also causes a sustained decrease in the levels of $[Ca^{2+}]_{er}$ (Fig. 3*B*). It has been reported previously that 4-chloro-*m*-cresol raises $[Ca^{2+}]_c$ in HIT-T15 cells (40). That this effect is exerted through activation of ryanodine receptors in the ER (24) is suggested by the results in Fig. 3*C*. At 500 μM, 4-chloro-*m*-cresol caused an almost complete and irreversible emptying of $[Ca^{2+}]_{er}$. In the presence of 0.1 mM 4-chloro-*m*-cresol the lowering of $[Ca^{2+}]_{er}$ was slower, but the steady state reached was similar to that observed with 0.5 mM (data not shown). As expected, the SERCA inhibitor, CPA, efficiently lowered $[Ca^{2+}]_{er}$. Removal of the inhibitor results in an almost complete refilling because a new steady state similar to that observed before the addition of CPA is reached again after approximately 3 min (Fig. 3*D*). It should be noted that the initial phase of emptying of the ER Ca^{2+} store was more rapid with the $InsP_3$-generating agent carbachol than with CPA, because 20% lowering of $[Ca^{2+}]_{er}$ was 50%

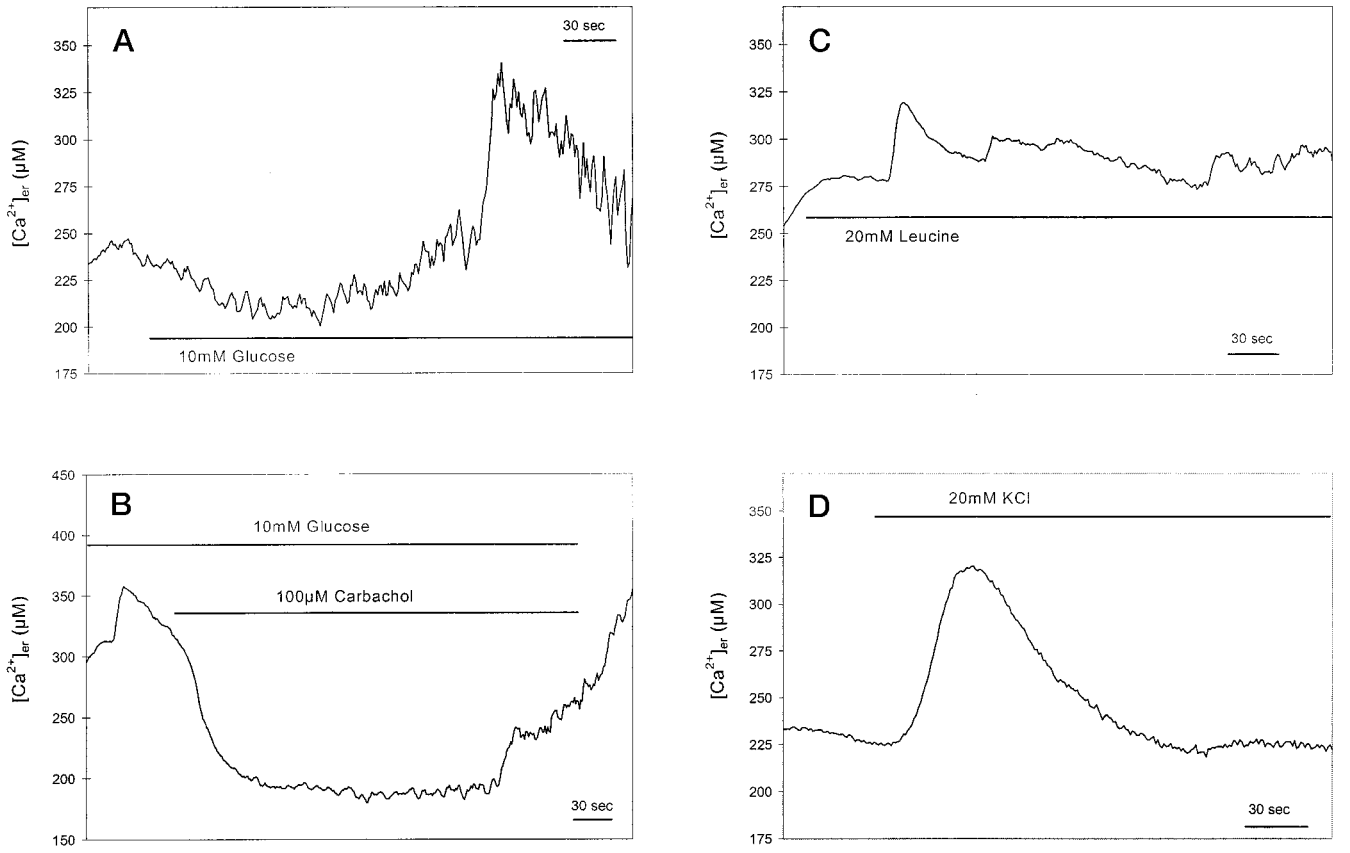

FIG. 4. **$[Ca^{2+}]_{er}$ changes induced by glucose, leucine, and KCl, all of which promote voltage-sensitive Ca^{2+} influx into INS-1 cells.** *A*, 10 mM glucose. *B*, 10 mM glucose plus 100 μM carbachol (see "Results" for comparative values of carbachol action at 2.8 and 10 mM glucose). *C*, 20 mM leucine. *D*, 20 mM KCl. The basal glucose concentration was 2.8 mM throughout. The *traces* are representative of four to six independent experiments.

slower with the latter agent. This is in accordance with observations using histamine in HeLa cells (12).

Although it has been established that ER refilling after Ca^{2+} mobilization is due to Ca^{2+} influx from the extracellular medium, it is less clear whether Ca^{2+} entry invariably causes filling of the ER compartment. To investigate this, the insulin secretagogues glucose, leucine, and KCl were used, which all raise $[Ca^{2+}]_c$ in β-cells by membrane depolarization and opening of voltage-sensitive Ca^{2+} channels (23, 32), thereby promoting Ca^{2+} influx from the extracellular space. Glucose caused an average increase in $[Ca^{2+}]_{er}$ of 35 ± 13 μM ($p < 0.02$; $n = 12$). These experiments comprised two cell preparations displaying either negligible or large responses. Considering only the latter experiments, the glucose-induced $[Ca^{2+}]_{er}$ rise was 64 ± 19 μM ($p < 0.02$; $n = 6$; Fig. 4*A*). The increased $[Ca^{2+}]_{er}$ induced by glucose results in a larger Ca^{2+} mobilization evoked by 100 μM carbachol as shown in Fig. 4*B*. Carbachol decreases $[Ca^{2+}]_{er}$ in control cells (2.8 mM glucose) by 63.8 ± 5.5 μM ($n = 4$) and by 91.6 ± 6.7 μM ($n = 8$) in cells stimulated with 10 mM glucose ($p < 0.05$).

Leucine, which acts like glucose (41), also causes a small increase in $[Ca^{2+}]_{er}$ as shown in Fig. 4*C* (incremental increase 41 ± 4 μM; $p < 0.001$; $n = 6$). This response had a more rapid onset time than that of glucose. KCl (20 mM) causes a rapid and pronounced increase in the $[Ca^{2+}]_{er}$ by approximately 100 μM (Fig. 4*D*). This increase is transient, and there is only a small sustained elevation despite the continued presence of KCl. This result might be expected from the known biphasic effect of KCl on $[Ca^{2+}]_c$ in these cells (32). The lag times for the onset of the $[Ca^{2+}]_{er}$ rises after the addition of the stimulus were calculated: glucose, 184 ± 12 s; leucine, 66 ± 11 s; and KCl, 12 ± 1 s. These values in part reflect the difference between the asynchronous metabolic stimulation of the cells with glucose and leucine and the synchronous stimulation with KCl. Because of artifactual decreases in $[Ca^{2+}]_{er}$ when rapid stimulus application was used, the lag times cannot be compared with those calculated in our previous study for the rises in cytosolic and mitochondrial $[Ca^{2+}]$ (32).

The increase in $[Ca^{2+}]_{er}$ evoked by glucose and leucine could theoretically be secondary to an increase in intracellular ATP (33, 42). Fig. 5*A* shows that 10 mM glucose causes a rapid increase in the cytosolic [ATP] monitored in living INS-1 cells stably expressing cytosolic luciferase. The increase in ATP, however, is not the cause of the $[Ca^{2+}]_{er}$ rise because in the absence of extracellular Ca^{2+} glucose elicits the same increase in cytosolic [ATP] (Fig. 5*B*) but has no effect on $[Ca^{2+}]_{er}$. As to the elevation in [ATP], this is not caused by the $[Ca^{2+}]_c$ rise given that depolarizing concentrations of KCl, which raise $[Ca^{2+}]_c$ to levels higher that 1 μM (32), actually decrease [ATP] by approximately 10% (Fig. 5*A*). It appears that basal ATP levels in resting cells efficiently ensure Ca^{2+} pumping into the ER and that the $[Ca^{2+}]_c$ rise but not increased ATP generation underlie the filling of the ER Ca^{2+} stores.

A series of experiments was performed in ER aequorin-expressing INS-1 cells permeabilized with *Staphylococcus* α-toxin to gain further insight into the regulation of $[Ca^{2+}]_{er}$. This approach allows the clamping of the concentration of ions, nucleotides, and glucose metabolites (33, 35) while leaving the organelle structures unperturbed. Fig. 6*A* illustrates that in the continued presence of the SERCA inhibitor CPA, an increase in the ambient free $[Ca^{2+}]$ from 0.1 μM to 1.3 μM only causes a small elevation in the $[Ca^{2+}]_{er}$. This gives a further

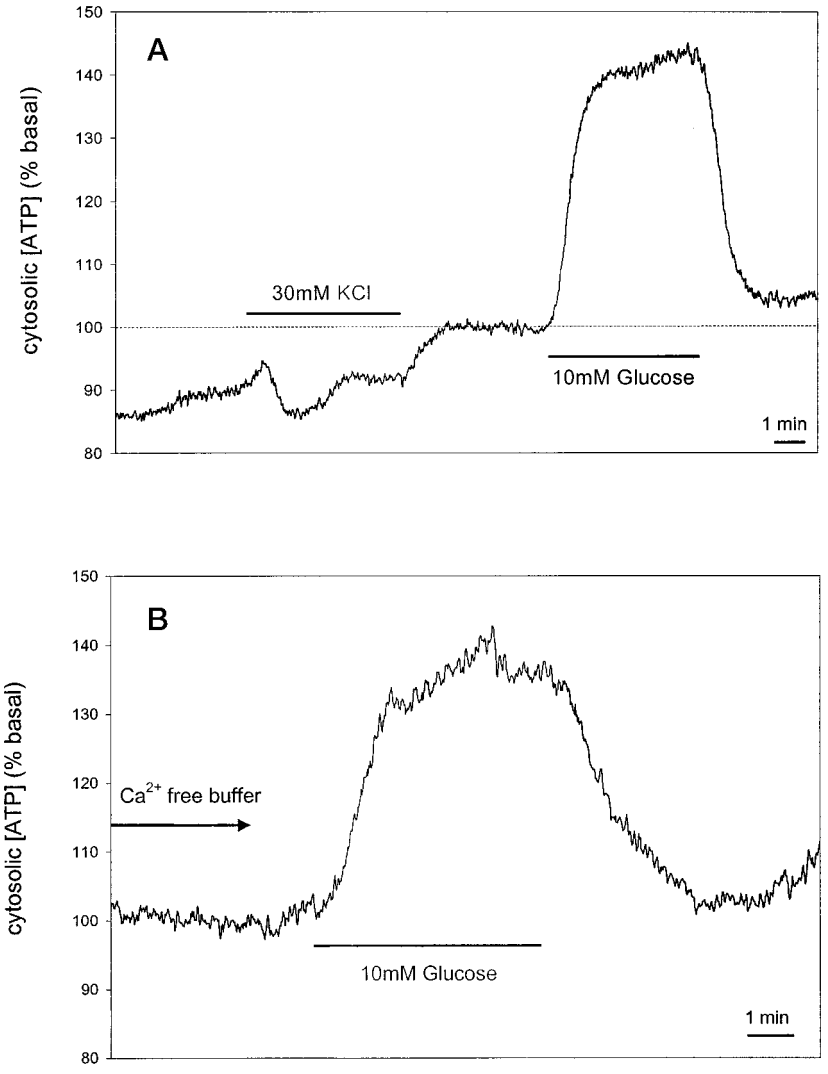

FIG. 5. **Effect of KCl and glucose on cytosolic ATP levels in luciferase-expressing INS-1 cells.** Luciferase luminescence was recorded under the same conditions as those of $[Ca^{2+}]_{er}$ measurements. *A*, effect of 30 mM KCl followed by stimulation with 10 mM glucose in normal KRBH (1.5 mM $CaCl_2$). *B*, effect of 10 mM glucose in a Ca^{2+}-free buffer (no $CaCl_2$, 0.5 mM EGTA). The *traces* are representative of four independent experiments.

functional indication of the ER localization of the recombinant aequorin. After removal of CPA, $[Ca^{2+}]_{er}$ rapidly rises to a new steady state value of approximately 180 μM. As in intact cells, the action of the SERCA inhibitor is thus reversible albeit not completely with this short wash period. This is demonstrated by comparing Fig. 6*A* with the steady state value of 457 ± 59 μM (n = 4) observed in the presence of 1.3 μM Ca^{2+} and 10 mM ATP (Fig. 7*B*). When the permeabilized cells were perifused with 0.1 μM Ca^{2+} and 10 mM ATP, the resulting average $[Ca^{2+}]_{er}$ was 215 ± 20 μM (n = 4). As expected, 5 μM $InsP_3$ elicits a pronounced emptying of the ER Ca^{2+} store (Fig. 6*B*). This response was almost as potent as that seen with CPA, although the agents were tested at a slightly different $[Ca^{2+}]$.

The rate-limiting reaction in glucose metabolism in the β-cell is the high K_m glucose phosphorylating enzyme, glukokinase. Its product, glucose 6-phosphate, has been suggested to promote Ca^{2+} sequestration by directly stimulating Ca^{2+} uptake by the ER in pancreatic islets (43). To investigate whether the effect of glucose on the $[Ca^{2+}]_{er}$ is mediated by glucose 6-phosphate, this metabolite (1 mM) was tested in the permeabilized cell system at ambient $[Ca^{2+}]$ of 500 nM. There was no significant effect on the filling of the ER (Fig. 6*C*).

Next we examined the impact of carbonyl cyanide *m*-chlorophenyl hydrazone (FCCP), which dissipates the mitochondrial proton gradient and thereby inhibits ATP production and Ca^{2+} uptake via the Ca^{2+} uniporter of these organelles (33, 41, 44, 45). As shown in Fig. 7, exposure of the intact cells to 1 μM FCCP evokes a pronounced loss of Ca^{2+} in the ER resulting in a new steady state that remained higher than the corresponding steady state reached in the presence of 10 μM CPA (Fig. 3*D*). This effect appears to be exerted on the $InsP_3$-sensitive ER store because the addition of carbachol failed to alter further the $[Ca^{2+}]_{er}$ (Fig. 7*A*), although a diminished pool of the phosphoinositide lipid precursor of $InsP_3$ cannot be excluded. In addition, FCCP does not change the ATP-dependent filling of the ER in the permeabilized cells, which suggests that the effect of the protonophore is at the level of the mitochondria rather than on the ER itself (Fig. 7*B*). The action of FCCP is reversible because the $[Ca^{2+}]_{er}$ rapidly increases upon its removal.

Finally, it was felt important to examine whether the conditions for the emptying of the ER Ca^{2+} stores alter the physiological response of INS-1 cells. This can be seen in Table I. Glucose (10 mM) or KCl (20 mM) still cause significant increases in insulin secretion after emptying/refilling of the ER Ca^{2+} stores. It should be noted that later protocol results in elevated basal insulin secretion (+42%, $p < 0.001$).

DISCUSSION

An insulin-secreting cell line was established that stably expresses aequorin in the ER. The measurement of Ca^{2+} within this organelle could shed light on the maintenance of calcium levels within the secretory cell as well as on putative interactions between organelles involved in Ca^{2+} homeostasis.

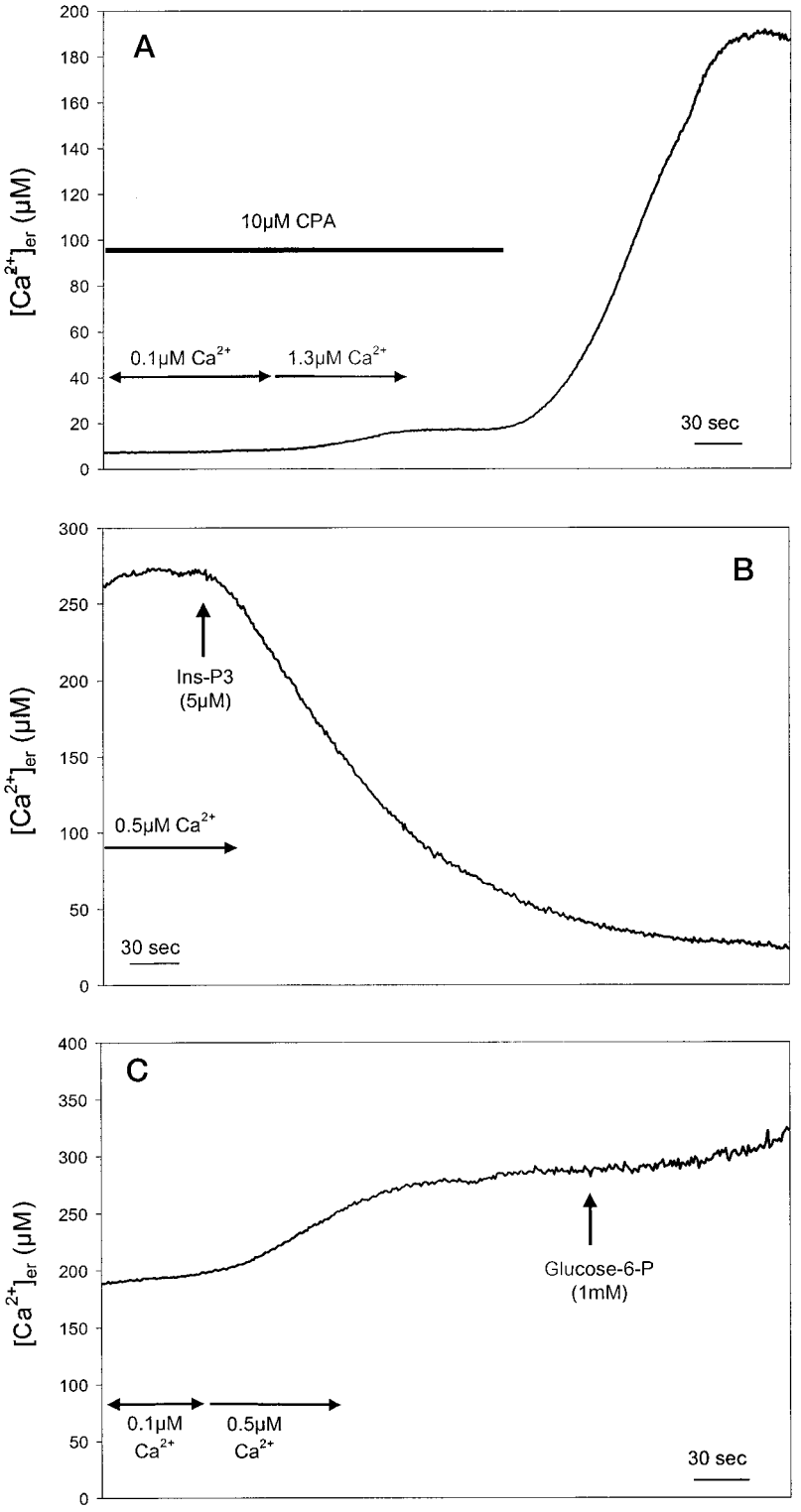

FIG. 6. **Regulation of $[Ca^{2+}]_{er}$ in α-toxin permeabilized INS-1 cells.** *A*, in the presence of the SERCA inhibitor CPA (10 μM), changes in the ambient $[Ca^{2+}]$ at 10 mM ATP have only minor effects on $[Ca^{2+}]_{er}$. The effect of CPA is partially reversible. *B*, effect of $InsP_3$ (5 μM) in the presence of 0.5 μM free Ca^{2+} and 10 mM ATP. *C*, effect of glucose 6-phosphate (1 mM) in the presence of 0.5 μM Ca^{2+} and 10 mM ATP. The *traces* are representative of four independent experiments.

The measurement of $[Ca^{2+}]_{er}$ with aequorin is more complicated than the corresponding measurements in the mitochondria and the cytosol because of the higher $[Ca^{2+}]$ in the former compared with the latter compartments. Because the consumption of the prosthetic group, coelenterazine, is avid in a high calcium environment, the calibration of $[Ca^{2+}]_{er}$ without the corrective procedure shown in Fig. 2 appears bell-shaped rather than reaching a steady state phase. Taking this approximately 2–4% missorted aequorin into account, steady state $[Ca^{2+}]_{er}$ levels around 300 μM were consistently observed in the INS-1 cell that lasted for about 5 min before the aequorin luminescence became limiting. This corresponds well to the values observed using the same method of detection in several cell types (12, 14). However, others employing a different chimeric aequorin that lacked the mutation in one of the Ca^{2+}-binding pockets have reported values in the low micromolar range in an embryonal kidney cell line (13). Single cell estimates of $[Ca^{2+}]_{er}$ employing targeted "cameleon" calmodulin-containing fusion proteins have yielded resting values of 60–400 μM (46). The low affinity fluorescence indicator, mag-fura2, has also been used in various cell types to measure $[Ca^{2+}]_{er}$. This approach has yielded estimates of $[Ca^{2+}]_{er}$ also in the

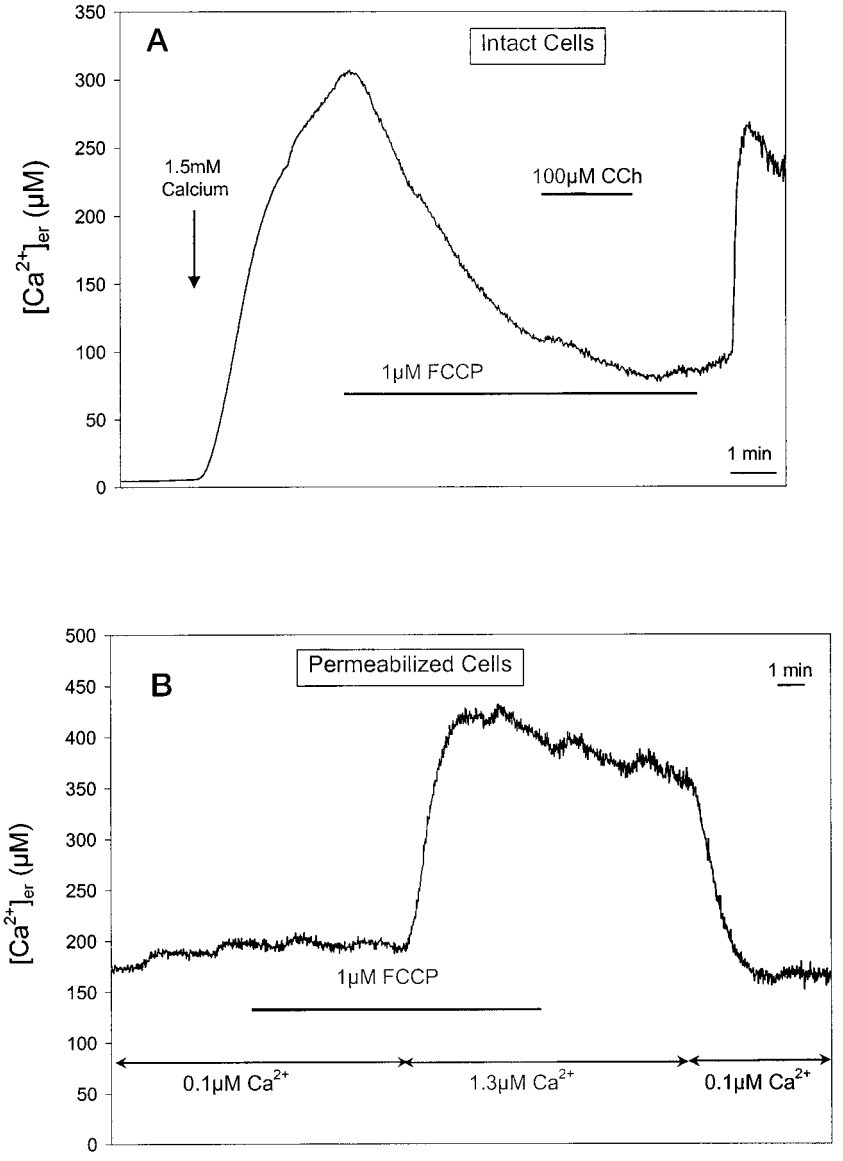

FIG. 7. **Effect of the mitochondrial uncoupler FCCP on $[Ca^{2+}]_{er}$ in intact and permeabilized INS-1 cells.** *A*, in intact cells 1 μM FCCP markedly lowered $[Ca^{2+}]_{er}$ and inhibited the action of carbachol (100 μM). *B*, in α-toxin permeabilized cells FCCP does not directly affect $[Ca^{2+}]_{er}$ in the presence of 10 mM ATP either at low (0.1 μM) or high (1.3 μM) ambient free Ca^{2+}. The *traces* are representative of three independent experiments.

TABLE I
Effect of depletion of ER Ca^{2+} on insulin secretion

Cells were seeded at a density of 10^5 in 24-well plates and allowed to settle for 2–3 days. Cellular Ca^{2+} stores were then emptied by the following incubations: (i) cells were washed twice briefly in Ca^{2+}-free KRBH; (ii) cells were then incubated for 5 min in Ca^{2+}-free KRBH containing 3 mM EGTA; (iii) following this, the ER stores were emptied for 5 min in Ca^{2+}-free KRBH containing 3 mM EGTA and 10 μM CPA. All cells were then preincubated for 30 min in KRBH (2.8 mM glucose) and subsequently stimulated for 15 min. Values of insulin secretion (percentage of cell content) are the means ± S.E. of the number of observations given in parentheses. Statistical analysis was performed by comparison with appropriate basal conditions.

Conditions	Control	ER Ca^{2+} store-depleted/repleted cells
Basal	2.87 ± 0.21 (17)	4.07 ± 0.29 (13)
Glucose (10 mM)	4.69 ± 0.46 (9) (+63%, $p < 0.001$)	5.91 ± 0.89 (8) (+45%, $p < 0.05$)
KCl (20 mM)	4.28 ± 0.44 (9) (+49%, $p < 0.01$)	7.32 ± 1.09 (9) (+80%, $p < 0.005$)

range of 300 μM (21, 47, 48). In permeabilized hepatocytes, a value of 500 μM has been reported under optimal conditions using fluorescent indicators (49). Thus our estimate of steady state $[Ca^{2+}]_{er}$ nicely agrees with those obtained in several cell types and further argues in favor of the idea that the very low values of $[Ca^{2+}]_{er}$ initially measured with recombinant aequorin were due to calibration artifacts (7, 9, 48).

The aequorin-containing ER compartment was potently emptied after treatment of permeabilized INS-1 cells with the SERCA inhibitor CPA, or $InsP_3$ by approximately 90%. In earlier work from this laboratory, the SERCA inhibitor thapsigargin caused a much greater release of Ca^{2+} than $InsP_3$ as measured with fluo-3 in the incubation medium of electropermeabilized INS-1 cells (38). The reason for this discrepancy is unknown but may relate to the different detection procedures and the more disruptive permeabilization method used in the earlier study. The resting levels of the $[Ca^{2+}]_{er}$ in the permeabilized aequorin-expressing INS-1 cells were not dissimilar (<20% lower) to values recorded in intact cells but depended on the prevailing ATP and Ca^{2+} concentrations. The conditions imposed for both of these parameters corresponded to the concentration ranges suggested to occur in intact cells (32, 33). A recent study employing the low affinity Ca^{2+} indicator furaptra in digitonin-permeabilized *ob/ob* mouse β-cells reported

$[Ca^{2+}]_{er}$ in the range 200–500 μM (50), in good agreement with the present results in INS-1 cells.

In intact cells the $InsP_3$-generating agonists carbachol and ATP used at maximal concentrations (39, 51) were efficient in causing Ca^{2+} mobilization. Carbachol, shown to exert its effect through muscarinic receptors, was the more potent of the two agonists. It should be noted that even in the presence of 100 μM carbachol only approximately 35% of the releasable Ca^{2+} is mobilized compared with the almost complete emptying seen in the presence of the SERCA inhibitor, CPA. Judging from the aforementioned data on permeabilized cells, this is not due to a subcompartment of the aequorin-containing ER being devoid of $InsP_3$ receptors but probably due either to low $InsP_3$ production or to its compartmentalization in intact cells.

In contrast to the $InsP_3$-mobilizing agents, the nutrient secretagogues glucose and leucine elicited Ca^{2+} sequestration by the ER. This effect was more pronounced with depolarizing concentrations of KCl. The nutrients promote Ca^{2+} influx following closure of ATP-sensitive K^+ channels and gating of voltage-sensitive Ca^{2+} channels in the plasma membrane (23). Both glucose and leucine generate ATP by driving oxidative phosphorylation in the mitochondria (33, 42). KCl does not activate cellular metabolism but directly promotes voltage-dependent Ca^{2+} influx. Therefore, the rise in $[Ca^{2+}]_{er}$ cannot be explained by an increase in ATP generation that in the resting state is sufficient to ensure efficient Ca^{2+} sequestration. This was substantiated by the measurements of cytosolic ATP showing a large increase in ATP in the presence of glucose but a slight decrease upon addition of KCl. This latter phenomenon probably reflects ATP consumption by the Ca^{2+}-ATPases in both ER and plasma membranes. Such consumption is to be expected with KCl, which raises cytosolic $[Ca^{2+}]$ to levels in excess of 1 μM in contrast to the 3–4-fold lower levels reached with glucose and leucine (32, 52). This confirms the reported lowering of the ATP/ADP ratio in K^+-stimulated mouse pancreatic islets (53). The pattern of Ca^{2+} uptake by the ER reflects the potency order for cytosolic $[Ca^{2+}]$ rises with KCl $\gg$ glucose $>$ leucine. This nicely confirms the role of the ER in Ca^{2+} homeostasis in β-cells.

Measurements of $[Ca^{2+}]_c$ in native β-cells have suggested that glucose could facilitate an increase in the calcium levels of the ER (28, 29, 54) that had previously been proposed from experiments using $^{45}Ca^{2+}$ (27, 43, 55). We tested directly the proposal that the sequestering effect of glucose is mediated by its immediate metabolite glucose 6-phosphate (43). However, glucose 6-phosphate had no significant effect on $[Ca^{2+}]_{er}$ when tested at 500 nM Ca^{2+} in the permeabilized INS-1 cell. This, taken together with the sequestering activities of KCl and leucine (which do not generate glucose 6-phosphate) precludes a role for this metabolite in $[Ca^{2+}]$ homeostasis in the β-cell.

It is of interest that, in a previous study from this laboratory, it was shown that in INS-1 cells stably expressing aequorin in the mitochondria, glucose not only increased the mitochondrial calcium concentration *per se* but that its preaddition could significantly augment the response to carbachol (32). That this phenomenon occurs can now, at least in part, be explained by the observed filling of the ER by glucose. We have now demonstrated that carbachol induces a more marked lowering of $[Ca^{2+}]_{er}$ in the presence of 10 mM compared with 2.8 mM glucose. This confirms measurements of cytosolic $[Ca^{2+}]$ that have anticipated these findings (28, 29). If, as has been shown for other cell types, mitochondria lie in close proximity to the ER release sites (44, 56), the elevated concentration of calcium will participate in the augmentation of oxidative metabolism during the potentiation of nutrient-induced insulin secretion (23).

Lowering of the cytosolic [ATP] with the mitochondrial uncoupler FCCP (33) caused a pronounced emptying of the ER Ca^{2+} store (approximately 60–70%). This is not due to a direct effect of FCCP on the ER because the uncoupler had no effect under conditions of clamped ATP in permeabilized INS-1 cells (Fig. 7*B*) or BHK-21 cells (20). The mobilizing effect of FCCP in intact BHK-21 cells and INS-1 cells is, therefore, the consequence of a lowering of the ATP/ADP ratio below a critical threshold, impairing the function of the SERCA-mediated uptake mechanism (20). This further demonstrates the intimate connection between mitochondrial metabolism and Ca^{2+} handling by the ER.

In many cell types calcium itself promotes Ca^{2+} mobilization from the ER by activation of ryanodine receptors (1, 5). In the β-cell and derived cell lines ryanodine, which can both activate and block its receptors, did not raise $[Ca^{2+}]_c$ (24, 40). However, other activators such as caffeine (24), NO (57), and 4-chloro-*m*-cresol (40) as well as related compounds (24) have been reported to raise $[Ca^{2+}]_c$. In the present study, 4-chloro-*m*-cresol indeed potently emptied the ER Ca^{2+} stores. This result and the presence of mRNA for type 2 ryanodine receptors in islets (24) suggest the implication of this Ca^{2+} release mechanism in the β-cell under certain physiological situations (24).

In conclusion we have established an insulin-secreting cell line in which it is possible to investigate changes in the lumenal $[Ca^{2+}]$ in the ER and found that glucose enhances filling of the ER Ca^{2+} store under conditions of stimulated insulin secretion. This may have implications in the treatment of noninsulin-dependent diabetes mellitus because it has been shown that the Ca^{2+} uptake and SERCA expression is lowered in the ER in pancreatic islets from two animal models of this disease (58, 59). The application of the targeted aequorin technology to β-cells from normal and diabetic animals should help clarify whether defective Ca^{2+} handling by the ER is a primary cause of impaired insulin secretion associated with diabetes mellitus. The feasibility of such measurements has become tangible in view of successful monitoring of intramitochondrial $[Ca^{2+}]$ in native rat islet cells (35).

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Secretagogues Modulate the Calcium Concentration in the Endoplasmic Reticulum of Insulin-secreting Cells: STUDIES IN AEQUORIN-EXPRESSING INTACT AND PERMEABILIZED INS-1 CELLS

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