The Endoplasmic Reticulum Can Act as a Functional Ca\(^{2+}\) Store in All Subcellular Regions of the Pancreatic Acinar Cell* 

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Stimulation of pancreatic acinar cells raises [Ca\(^{2+}\)], via Ca\(^{2+}\) release from inositol-1,4,5-trisphosphate (InsP\(_3\))-sensitive intracellular Ca\(^{2+}\) stores, generally considered to reside within the endoplasmic reticulum (ER). However, with physiological doses of cholinergic agonists, the [Ca\(^{2+}\)], increase is localized to the apical (secretory) pole of the cell, leading to suggestions that zymogen (secretory) granules themselves may constitute an InsP\(_3\)-sensitive Ca\(^{2+}\) store responsible for localized Ca\(^{2+}\) release. We have therefore re-investigated whether the ER in pancreatic acinar cells is capable of acting as a functional Ca\(^{2+}\) store in all, or only some, cellular regions. In streptolysin O-permeabilized cells, the ER accumulated up to 25 mmol of 45Ca\(^{2+}\) per liter ER volume by an ATP-dependent, thapsigargin-sensitive, fast exchange process. This tracer Ca\(^{2+}\) uptake was dependent on ambient (loading) [Ca\(^{2+}\)], as was the intra-ER free [Ca\(^{2+}\)], assessed by imaging the fluorescence of Magfura-2-AM. Comparison of the intracellular [Ca\(^{2+}\)] and total intra-ER [Ca\(^{2+}\)] indicated that 200-300 Ca\(^{2+}\) ions are bound within the ER lumen for every Ca\(^{2+}\) ion remaining free. Subcellular analysis showed that ER stores in all regions of the permeabilized cell took up Ca\(^{2+}\) at loading [Ca\(^{2+}\)] between 60 nm and 1 m\(\mu\)M. Thapsigargin-stimulated Ca\(^{2+}\) release from stores in all cellular regions, as did InsP\(_3\). Immunofluorescence with antibodies against sarco(endoplasmic reticulum-2b type Ca\(^{2+}\),Mg\(^{2+}\) -ATPase or calreticulin confirmed that ER Ca\(^{2+}\) stores were present throughout the cytoplasm. In summary, these results clearly show that the endoplasmic reticulum can act as a functional Ca\(^{2+}\) store in all regions of the acinar cell, including the apical pole.

Intracellular Ca\(^{2+}\) stores play a dominant role in Ca\(^{2+}\) signaling in pancreatic acinar cells. Indeed, the Ca\(^{2+}\)-mobilizing action of the intracellular messenger inositol-1,4,5-trisphosphate (InsP\(_3\))\(^{3}\) was first demonstrated using a permeabilized pancreatic acinar cell preparation (1). This initial work also identified the endoplasmic reticulum (ER) as the intracellular Ca\(^{2+}\) store responsible for agonist-induced increases in [Ca\(^{2+}\)], (1). Recently, however, zymogen granules have been proposed to act as a Ca\(^{2+}\) store in pancreatic acinar cells. (2) This hypothesis stemmed initially from the observation that stimulation of pancreatic (and other) acinar cells with acetylcholine results in a polarized rise in cytosolic free [Ca\(^{2+}\)], with the [Ca\(^{2+}\)], increase being initiated at the apical pole of the cell where zymogen granules are clustered (3–6). Subsequently the rise in [Ca\(^{2+}\)], spreads to the basal pole of the acinar cell (3–6). The role of the proposed zymogen granule Ca\(^{2+}\) store would be to act as the releasable Ca\(^{2+}\) store responsible for the initiation of the intracellular Ca\(^{2+}\) signal at the apical pole (2, 7). Propagation of the increase in [Ca\(^{2+}\)], toward other regions of the cell could then be mediated by the ER Ca\(^{2+}\) stores, since the rough endoplasmic reticulum is found throughout the acinar cell (see e.g., Ref. 8). This zymogen granule Ca\(^{2+}\) store model is attractive since it can explain the restriction of the [Ca\(^{2+}\)], signal to only the luminal (apical) region when low physiological doses of cholinergic agonists are applied (7, 9). However, there is as yet no conclusive evidence on whether zymogen granules are equipped with intracellular messenger-triggered Ca\(^{2+}\) release. Indeed, recent evidence suggests that the report of InsP\(_3\)-sensitive Ca\(^{2+}\) release from granules (2) may be an artifact produced by the impurity of the zymogen granule preparation employed (10).

A further problem with the report of zymogen granule Ca\(^{2+}\) stores (2) is that no evidence was found for a Ca\(^{2+}\) uptake mechanism in the proposed zymogen granule Ca\(^{2+}\) store. However, an active Ca\(^{2+}\)-sequestering mechanism is essential to explain the refilling of Ca\(^{2+}\) stores and hence the repetitive nature of the agonist-induced [Ca\(^{2+}\)], transients. These conflicting results have prompted us to re-evaluate the suggestion that the endoplasmic reticulum can act as a functional Ca\(^{2+}\) store in all subcellular regions of the pancreatic acinar cell. Our results demonstrate that the endoplasmic reticulum can indeed act as a Ca\(^{2+}\) store in all subcellular regions, including the apical pole.

**EXPERIMENTAL PROCEDURES**

Cell Preparation—Small clusters of acinar cells were prepared from the pancreas of one 200-g male Sprague-Dawley rat by the same enzymatic digestion procedure described in Ref. 11. After isolation, cells were resuspended in a HEPES/Tris-buffered physiological saline containing 133 mM NaCl, 4.2 mM KCl, 1.0 mM CaCl\(_2\), 1.0 mM MgCl\(_2\), 5.8 mM glucose, 0.2 mM L-glutamine, 10 mM HEPES, 0.5 mM bovine serum albumin, and 10 mM L-glutamine. The pH of this medium was set at 7.4 with Tris. Cells were either used immediately or stored in 1-ml portions on ice until use.

Loading with Magfura-2 and Fluorescence Imaging—Cells were loaded with 5 \(\mu\)M Magfura-2-AM for 30 min at 37 °C, as described previously (11), and allowed to settle on a polystyrene-coated glass coverslip which formed the bottom of a perfusion chamber. Fluorescence was imaged using a system based on an inverted epifluorescence Nikon Diaphot microscope and a slow scan CCD camera (Digital Pixel Ltd, Brighten, UK). Details of the imaging system were described in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: InsP\(_3\), d-myo-inositol 1,4,5-trisphosphate; [Ca\(^{2+}\)]\(_{\text{int}}\), free intracellular Ca\(^{2+}\); [Ca\(^{2+}\)]\(_{m}\), free cytosolic Ca\(^{2+}\); FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; SERCA-2b, Ca\(^{2+}\)-ATPase sarco(endoplasmic reticulum-2b type Ca\(^{2+}\),Mg\(^{2+}\)–ATPase; SLO, streptolysin O; HEDTA, N-hydroxyethylendiaminetriacetic acid; ER, endoplasmic reticulum.
previously (11). The microscope objective was a Nikon 40× oil immersion lens (Numerical Aperture 1.3), which allowed images of a field 90 × 135 μm to be captured. For imaging Magfura-2 fluorescence in permeabilized cells, a 3 × 3 binning was applied to the individual pixels on the image sensor to give a theoretical spatial resolution of 0.67 × 0.67 μm². Background-subtracted 340-380 images were calculated off-line.

**Permeabilization and Perfusion of Pancreatic Acinar Cells**—Before initiating permeabilization acinar cells were perfused with Ca²⁺ uptake medium containing 135 mM KCl, 1.2 mM KH₂PO₄, 0.5 mM EGTA, 0.5 mM HEDTA, 0.5 mM nitritotriacetic acid, and 20 mM HEPEs/KOH, pH 7.1. The free Mg²⁺ concentration was 0.9 mM and was adjusted as described by Schoenmakers et al. (12). SLO (0.4 IU/ml final concentration) was added directly to the perfusion chamber to permeabilize acinar cells as described previously (11). When permeabilization was achieved (as judged by a drop in fluorescence at the dye isosbestic wavelength) cells were re-perfused with the Ca²⁺ uptake medium as described above but devoid of SLO. Perfusion continued for the duration of the experiment. All experiments were performed at room temperature.

Calcium uptake by intracellular Ca²⁺ stores was initiated by superfusing cells with Ca²⁺ uptake medium containing 1 mM ATP, free Ca²⁺ concentration as indicated in the text and figures, and free Mg²⁺ concentration of 0.9 mM (free divalent cation concentrations calculated according to Ref. 12). When free Ca²⁺ concentration was set to 150–200 nM, no mitochondrial inhibitors were included in the medium since mitochondrial Ca²⁺ uptake has previously been shown not to occur at this ambient free Ca²⁺ concentration (13). The mitochondrial Ca²⁺ uptake inhibitors oligomycin (5 μM) and antimycin (5 μM) were included when a free Ca²⁺ concentration of 1.0 μM was applied. Calcium uptake was monitored for a period of 20 min with ratio images acquired at 15-s intervals.

**Radioactive ¹⁴C³⁺ Uptake Experiments in Permeabilized Cells**—Isolated pancreatic acinar cells in suspension were permeabilized by treatment with SLO (0.4 IU/ml) for 10 min as described previously (14), washed twice, resuspended in (Ca²⁺-free) Ca²⁺ uptake medium (see above), and kept at 0 °C until use. Cell density was adjusted to give a protein concentration of around 4 mg of protein/ml. At the beginning of the radiotracer uptake experiment 10 μl of permeabilized cell suspension was added to 87 μl of Ca²⁺ uptake medium which contained 10 mM phosphocreatine, 10 units of creatine kinase/ml, 1 μM thapsigargin or 1% (v/v) Me₂SO, and 5 μCi of ¹⁴C³⁺/ml. The free Mg²⁺ (0.9 mM) and Ca²⁺ (as indicated) concentrations were adjusted as described above. After 3 min, the incubation was started by adding 3 μl of MgATP stock solution to give a final MgATP concentration of 1 mM. The incubation was terminated after 15 min by adding 1.0 ml of ice-cold stop solution containing 150 mM KCl, 5.0 mM MgCl₂, 1.0 mM EGTA, and 20 mM HEPEs/KOH, pH 7.1, and the suspension was rapidly filtered (GFC glass microfiber filters, Whatman, Kent, UK). The filters were washed with 2 × 1.0 ml of ice-cold stop solution, dissolved in scintillation fluid, and counted for radioactivity. Total Ca²⁺ was calculated and expressed as nmol/mg protein or as mol per liter of endoplasmic reticulum. For the former, cellular protein was determined (after treatment of the cells with 0.1% Triton X-100) with a commercial Coomassie Blue kit (Bio-Rad), using gamma globulin (Bio-Rad) as a standard. For the latter, cell density was determined using a hemocytometer (Weber Scientific International Ltd., Teddington, UK), and the volume of endoplasmic reticulum per incubation was calculated using the number of cells combined with morphological data on pancreatic acinar cells given by Bølender (8). Calcium actively stored by the endoplasmic reticulum was defined as the difference in total Ca²⁺ retained on the filter after incubation in the absence and presence of the endoplasmic reticulum Ca²⁺ uptake inhibitor thapsigargin.

**Immunohistochemistry**—A small drop of cell suspension in physiological saline (see above) was placed on a slide-coated slide, and the cells were allowed to adhere for 10 min in a moist environment. The cells were then fixed for 15 min in a freshly prepared paraformaldehyde solution (2% w/v in phosphate-buffered saline (PBS)). Cells were washed twice in PBS and were permeabilized using 1% (v/v) Triton X-100 in PBS dissolved in PBS. The slides were subsequently incubated with primary antibody or preimmune serum in the presence of 0.1% Triton and 1% normal serum for 1 h at room temperature. Rabbit polyclonal antisera against calreticulin (15) was diluted 1:100 and rabbit polyclonal antisera to SERCA-2b Ca²⁺-ATPase (16-18) were used diluted 1:1000. Slides were washed three times with PBS and were then incubated for 1 h at room temperature with FITC-conjugated swine anti-rabbit polyclonal antisera diluted 1:200 in PBS containing 0.1% (v/v) Triton and 1% (v/v) normal goat serum. Cells were washed three times and mounted in glycerol, which contained SlowFade-Light to prevent photobleaching. Slides were analyzed using the same imaging system employed for Magfura-2 imaging (see above and Ref. 11), except that a FITC dichroic filter set (Chroma Technologies) was used to observe FITC fluorescence. In addition, the resolution of the cooled CCD camera was increased to its maximum by applying a binning of 1 × 1 (i.e. no summation of pixels) to give a theoretical spatial resolution of 0.22 × 0.22 μm per pixel.

**Materials**—Polyclonal antisera against calreticulin was generously supplied by Drs. D. H. Llewellyn and L. Roderick (UWMC, Cardiff, UK). Two different polyclonal antisera against SERCA-2b Ca²⁺-ATPase were used, kindly supplied by Dr. F. Wuytack (Katholieke Universiteit Leuven, Leuven, Belgium), and by Dr. R. L. Dormer (UWCM, Cardiff, UK). Normal goat serum and FITC-conjugated swine anti-rabbit immunoglobulins were from DAKO, Glostrup, Denmark. Other chemicals were obtained from the following suppliers: SLO from Difco; thapsigargin from Calbiochem; insulin 1, 4, 5-P₃ from Sigma; Magfura-2-AM and Slow-Fade Light from Molecular Probes; ⁴⁰Ca²⁺ (20 mCi/ml) from NEN Life Science Products. All other chemicals were of analytical grade.

**RESULTS**

**Total Ca²⁺ Uptake by Endoplasmic Reticulum in Permeabilized Pancreatic Acinar Cells**—The total exchangeable Ca²⁺ uptake capacity of the thapsigargin-sensitive intracellular Ca²⁺ stores in permeabilized pancreatic acinar cells was determined using the radioactive ⁴⁰Ca²⁺ technique. The effect of the ambient (loading) [Ca²⁺]ᵢ on the total exchangeable uptake capacity of intracellular Ca²⁺ stores was examined over the range 60 nM to 1.0 μM. Total thapsigargin-sensitive Ca²⁺ uptake rose from 1.71 nmol per mg of protein to 6.11 nmol per liter of endoplasmic reticulum (see “Experimental Procedures” for details).

**Subcellular Imaging of Ca²⁺ Uptake Mechanisms**—As in our previous paper (11), we used the low affinity Ca²⁺ indicator Magfura-2 to monitor the free Ca²⁺ concentration inside Ca²⁺ storage compartments in real time in individual cells. Calcium uptake was ATP-dependent and reached steady-state levels within 15 min. The Magfura-2 ratio at steady state was dependent on the ambient (loading) [Ca²⁺]ᵢ and rose from 0.79 to 2.08 as [Ca²⁺]ᵢ was increased from 60 nM to 1 μM (Fig. 2A). The
initial rate of Ca\textsuperscript{2+} uptake into the stores was very steeply dependent on ambient [Ca\textsuperscript{2+}], increasing more than 6-fold with the change in [Ca\textsuperscript{2+}], (Fig. 2A). Because of the greatly enhanced initial Ca\textsuperscript{2+} uptake rate, the time taken to reach steady state [Ca\textsuperscript{2+}]\textsubscript{hwm} was reduced at higher [Ca\textsuperscript{2+}].

Fig. 2B shows the relationship between steady-state free [Ca\textsuperscript{2+}]\textsubscript{hwm} (as indicated by the steady-state Magfura-2 ratio from Fig. 2A) and total thapsigargin-sensitive exchangeable stored Ca\textsuperscript{2+} (taken from Fig. 1) in pancreatic acinar cells. The two parameters change in parallel over the entire range of loading [Ca\textsuperscript{2+}], suggesting that increased Ca\textsuperscript{2+} uptake at the higher loading [Ca\textsuperscript{2+}], values does not saturate the intra-store Ca\textsuperscript{2+} buffering system. In addition, the parallelism of total and free [Ca\textsuperscript{2+}]\textsubscript{hwm} within the stores implies that the free [Ca\textsuperscript{2+}]\textsubscript{hwm}, in any given part of the cell can be used to infer the total exchangeable Ca\textsuperscript{2+} in the same compartment.

We proceeded to study Ca\textsuperscript{2+} uptake in more detail by comparing uptake in apical and basal areas of acinar cells. This was achieved by applying the same method of subcellular regional analysis used in our previous study (11), i.e. selecting regions of interest in the apical area and in the basal area of the same cell(s). At 60 and 100 nM [Ca\textsuperscript{2+}], both initial uptake rates and steady-state ratio values were almost identical between apical and basal regions (Fig. 3, A and B). At higher values of [Ca\textsuperscript{2+}], namely 200 nM and 1 \mu M, initial uptake rates were also similar for the two regions. Interestingly, however, the [Ca\textsuperscript{2+}]\textsubscript{hwm} reached at steady state at these higher values of [Ca\textsuperscript{2+}] was noticeably greater in the basal area of the cell (Fig. 3, C and D).

Thapsigargin has been widely used in intact acinar cells to induce "global" [Ca\textsuperscript{2+}] release (see e.g. Ref. 4). In contrast to the apical pole [Ca\textsuperscript{2+}], signals observed with acetylcholine and thapsigargin (and other organellar Ca\textsuperscript{2+}-ATPase inhibitors) evoke a homogeneous elevation in cytosolic [Ca\textsuperscript{2+}] (4 or, in some cases, a [Ca\textsuperscript{2+}], rise which is largest in the basolateral pole (6). In permeabilized cells, thapsigargin completely prevents ATP-driven 45Ca\textsuperscript{2+} accumulation (13). We tested whether thapsigargin could deplete previously loaded Ca\textsuperscript{2+} stores in permeabilized pancreatic acinar cells. Fig. 4A shows that, as expected, thapsigargin caused a slow depletion of Ca\textsuperscript{2+} stores which was similar in both the apical and basolateral poles. Prevention of Ca\textsuperscript{2+}-ATPase activity by removal of ATP had essentially similar results (data not shown). The kinetics of thapsigargin-induced depletion were examined by normalizing the data shown in Fig. 4A to the total size of the ATP-sensitive Ca\textsuperscript{2+} pool (i.e. taking the ratio before loading the stores in each experiment as 0% and the steady-state ratio following loading as 100%). This gave depletion curves for apical and basolateral stores which were not significantly different (data not shown), again indicating that thapsigargin has equal actions on Ca\textsuperscript{2+} stores in the two cellular regions.

We have previously shown that thapsigargin enhances the rate of InsP\textsubscript{3}-evoked Ca\textsuperscript{2+} release from loaded intracellular Ca\textsuperscript{2+} stores and also converts apparent "quantal" release of Ca\textsuperscript{2+} from stores into an essentially monophasic release process (11). Fig. 4B presents subcellular regional analysis of the effects of sequential addition of thapsigargin and InsP\textsubscript{3}. To facilitate comparison between the apical and basolateral poles, data were normalized as described above. It is again clear that the kinetics of Ca\textsuperscript{2+} store depletion by thapsigargin and InsP\textsubscript{3} are identical for Ca\textsuperscript{2+} stores in the apical and basolateral poles.

SERCA-2b Ca\textsuperscript{2+}-ATPase and Calreticulin Distribution in Pancreatic Acinar Cells—We used immunohistochemistry to study the distribution of SERCA-2b Ca\textsuperscript{2+}-ATPase and of calreticulin in pancreatic acinar cells. Both SERCA-2b Ca\textsuperscript{2+}-ATPase (Fig. 5A) and calreticulin (Fig. 5B) appeared to be present in all regions of the cell. Immunostaining for the SERCA-2b Ca\textsuperscript{2+}-ATPase was slightly weaker in the apical area than in other regions of the cell (Fig. 5A). Weak decoration with the Ca\textsuperscript{2+}-ATPase antibody in the central portion of the basal areas of the cells indicated the presence of the nucleus, which was verified by staining DNA with 4,6-diamidino-2-phenylindole (results not shown). The polyclonal antibody against calreticulin decorated acinar cells in essentially the same pattern as observed with Ca\textsuperscript{2+}-ATPase antisemur. The only major difference was that the anti-calreticulin staining was slightly more punctate. As with the SERCA-2b antibody, both the apical region and the nucleus appeared less decorated than the basolateral cytoplasm. Since SERCA-2B Ca\textsuperscript{2+}-ATPase and calreticulin are both markers of the endoplasmic reticulum in pancreatic acinar cells, the less intense decoration of the apical cytoplasm probably reflects the fact that the apical region contains relatively less endoplasmic reticulum compared with other areas of the cell. This is well known from numerous morphological studies (see e.g. Ref. 8) and arises from the fact that a large part of the apical cytoplasm is occupied by zymogen granules.
Localizing ER Calcium Stores in Pancreatic Acinar Cells

**FIG. 3. Comparison of ATP-driven Ca\(^{2+}\) uptake into Ca\(^{2+}\) stores in apical and basolateral regions of permeabilized pancreatic acinar cells.** For every field of cells analyzed to obtain the Ca\(^{2+}\) uptake data in Fig. 2A, we selected those cells where apical and basal regions could be unambiguously distinguished on the basis of light microscopic imaging (between three and five cells depending on the experiment). A region of interest was then defined in the apical and basolateral region of each selected cell, and a time course of the averaged 340/380 ratio was then computed for apical regions and basolateral regions in each experiment. Finally, time courses from individual experiments were averaged to give the data shown. The data follow the same format as Fig. 2A (i.e. n = three experiments on different cell preparations) but with uptake separated into apical or basolateral regions. ATP-driven Ca\(^{2+}\) uptake is compared for apical (broken lines) and basolateral (solid lines) regions at ambient free Ca\(^{2+}\) concentrations of 60 nM (A), 0.1 \(\mu\)M (B), 0.2 \(\mu\)M (C), or 1 \(\mu\)M (D). Bars show \(\pm\) S.E.

**DISCUSSION**

This study describes the Ca\(^{2+}\) sequestering properties of intracellular Ca\(^{2+}\) stores in pancreatic acinar cells at the subcellular level. In situ imaging of intracellular Ca\(^{2+}\) stores revealed that Ca\(^{2+}\) can be accumulated in all regions of this polarized cell type. The [Ca\(^{2+}\)]\(_{\text{lumen}}\) levels reached at steady state were dependent on the ambient [Ca\(^{2+}\)] used to load the stores. The total Ca\(^{2+}\) taken up by the Ca\(^{2+}\) stores also rose with increasing [Ca\(^{2+}\)]\(_{i}\). These observations show that increased Ca\(^{2+}\) uptake does not result in saturation of the intravesicular Ca\(^{2+}\)-buffering system, since both total Ca\(^{2+}\) and [Ca\(^{2+}\)]\(_{\text{lumen}}\) increased upon elevation of [Ca\(^{2+}\)]\(_{i}\). In addition, these data tend to suggest that a single type of intra-store Ca\(^{2+}\) buffer with a single class of binding site is highly unlikely to account for all intra-luminal Ca\(^{2+}\) buffering.

Detailed subcellular analysis of the Ca\(^{2+}\) uptake process showed that ATP-driven Ca\(^{2+}\) sequestration occurred in both apical and basal regions of the cell. The two subcellular regions did not differ in their capacity to accumulate Ca\(^{2+}\) at lower values of [Ca\(^{2+}\)]\(_{i}\). At 0.2 and 1.0 \(\mu\)M [Ca\(^{2+}\)]\(_{i}\), however, stores in the basal area were able to accumulate significantly more Ca\(^{2+}\) than stores in the apical region, as judged by a higher steady-state Magfura ratio. Immunohistochemistry revealed a higher density of SERCA-2b Ca\(^{2+}\)-ATPases in the basal area. However, this most probably reflects the simple fact that the basal area contains relatively more endoplasmic reticulum (8). We conclude that intracellular endoplasmic reticulum stores in the apical and basal area are able to accumulate Ca\(^{2+}\) but that stores in the basal area have an increased capacity for storing Ca\(^{2+}\).

In our previous study we showed that, at an ambient [Ca\(^{2+}\)]\(_{i}\) of 0.2 \(\mu\)M, the steady-state intravesicular [Ca\(^{2+}\)] was 70 \(\mu\)M (11). This figure can be compared with the measured total thapsigargin-sensitive Ca\(^{2+}\) uptake under identical conditions, which was 19 mmol of Ca\(^{2+}\) per liter of endoplasmic reticulum (Fig. 1B). From this comparison we conclude that around 270 Ca\(^{2+}\) ions are bound for every free Ca\(^{2+}\) ion. This calculation confirms that most stored Ca\(^{2+}\) is buffered within intracellular Ca\(^{2+}\) stores. Despite this heavy Ca\(^{2+}\) buffering, large amounts of Ca\(^{2+}\) are immediately accessible for rapid mobilization from these stores in both intact and permeabilized cell systems. This indicates that stored Ca\(^{2+}\) is not tightly bound or trapped once accumulated.

Calreticulin is proposed to act as a major Ca\(^{2+}\) buffer within Ca\(^{2+}\) stores, as well as having an important role as an intra-ER molecular chaperone (19). The calreticulin molecule has the ability to bind up to 25 mol of Ca\(^{2+}\) per mol with a low milliolar affinity. Pancreatic tissue is the richest known source of calreticulin (calreticulin content of the pancreas is 540 \(\mu\)g/g of tissue (20)), presumably because the high protein synthesis rate of acinar cells imposes a requirements for molecular chaperones. Simple calculation reveals that the calreticulin concentration within the pancreatic endoplasmic reticulum is about 52 \(\mu\)mol/liter ER.\(^2\) Our estimate of [Ca\(^{2+}\)]\(_{\text{lumen}}\) indicates that calreticulin will not be saturated under these conditions. Even if one assumes a [Ca\(^{2+}\)]\(_{\text{lumen}}\) of 1 \(\mu\)M (instead of our 70 \(\mu\)M

\(^2\) Eighty-two percent of pancreatic volume consists of acinar cells (8). To simplify the calculation we have assumed that all calreticulin in the pancreas is contained in acinar cells.
estimate), as has been suggested for other cell types on the basis of work with ER-targeted aequorin (21), calreticulin can only account for the binding of 3–4% of the total amount of stored Ca\(^{2+}\). This calculation shows that calreticulin may not be as important in organellar Ca\(^{2+}\) buffering as originally proposed. This is in agreement with recent work on fibroblasts from calreticulin null mice, which showed no differences in ER Ca\(^{2+}\) storage from wild-type cells (22). In fact, recent studies have provided evidence that calreticulin may have other func-

![Image](image1)

**FIG. 4.** Thapsigargin, or thapsigargin followed by InsP\(_3\), releases Ca\(^{2+}\) from loaded intracellular Ca\(^{2+}\) stores in both apical and basolateral regions of permeabilized pancreatic acinar cells. Permeabilized pancreatic acinar cells were superfused with a solution containing ATP and 200 nM free [Ca\(^{2+}\)] for 15 min prior to the start of the record to enable ATP-driven Ca\(^{2+}\) uptake to reach a steady state. Thapsigargin (1 \(\mu\)M) was then added (in the continued presence of ATP and Ca\(^{2+}\)) to block SERCA Ca\(^{2+}\) pumps and induce Ca\(^{2+}\) leak from the stores. Ratio images were acquired every minute in A or every 15 s in B, and data were obtained as described in the legend to Fig. 3. The format follows that of Fig. 3, with the averaged Magfura-2 ratio ([Ca\(^{2+}\)\(_{human}\) shown for apical (broken lines) or basolateral (solid lines) regions. Bars show S.E. A shows Ca\(^{2+}\) release evoked by prolonged exposure to thapsigargin alone and represents analysis of 14 cells derived from three experiments on different cell preparations. B shows the effect of addition of InsP\(_3\) after thapsigargin and represents analysis of 21 cells derived from three experiments on different cell preparations. In B the data were normalized to the maximal store size, defined as the difference between the ratio before and after Ca\(^{2+}\) loading (see text for details).

**FIG. 5.** Distribution of SERCA-2B Ca\(^{2+}\)-ATPases and calreticulin in isolated pancreatic acinar cells. Immunofluorescence localization of SERCA-2B Ca\(^{2+}\)-ATPases (A) and calreticulin (B) in isolated pancreatic acinar cells by conventional fluorescence microscopy using polyclonal antisera. In each panel two separate acinar cell clusters are shown; the left-hand images show the cells in transmitted light, while the right-hand images show the immunofluorescence micrographs. Both the SERCA-2B Ca\(^{2+}\)-ATPase and calreticulin appeared to be present in all regions of the acinar cell, with the strongest staining in the basolateral cytoplasm. The less intensely stained regions in the basolateral areas of the cells indicate the presence of the nucleus. Calreticulin distribution was noticeably more punctate than was SERCA-2B distribution. Both panels are representative of at least three experiments on different cell preparations. Results shown in A were obtained using SERCA-2B Ca\(^{2+}\)-ATPase antiserum provided by Dr. F. Wuytack (Leuven) (16, 17). Essentially identical results (not shown) were obtained using a different polyclonal antiserum provided by Dr. R. L. Dormer (Cardiff) (18). Scale bar = 10 \(\mu\)m.
tions in Ca\(^{2+}\) signaling than organellar Ca\(^{2+}\) buffering. For instance, although calreticulin overexpression inhibited Ca\(^{2+}\) waves in *Xenopus* oocytes, this action was found to be associated with the high affinity, low capacity, Ca\(^{2+}\)-binding site, rather than with the low affinity, high capacity binding site which confers Ca\(^{2+}\) buffering properties (23). In addition, recent overexpression studies in epithelial and fibroblast cell lines revealed that calreticulin may play an important role in the regulation of Ca\(^{2+}\) influx (24, 25). Finally, the knockout studies indicate that calreticulin plays a critical role in transmitting signals from integrin extracellular matrix receptors to the cell interior (22).

As already discussed above, we can use morphological data to express our permeabilized cell Ca\(^{2+}\) uptake data in terms of Ca\(^{2+}\) uptake per unit volume of rough endoplasmic reticulum. It is interesting to compare these data with Ca\(^{2+}\) fluxes observed in intact acinar cell systems. In isolated intact pancreas, the neurotransmitter acetylcholine can mobilize 0.5 μmol of Ca\(^{2+}\) per g of tissue.\(^5\) Assuming the endoplasmic reticulum is the agonist-sensitive intracellular Ca\(^{2+}\) storage site, this value suggests the Ins\(_P_3\)-sensitive store holds approximately 2.6 mmol of Ca\(^{2+}\) per liter of endoplasmic reticulum. Dormer et al. (27) reported that agonists mobilized around 3 nmol of Ca\(^{2+}\) per mg of protein from intact acini, equal to approximately 1.8 mmol of Ca\(^{2+}\) per liter rough endoplasmic reticulum.\(^4\) In permeabilized pancreatic acinar cells, 60% of the thapsigargin-sensitive Ca\(^{2+}\) store is Ins\(_P_3\)-sensitive (13, 14). Although the corresponding value for the thapsigargin-sensitive store in intact pancreas is not known, the thapsigargin-sensitive Ca\(^{2+}\) store is known to be bigger than the agonist-sensitive intracellular store (29). If we assume that the Ins\(_P_3\)-sensitive store in intact tissues accounts for 60% of the thapsigargin-sensitive store, then the thapsigargin-sensitive intracellular Ca\(^{2+}\) stores of intact cells would contain about 4 mmol of Ca\(^{2+}\) per liter endoplasmic reticulum. These calculations show that intracellular Ca\(^{2+}\) stores in permeabilized cell systems are not by any means reduced compared with those in intact cells. In fact, under the standard conditions used in our study (0.2 mM free Ca\(^{2+}\)) intracellular Ca\(^{2+}\) stores in permeabilized cells accumulate considerably more Ca\(^{2+}\) than in intact cells. The most likely explanation for this difference is that the ambient free [Ca\(^{2+}\)] which we have used to load the stores is higher than the free [Ca\(^{2+}\)] in unstimulated intact cells. At the lowest ambient (loading) [Ca\(^{2+}\)] used in this study, 60 mM, the stores accumulated around 6 mmol of Ca\(^{2+}\) per liter endoplasmic reticulum. This suggests, albeit indirectly, that the cytosolic [Ca\(^{2+}\)], under resting conditions in intact cells is below 60 nM.

It well established that the endoplasmic reticulum can be found in all regions of the pancreatic acinar cell, including the apical pole (e.g. Refs. 8 and 11). The ER is, therefore, a plausible candidate for an apical pole Ca\(^{2+}\) store. The same is true for other acinar cell types, for instance lacrimal cells (see Ref. 6 for references). In our previous work we have shown that all ATP-dependent Ca\(^{2+}\) uptake in permeabilized acinar cells can be inhibited by the SERCA Ca\(^{2+}\)-ATPase inhibitor thapsigargin, indicating that the endoplasmic reticulum is wholly responsible for ATP-driven Ca\(^{2+}\) sequestration (13). The *in situ* imaging experiments in the present study confirm that ATP-driven Ca\(^{2+}\) uptake can indeed be observed in all regions of the acinar cell. To seek further evidence that the endoplasmic reticulum can act as Ca\(^{2+}\) stores in all cellular regions, we mapped the distribution of the SERCA-2B isoform of the microsomal Ca\(^{2+}\) pump, known to be present in acinar cells (18), at low (non-confocal) resolution. Immunofluorescence demonstrated that SERCA-2B Ca\(^{2+}\)-ATPases are present in all regions of the pancreatic acinar cell. In keeping with this result, addition of thapsigargin caused depletion of Ca\(^{2+}\) stores in all cellular regions. We can therefore conclude that Ca\(^{2+}\) can be actively reaccumulated by the endoplasmic reticulum in any area of the acinar cell. Calcium accumulation will occur during rises in [Ca\(^{2+}\)]\(_i\), regardless of whether the increase in [Ca\(^{2+}\)]\(_i\) derives from intracellular stores or from influx from the extracellular environment.

A functional agonist-sensitive Ca\(^{2+}\) store minimally requires an active Ca\(^{2+}\) uptake mechanism, an intra-store Ca\(^{2+}\) buffering system, and a Ca\(^{2+}\) release mechanism. The present work clearly shows that the first two are ubiquitous in the acinar cell cytoplasm. In our previous work we demonstrated that the Ins\(_P_3\)-sensitive release mechanism is also present in all subcellular regions of the acinar cell (11). This latter result is re-emphasized in Fig. 4B, which shows that thapsigargin alters the kinetics of Ins\(_P_3\)-evoked Ca\(^{2+}\) release in both apical and basolateral poles (contrast Fig. 4B with Fig. 6B of Ref. 11). This shows that the Ca\(^{2+}\) store compartment(s) where the Ins\(_P_3\)-evoked Ca\(^{2+}\) release mechanism is located are loaded by a thapsigargin-sensitive uptake mechanism. It is thus clear that endoplasmic reticulum stores able to take up store, and release Ca\(^{2+}\) are present in the apical pole of pancreatic acinar cells.

The present study and the recent work of Mogami et al. (30) show unequivocally that the endoplasmic reticulum in the apical pole can act as a functional Ca\(^{2+}\) store. Zymogen granules have also been suggested to be an Ins\(_P_3\)-sensitive Ca\(^{2+}\) storage compartment in this cellular region, a view based largely on work on an isolated pancreatic zymogen granule preparation (2). However, a very recent cell fractionation study concluded that zymogen granules did not show Ins\(_P_3\)-sensitive Ca\(^{2+}\) release (10). In fact, Yule et al. (10) showed that the zymogen granule preparation used by Gerasimenko et al. (2) was contaminated with mitochondria, nuclei, and endoplasmic reticulum. This “mixed” preparation showed Ca\(^{2+}\) release on treatment with Ins\(_P_3\), although Ca\(^{2+}\) release was not evoked by thapsigargin (2, 10).\(^5\) Further purification resulted in a homogenous zymogen granule preparation that had lost its Ins\(_P_3\) sensitivity, although the granules did contain Ca\(^{2+}\), which could be released using Ca\(^{2+}\) ionophores (10). Immunolocalization studies in intact cells also failed to detect any Ins\(_P_3\) receptors in the granule region (32), and it has been suggested very recently that some zymogen granules may “acquire” Ins\(_P_3\) sensitivity during isolation by fusion with nearby membrane domains containing high concentrations of Ins\(_P_3\) receptors (33).

In addition to the recent work of Yule et al. (10) discounting the role of zymogen granules in [Ca\(^{2+}\)]\(_i\) signaling, several older studies employing radioactive 45Ca\(^{2+}\) in isolated intact pancreas also specifically ruled out zymogen granules as an agonist-mobilizable Ca\(^{2+}\) store (34–36). In particular, zymogen granules were unable to exchange Ca\(^{2+}\) for radioactive 45Ca\(^{2+}\) despite prolonged labeling protocols (34–36). The authors of

\(^5\) From the lack of effect of thapsigargin, it might be argued that Ins\(_P_3\) sensitivity in the mixed granule preparation resides in an organelle other than the endoplasmic reticulum. However, this argument must be regarded with caution, since it is well known that cell homogenization and purification of the microsomal fraction of many different tissues results in a separation of Ca\(^{2+}\) release and accumulation sites. For instance, only 10% of the Ca\(^{2+}\) accumulated by ATP-dependent uptake in pancreatic microsomes is released by Ins\(_P_3\) (31), compared with a corresponding figure of 60% in permeabilized acinar cell preparations (13, 14).

Data from Fig. 2 of Ref. 26. The pancreas primarily consists of acinar cells (82% of its volume (8)), and we assume for the sake of simplicity that all agonist-induced Ca\(^{2+}\) fluxes originate from acinar cells.

Calculated by assuming 1 g of pancreas contains 144 mg of protein (28).
these studies concluded that during protein processing the divalent cations Ca\(^{2+}\) and Mg\(^{2+}\) become trapped inside zymogen granules (34–36). This is consistent with the classical observation that, during agonist stimulation, pancreatic enzyme secretion is tightly correlated with the secretion of Ca\(^{2+}\) and Mg\(^{2+}\) into the luminal space (where divalent cations are believed to be necessary for the activation of digestive enzymes) (37, 38).

In conclusion, InsP\(_3\)-sensitive Ca\(^{2+}\) stores can only act as fully functional Ca\(^{2+}\) stores when they are equipped with a Ca\(^{2+}\)-pumping mechanism. This is mandatory in order for the stores to refill following depletion. The combination of techniques applied in this study to investigate organellar Ca\(^{2+}\) uptake provides clear evidence that the endoplasmic reticulum can act as a functional Ca\(^{2+}\) store in all subcellular regions of the pancreatic acinar cell.

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