Imaging of Intracellular Calcium Stores in Individual Permeabilized Pancreatic Acinar Cells

APPARENT HOMOGENEOUS CELLULAR DISTRIBUTION OF INOSITOL 1,4,5-TRISPHOSPHATE-SENSITIVE STORES IN PERMEABILIZED PANCREATIC ACINAR CELLS*

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Several lines of evidence suggest that the existence of a heterogeneous population of inositol 1,4,5-trisphosphate (Ins(1,4,5)P3)-sensitive Ca2+ stores underlies the polarized agonist-induced rise in cytosolic Ca2+ concentration ([Ca2+]i) in pancreatic acinar cells (Kasai, H., Li, Y. X., and Miyashita, Y. (1993) Cell 74, 669–677; Thorn, P. M., Lawrie, A. M., Smith, P. M., Gallacher, D. V., and Petersen, O. H. (1993) Cell 74, 661–668). To investigate whether the apical pole of acinar cells contains Ca2+ stores which are relatively more sensitive to Ins(1,4,5)P3 than those in basolateral areas, we studied Ca2+ handling by Ca2+ stores in individual streptolysin O (SLO) permeabilized cells using the low affinity Ca2+ indicator Magfura-2 and an in situ imaging technique. The uptake of Ca2+ by intracellular Ca2+ stores was ATP-dependent. A steady-state level was reached within 10 min, and the free Ca2+ concentration inside loaded Ca2+ stores was estimated to be 70 μM. Ins(1,4,5)P3 induced Ca2+ release in a dose-dependent, “quantal” fashion. The kinetics of this release were similar to those reported for suspensions of permeabilized pancreatic acinar cells. Interestingly, the permeabilized acinar cells showed no intracellular variation in Ins(1,4,5)P3 sensitivity. Although SLO treatment is known to result in a considerable loss of cytosolic factors, permeabilization did not result in a redistribution of zymogen granules, as judged by electron microscope analysis. These results suggest that Ins(1,4,5)P3-sensitive Ca2+ stores are unlikely to be redistributed as a result of SLO treatment. The effects of Ins(1,4,5)P3 were therefore subsequently studied at the subcellular level. Detailed analysis demonstrated that no regional differences in Ins(1,4,5)P3 sensitivity exist in this permeabilized cell system. Therefore, we propose that additional cytosolic factors and/or the involvement of ryanodine receptors underlie the polarized pattern of agonist-induced Ca2+ signaling in intact pancreatic acinar cells.

In many non-excitable cell types, agonist stimulation results in repetitive oscillations of the free cytosolic Ca2+ concentration ([Ca2+]i), arising largely from Ca2+ release from intracellular stores (1). In the polarized exocrine acinar cell of exocrine glands, these agonist-induced intracellular Ca2+ signals are not spatially homogeneous. Thus acetylcholine- or cholecystokinin-octapeptide-induced [Ca2+]i rises are initiated in the luminal pole of the cell with the Ca2+ wave subsequently spreading into the basolateral areas of the cell (2–4). In some cases little or no [Ca2+]i increase at all is observed in basolateral regions (5, 6). This spatial pattern of [Ca2+]i signaling has been suggested to be important for both unidirectional fluid secretion (2, 7) and exocytosis (8).

In permeabilized pancreatic acinar cells, as in many other permeabilized cell systems, the intracellular messenger 3,5-di-O-myo-inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) mobilizes Ca2+ from non-mitochondrial intracellular Ca2+ stores (1). Recent evidence in both permeabilized (10–12) and intact cells (5, 6) favors the existence of a heterogeneous population of Ins(1,4,5)P3-sensitive Ca2+ stores. In a recent study (12) it was suggested that the Ca2+ imaging data obtained by several laboratories (see above) might be explained by the existence of stores more sensitive to Ins(1,4,5)P3 localized in the apical pole with stores less sensitive to Ins(1,4,5)P3 being located in basolateral areas.

To address this question we have now imaged the Ca2+ concentration within Ca2+ stores in pancreatic acinar cells using the in situ imaging technique originally described by Hofer and Machen (13). The low affinity Ca2+ indicator Magfura-2 was loaded into intracellular stores and the properties of these stores were studied in streptolysin O-permeabilized cells. ATP-driven Ca2+ uptake and Ins(1,4,5)P3-dependent Ca2+ release could be clearly demonstrated within individual permeabilized pancreatic acinar cells. However, we were unable to detect any subcellular regional differences in Ins(1,4,5)P3 sensitivity. This may indicate that cytosolic modulators of Ins(1,4,5)P3-operated Ca2+ channels and/or the involvement of ryanodine receptors, underlie the polarized nature of [Ca2+]i signaling patterns in acinar cells.

**EXPERIMENTAL PROCEDURES**

Pancreatic Acinar Cells—Acinar cells were prepared from the pancreas of one 200-g male Sprague-Dawley rat. The cell isolation procedure used was the same as described previously for rabbit pancreas (12). After isolation, acinar cells were resuspended in 6 ml of a HEPES/Tris-buffered (pH 7.4) physiological medium which contained: 133 mM NaCl, 4.2 mM KCl, 1.0 mM CaCl2, 1.0 mM MgCl2, 5.8 mM glucose, 0.2 mg/ml soybean trypsin inhibitor, an amino acid mixture according to Eagle (14), 1% (w/v) bovine serum albumin, and 10 mM HEPES. The pH of the medium was set at 7.4 with Tris. Cells were either used immediately or stored in 1-ml portions on ice until use.

Loading of Pancreatic Acinar Cells with Magfura-2—Pancreatic acinar cells were resuspended in the physiological medium described above with the addition of 5 μM Magfura-2-AM. After a 30-min incubation at 37 °C, cells were washed twice with physiological medium containing 0.1% bovine serum albumin. Cells were allowed to settle on a
poly-L-lysine-coated glass coverslip which formed the bottom of a perfusion chamber.

Imaging of Magfura-2-loaded Pancreatic Acinar Cells—The imaging system used was based on an inverted epifluorescence Nikon Diaphot microscope and a ×40 oil immersion lens (numerical aperture 1.3). A field containing 5–15 cells was selected and the dye-loaded cells were excited alternately with light at 340 and 380 nm using a filterwheel (Lambda 10, Sutter Instruments; 340- and 380-nm band-pass filters were from Ealing Electro-Optics) and a dichroic mirror (400-nm dichroic mirror, 420-nm barrier filter). The emitted fluorescence was captured and digitized at 12-bit resolution by a slow scan CCD camera (Digital Pixel Ltd., Brighton, United Kingdom). An IBM-compatible personal computer with an imaging software package (Kinetic Imaging Ltd., Liverpool, UK) was used to drive the filterwheel and camera and store acquired images. The size of the silicon sensor of the camera and the ×40 objective allowed images of a field of 90 × 135 μm to be captured.

A 3 × 3 binning was applied to the individual pixels on the image sensor to give a spatial resolution of 0.67 μm/pixel. Since acinar cells have virtually no detectable autofluorescence (results not shown) an empty area was background corrected. The ratio of the 340 nm and 380 nm excitations of paired, background-subtracted images were calculated offline. All experiments were performed at room temperature.

Permeabilization of Pancreatic Acinar Cells—Acinar cells were perfused with Ca2+ uptake medium containing: 135 mM KCl, 1.2 mM KH2PO4, 0.5 mM EGTA, 0.5 mM Na3citrate, 0.5 mM nitrolitratricarboxylic 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. (15). SLO (0.4 IU/ml) was used to permeabilize acinar cells.

Since a considerable amount of the total accumulated Magfura-2 was present in the cytotoxic compartment, the permeabilization process could be followed on-line (as loss of cytosolic dye) by using the imaging system. At the start of the permeabilization procedure, loaded cells were excited at the isosbestic wavelength for Magfura-2, i.e. 360 nm. Permeabilization was achieved within 10 min and, as a consequence, a significant drop in fluorescence was observed as cytotoxic Magfura-2 was released into the incubation medium (results not shown). Perfusion of the permeabilized cells was subsequently continued with the Ca2+ uptake medium devoid of SLO, as described above.

Ca2+ Uptake and Release Experiments—Permeabilized cells were continuously perfused throughout the experiments. Ca2+ uptake by intracellular Ca2+ stores was initiated by superfusing cells with a medium containing 1 mM ATP and a free Ca2+ concentration of 0.2 μM; the Ca2+ concentration in the incubation medium remained 0.9 mM (free divalent cation concentrations were calculated again according to Schoenmakers et al. (15)). Mitochondrial Ca2+ uptake inhibitors were not included in the medium, since mitochondrial Ca2+ uptake has previously not been shown not to occur at this ambient free Ca2+ concentration (16). After a loading period of 15 min, permeabilized cells were stimulated with Ins(1,4,5)P3, thapsigargin, thapsigargin-cyclic ADP-ribose from Amersham International plc, Buckinghamshire, UK. All other chemicals were of analytical grade.

### RESULTS

Effect of Streptolysin O Treatment on Pancreatic Acinar Cells—The morphology of pancreatic acinar cells was altered after permeabilization. Acinar cells had a more swollen appearance in the light microscope, and the cytosol became paler, reflecting loss of proteins. Permeabilized cells coupled to each other in doublets or triplets clearly retained their polarized morphology, whereas the polarity in individual cell was, just as in intact cells, largely lost (see e.g. Fig. 5B for an example). To study the effect of SLO treatment on the cellular structure in more detail, electron microscopy was performed on both intact and permeabilized cells. Fig. 1A shows a representative triplet of intact acinar cells. Cells retained a polarized morphology, since the large densely stained secretory granules were restricted to the apical pole. The endoplasmic reticulum retained its typical tubular arrangement and was present in all parts of the cell, although it was more abundant in basolateral regions. A representative example of permeabilized pancreatic acinar cells is shown in Fig. 1B. The cells had a much brighter appearance, reflecting loss of the majority of the cytosolic content after permeabilization. The picture shows two coupled cells which were, similar to intact cells, polarized. The endoplasmic reticulum was rearranged but appeared to be continuous. The changes in endoplasmic reticulum morphology may be due to the swelling of the cells after permeabilization.

### Effect of Streptolysin O on Intracellular Calcium Stores

- **Results:**
  - The mean ratio values mentioned under “Results” were calculated by determining ratio values for each of the individual cells in a field. From these values, an average value for a particular experiment was determined. These values were in turn averaged between preparations to give the values presented in the text. The value of n given thus refers to the number of cell preparations on which a given experiment was performed, although the total number of individual cells analyzed was between 19 and 180 for different experimental procedures.

### Materials—Chemicals

- SLO from Difco; thapsigargin from Calbiochem; Ins(1,4,5)P3 and 4-Br-A23187 from Sigma; Magfura-2-AM from Molecular Probes, Eugene, OR; cyclic ADP-ribose from Amersham International plc, Buckinghamshire, UK. All other chemicals were of analytical grade.

### Effect of Streptolysin O Treatment on Pancreatic Acinar Cells—The morphology of pancreatic acinar cells was altered after permeabilization. Acinar cells had a more swollen appearance in the light microscope, and the cytosol became paler, reflecting loss of proteins. Permeabilized cells coupled to each other in doublets or triplets clearly retained their polarized morphology, whereas the polarity in individual cell was, just as in intact cells, largely lost (see e.g. Fig. 5B for an example). To study the effect of SLO treatment on the cellular structure in more detail, electron microscopy was performed on both intact and permeabilized cells. Fig. 1A shows a representative triplet of intact acinar cells. Cells retained a polarized morphology, since the large densely stained secretory granules were restricted to the apical pole. The endoplasmic reticulum retained its typical tubular arrangement and was present in all parts of the cell, although it was more abundant in basolateral regions. A representative example of permeabilized pancreatic acinar cells is shown in Fig. 1B. The cells had a much brighter appearance, reflecting loss of the majority of the cytosolic content after permeabilization. The picture shows two coupled cells which were, similar to intact cells, polarized. The endoplasmic reticulum was rearranged but appeared to be continuous. The changes in endoplasmic reticulum morphology may be due to the swelling of the cells after permeabilization.

**Ca2+ Uptake by Permeabilized Pancreatic Acinar Cells—** After permeabilization, cells were exposed to an “intracellular” medium with an ambient free Ca2+ concentration of 0.2 μM Ca2+ and 1 mM ATP. This resulted in an increase in the Magfura ratio, presumably reflecting an increase in the free Ca2+ concentration in intracellular Ca2+ stores (Fig. 2). To rule out the possibility that changes in the Magfura ratio were due to changes in extra-organelle [Mg2+]i, rather than [Ca2+]i, the experimental protocol was adapted by perfusing cells initially with Ca2+ uptake medium devoid of Ca2+ but containing ATP and Mg2+. Fig. 2 shows that the ratio remained unaltered and could only be increased by including Ca2+ in the medium. The combined addition of Ca2+ and ATP increased the Magfura ratio from 0.53 (S.E. = 0.04; n = 14 cell preparations and 137 individual cells analyzed) to 1.44 (S.E. = 0.04; n = 16 cell preparations and 178 individual cells analyzed) within 10 min. This increased level remained constant for a considerable period thereafter (at least 10 min).

To demonstrate that the Magfura signal was not saturated in loaded Ca2+ stores under these conditions, the Ca2+ ionophore 4-Br-A23187 (2 μM) was included and the ambient free Ca2+ concentration in the medium was increased to 1 mM (for details of the medium used, see “Experimental Procedures”). This treatment resulted in a further increase in the ratio to 3.43 (S.E. = 0.02; n = 3 cell preparations and 19 individual cells analyzed; results not shown, but see also Fig. 3). This indicates that the free Ca2+ concentration of steady-state loaded intracellular Ca2+ stores of pancreatic acinar cells is not in the millimolar range. A tentative calibration was made by perfus-
ing permeabilized cells with Ca\(^{2+}\) ionophore and a medium containing different ambient free Ca\(^{2+}\) concentrations. Fig. 3 shows the result of this calibration. The average Magfura ratio in stores loaded at steady-state was 1.44 (see above), and therefore the free Ca\(^{2+}\) concentration in loaded intracellular Ca\(^{2+}\) stores is estimated to be 70 \(\mu\)M. The minimum ratio, obtained with permeabilized cells in a Ca\(^{2+}\) free medium, including ionophore, was 0.37 (S.E. = 0.02; n = 4 cell preparations and 37 individual cells analyzed). The average ratio in unloaded stores without ionophore in the same set of experiments was 0.47 (S.E. = 0.03; n = 4 cell preparations and 37 cells analyzed). This shows that, before initiation of Ca\(^{2+}\) uptake, the Ca\(^{2+}\)
stores in the permeabilized cells were virtually depleted of their Ca\(^{2+}\) content.

Effects of Ins(1,4,5)P\(_3\) on Loaded Intracellular Ca\(^{2+}\) Stores—Perfusion of permeabilized pancreatic acinar cells with an Ins(1,4,5)P\(_3\) containing medium resulted in a release of Ca\(^{2+}\), indicated by a fall in the Magfura ratio. Fig. 4, A and B, show the effect of 0.3 and 1.0 \(\mu\)M Ins(1,4,5)P\(_3\), respectively, on loaded stores in permeabilized cells. Ins(1,4,5)P\(_3\) induced a release of Ca\(^{2+}\) in both cases. The rate of release, and the new reduced intravesicular [Ca\(^{2+}\)] achieved, both varied with the concentration of Ins(1,4,5)P\(_3\) applied. The intravesicular Ca\(^{2+}\) levels could be further reduced by increasing the Ins(1,4,5)P\(_3\) concentration, which also indicated that the release process was not desensitized despite the 12 min presence of submaximal doses of Ins(1,4,5)P\(_3\). At 10 \(\mu\)M Ins(1,4,5)P\(_3\), a maximal Ca\(^{2+}\) releasing effect was obtained and the ratio was reduced to 0.62 (S.E. = 0.02, \(n = 5\) cell preparations and 52 individual cells analyzed). This level was close to the ratio in unloaded stores, indicating that the Ca\(^{2+}\) stores were virtually emptied by a maximally effective dose of Ins(1,4,5)P\(_3\) (10 \(\mu\)M shown to be a maximally effective dose, since 30 \(\mu\)M did not induce any further Ca\(^{2+}\) release). The half-maximal effect of Ins(1,4,5)P\(_3\) occurred at 0.3 \(\mu\)M, a value slightly lower than, but in a range similar to that reported by others using permeabilized pancreatic acinar cell preparations (e.g. Refs. 9, 10, 19, and 20).

In permeabilized gastric epithelial cells, mitochondrial uptake of Ca\(^{2+}\) contributed substantially to the ATP- and Ca\(^{2+}\)-dependent increase in the signal from compartmentalized Magfura-2 (21). Thus a considerable part of the ATP-dependent Ca\(^{2+}\) pool in this cell type was Ins(1,4,5)P\(_3\)-insensitive but sensitive to mitochondrial Ca\(^{2+}\) uptake inhibitors. In pancreatic acinar cells, however, a maximal dose of Ins(1,4,5)P\(_3\) released virtually all the Ca\(^{2+}\) that had been taken up in an ATP-dependent manner, maintaining that mitochondria were not active under the conditions used (see above). In addition, when Ca\(^{2+}\) uptake was performed in the presence of the mitochondrial inhibitors antimycin and oligomycin, no change in uptake characteristics was observed (results not shown). These observations confirm previous studies with radiotracer techniques on permeabilized pancreatic acinar cells, in which it was shown that mitochondrial Ca\(^{2+}\) uptake was inactive at an ambient free Ca\(^{2+}\) concentration identical to that used in the present study (16).

Effects of Ins(1,4,5)P\(_3\) on Cellular and Subcellular Level—We went on to study the kinetics and sensitivity of Ins(1,4,5)P\(_3\)-induced Ca\(^{2+}\) release in individual cells in more detail. Fig. 5A shows the effect of sequential additions of increasing Ins(1,4,5)P\(_3\) concentrations. The addition of 0.1 \(\mu\)M Ins(1,4,5)P\(_3\) resulted in a small further reduction of the Ca\(^{2+}\) levels in the intracellular Ca\(^{2+}\) stores. Subsequent addition of 0.3 \(\mu\)M induced a more pronounced release, which leveled off within 5 min of application (see also Fig. 4A). The addition of 1.0 \(\mu\)M Ins(1,4,5)P\(_3\) nearly completed the Ca\(^{2+}\) release process, since the maximal effective dose of 10.0 \(\mu\)M Ins(1,4,5)P\(_3\) induced only a small further reduction of the Magfura ratio. The “steps” in store Ca\(^{2+}\) content on increasing Ins(1,4,5)P\(_3\) resembled the so-called “quantal” Ca\(^{2+}\) release previously observed in these and other cells (10, 12, 22). The effect of Ins(1,4,5)P\(_3\) was reversible, since reperfusion with control medium resulted in a reuptake of Ca\(^{2+}\) (this reversibility was tested in four experiments and was observed in all cases). Fig. 5A also shows that the effects of Ins(1,4,5)P\(_3\) were observed in all seven cells analyzed from this field. Ins(1,4,5)P\(_3\) evoked a simultaneous and equal response in all cells, indicating that there were no intercellular differences in Ins(1,4,5)P\(_3\) sensitivity.

The action of Ins(1,4,5)P\(_3\) was studied in more detail by analyzing the ratio images of the experiment. Fig. 5, B and C show a brightfield and a fluorescence image respectively of the selected field of cells. The brightfield image shows again that permeabilized cells organized in triplets clearly maintained their polarized morphology. The first ratio image (Fig. 5D, image 1) shows Ca\(^{2+}\) stores in permeabilized cells loaded to a steady-state level. The ratio intensity throughout the different regions of the cells was not homogeneous, although virtually all regions had a ratio value of about 1 or higher (i.e. the free [Ca\(^{2+}\)] was 40 \(\mu\)M or higher). Stimulation with a low dose of Ins(1,4,5)P\(_3\) (0.3 \(\mu\)M) resulted in a simultaneous decrease of Ca\(^{2+}\) levels in all regions of the permeabilized cells (Fig. 5D, images 2 and 3). Elevation of the dose to 1.0 \(\mu\)M (Fig. 5D, image 4) and then to 10.0 \(\mu\)M (Fig. 5D, image 5) resulted again in a simultaneous reaction in all subcellular regions in all permeabilized acinar cells. Subsequent removal of Ins(1,4,5)P\(_3\) resulted in Ca\(^{2+}\) reuptake in all regions of the permeabilized cells (Fig. 5D, image 6). (The regional analysis, as presented in Fig. 5D, was performed in four additional experiments; in two experiments sequential Ins(1,4,5)P\(_3\) additions were made as presented in Fig. 5, and in the other two experiments, 0.3 \(\mu\)M Ins(1,4,5)P\(_3\) added to loaded stores of permeabilized cells. All
FIG. 5. Effect of stepwise increases in Ins(1,4,5)P₃ concentration on loaded intracellular Ca²⁺ stores. Intracellular stores of permeabilized pancreatic acinar cells loaded Ca²⁺ in an ATP-dependent fashion for 15 min. The experiment was started and image pairs were collected at 15 s intervals. After 1.5 min 0.1 μM Ins(1,4,5)P₃ was included for 5 min in the medium and its concentration was increased at 5-min intervals to 0.3, 1.0, and 10 μM. Finally, permeabilized cells were reperfused with control medium until the end of the experiment. A, individual responses of seven acinar cells plotted in a similar format to that of Fig. 2. B and C show, respectively, a brightfield image and a fluorescence image (340 nm excitation). Six ratio images are presented in D. The ratio values were converted to pseudocolor to clarify the ratio levels measured. The pseudocolor scale used is shown at the side. The images shown are taken at time points indicated with the corresponding numbers in A. The calibration squares in B and C, and the sizes of the boxes used to number the images in D, represent 25 μm. The results shown are representative for three independent experiments.

experiments analyzed gave similar results to those presented in Fig. 5). To further demonstrate the uniform Ins(1,4,5)P₃ sensitivity, the kinetics of Ins(1,4,5)P₃-induced Ca²⁺ release were compared between selected areas of interest in apical and basolateral regions in the same field of cells. Fig. 6A shows the selected areas and Fig. 6B shows the averaged and normalized kinetics of Ca²⁺ release induced by Ins(1,4,5)P₃ in apical and basolateral regions. Again, the results demonstrate that both regions were equally sensitive to Ins(1,4,5)P₃. Taken together, the results demonstrate that Ca²⁺ stores in SLO-permeabilized acinar cells display neither regional nor intercellular differences in their sensitivity toward Ins(1,4,5)P₃.

Effect of Thapsigargin on Ins(1,4,5)P₃-induced Ca²⁺ Release—To study the effect of Ca²⁺ pump activity on Ins(1,4,5)P₃-induced Ca²⁺ release, thapsigargin was used to completely block all Ca²⁺ pumping into the intracellular Ca²⁺ stores (12, 16). Addition of thapsigargin (1 μM) resulted in a slow but sustained efflux of Ca²⁺ from the stores with kinetics similar to that observed in permeabilized acinar cell suspensions (Fig. 7). Addition of 0.1 μM Ins(1,4,5)P₃ resulted in an increased efflux rate, indicating that Ins(1,4,5)P₃-operated Ca²⁺ channels were indeed activated at this low dose. After 5 min the Ins(1,4,5)P₃ concentration was increased to 0.3 μM; the efflux rate increased again and remained elevated. Under these conditions suboptimal doses of Ins(1,4,5)P₃ were indeed more effective in depleting stores compared with the situation where Ca²⁺ pumps remained active. The Ins(1,4,5)P₃-sensitive Ca²⁺ stores in the acinar cells were virtually depleted after prolonged treatment with 0.3 μM Ins(1,4,5)P₃ since a further increase of the Ins(1,4,5)P₃ concentration to 1.0 μM evoked only a minor further release of Ca²⁺.

DISCUSSION

The major aim of the present study was to characterize the spatial organization of intracellular Ca²⁺ stores in pancreatic acinar cells. To address this question we investigated Ca²⁺ stores in individual permeabilized pancreatic acinar cells using a Ca²⁺-sensitive dye compartmentalized in organelles. Our main finding is that Ins(1,4,5)P₃-sensitive Ca²⁺ stores are located throughout the acinar cell cytoplasm and that no regional differences in Ins(1,4,5)P₃ sensitivity exist, at least in the absence of cytosolic modulatory factors.

During the cell permeabilization process, cytosolic factors are lost via the pores created by SLO in the plasma membrane (23). One of our concerns was that SLO treatment affected intracellular structures. Both conventional and electron microscopy demonstrated that SLO permeabilized pancreatic acinar cells had a more swollen appearance. However, the cell architecture remained polarized, since the localization of zymogen granules remained restricted to the apical pole of the cells. Other studies on SLO-permeabilized pancreatic acinar cells have also shown that they retain their polarity and remain functionally active, both in terms of agonist- or Ins(1,4,5)P₃-stimulated Ca²⁺ release from intracellular stores and in terms of agonist- or Ins(1,4,5)P₃- or Ca²⁺-stimulated enzyme secretion (24, 25). Electron microscopy revealed that the endoplasmic reticulum was less strictly arranged compared with intact cells, an effect that was most likely caused by the swelling. We therefore cannot rule out the possibility that some rearrangement of the endoplasmic reticulum Ca²⁺ stores may have occurred. However, our hypothesis is that the relative position of the components of the Ca²⁺ stores is most likely not altered given that the permeabilized cells clearly retained their polarized morphology.

The characteristics of store loading, and the ratio values in unloaded and loaded stores of permeabilized pancreatic acinar cells, were similar to those observed in permeabilized gastric epithelial cells by Hofer and Machen (13). Our estimate of the free intra-organelar Ca²⁺ concentration in steady-state loaded intracellular Ca²⁺ stores as 70 μM is also in broad agreement with the value of 127 μM found in gastric epithelial cells. It might be argued that Mg²⁺ interferes to some extent with the Ca²⁺ signals reported by Magfura-2. However, this seems unlikely, since (i) Mg²⁺ is not transported in an ATP-dependent manner and (ii) the resting Magfura ratio in unloaded stores was very low, i.e. nearly equivalent to the minimum ratio for the dye in the total absence of all divalent cations. In addition,
Fig. 6. Comparison of Ins(1,4,5)P₃ sensitivity in apical and basolateral regions of permeabilized pancreatic acinar cells. A, in the same field of seven selected cells shown in Fig. 5, a region of interest was selected in the apical (black square symbols) and basolateral part of each cell (white square symbols). The selected regions of interest are shown on the corresponding brightfield image. B shows the effect of stepwise increases in Ins(1,4,5)P₃ on the averaged and normalized size of the Ins(1,4,5)P₃-sensitive store in the apical regions (solid line) and in the basolateral (dashed line) regions. Maximal store size was defined as the difference in ratio between unstimulated and maximally Ins(1,4,5)P₃-stimulated Ca²⁺ stores and the averaged results were normalized to this value; this is directly analogous to the definition of “ins(1,4,5)P₃-sensitive Ca²⁺ pools” commonly employed in radio-tracer experiments. The results presented are from the same experiment as shown in Fig. 5 and are typical for three independent experiments.

If Mg²⁺ was present inside stores its free concentration would need to exceed a value of 150 μM to give a significant contribution to the Magfura-2 signal, since the apparent affinity of the dye for Mg²⁺ is very low, i.e., 1.5 mM (26). The Magfura-2 signal in organelles was clearly not saturated with Ca²⁺ under normal conditions, since the ratio was increased markedly by exposure of Ca²⁺ stores to the Ca²⁺ ionophore 4-Br-A23187 in the presence of 1 mM ambient Ca²⁺.

Recent developments have allowed measurements of Ca²⁺ levels inside the endoplasmic reticulum by targeting the Ca²⁺-sensitive bioluminescent protein aequorin to this organelle (27–29). In the first work of this type, Kendall et al. (27, 28) have reported that in COS-7 cells free Ca²⁺ inside the endoplasmic reticulum was around 1–5 μM, approximately 5–20 times the free cytosolic Ca²⁺ concentration. Very recently, however, it has been reported by Montero et al. (29) that Ca²⁺ concentrations inside the endoplasmic reticulum of HeLa cells exceeded 100 μM. By using the Ca²⁺-surrogate Sr²⁺, these workers concluded that even millimolar free concentrations of divalent cations could occur within the endoplasmic reticulum and they argued from this that Ca²⁺ levels might reach similar values. Taken at face value, however, the widely differing estimates reported using the aequorin technique suggest that a ubiquitous conclusion about Ca²⁺ levels inside Ca²⁺ stores cannot be reached. Although, as discussed above, we cannot exclude the possibility that properties of the endoplasmic reticulum are altered during permeabilization, we suggest that our estimate of a free Ca²⁺ concentration within the endoplasmic reticulum of 70 μM might well be applicable to loaded Ca²⁺ stores in intact pancreatic acinar cells.

The second messenger Ins(1,4,5)P₃ released Ca²⁺ from intracellular Ca²⁺ stores in permeabilized pancreatic acinar cells. The characteristics of this release were similar to the fluxes observed in suspensions of permeabilized acinar cells using the radioactive tracer ⁴⁵Ca²⁺ (10, 12). Ca²⁺ release was of a quantal nature, apparently due to the compensatory action of the organelle Ca²⁺ pump during suboptimal stimulation. Thus, suboptimal concentrations of Ins(1,4,5)P₃ were much more efficient in releasing Ca²⁺ in the absence of Ca²⁺ pump activity. An interesting observation was that all permeabilized cells showed similar sensitivities to Ins(1,4,5)P₃. This observation rules out the often raised possibility that intercellular differences in Ins(1,4,5)P₃ sensitivity determine the quantal nature of Ins(1,4,5)P₃-induced Ca²⁺ release.

Compartmentalized dye techniques similar to those applied here have been employed in a number of cell types, including hepatocytes, gastric epithelial cells, AR4–2J pancreatic cells, and smooth muscle cells (13, 21, 30–32). In both hepatocytes (31) and DDT-MF-2 smooth muscle cells (32) the intracellular Ca²⁺ stores function as a single homogeneous pool, and electron microscopy has shown that the endoplasmic reticulum, which presumably acts as the intracellular Ca²⁺ storage compartment, is a continuous compartment. However, the properties of Ins(1,4,5)P₃-induced Ca²⁺ release from intracellular Ca²⁺ stores clearly differed between the two cell types. In permeabilized hepatocytes attached to coverslips, the Ins(1,4,5)P₃-induced Ca²⁺ release was non-quantal, in that, while the kinetics of Ca²⁺ release depended on the concentration of Ins(1,4,5)P₃ used, all doses of Ins(1,4,5)P₃ eventually induced total depletion of the Ca²⁺ stores. In smooth muscle cells, in contrast, Ca²⁺ release induced by Ins(1,4,5)P₃ was of a quantal nature, similar to what we have observed for pancreatic acinar cells.
Agonist stimulation of intact acinar cells initiates a rise of cytosolic Ca\(^{2+}\) in the apical pole of the cell, with the increase in Ca\(^{2+}\) rising uniformly throughout acinar cells upon agonist stimulation (33, 34). In recent studies on intact acinar cells, employing the combination of imaging techniques and patch-clamp recording, evidence was obtained for a heterogeneous distribution of Ca\(^{2+}\) stores (5, 6). By infusing acinar cells with a low dose of Ins(1,4,5)P_3, or its non-metabolizable analogue inositol 1,4,5-triphosphorothioate, it was shown that Ca\(^{2+}\) spikes could be generated exclusively in the apical pole. These results therefore strongly support the idea that a heterogeneous population and distribution of Ins(1,4,5)P_3-sensitive Ca\(^{2+}\) pools do exist in individual pancreatic acinar cells. Biochemical evidence suggested that this store heterogeneity might be explained by differences in numbers of Ins(1,4,5)P_3-operated Ca\(^{2+}\) channels and/or by differences in sensitivity to Ins(1,4,5)P_3 (12). In particular, it was suggested that, during suboptimal stimulation, the most sensitive stores were completely depleted, whereas less sensitive stores remained partially filled due to a compensatory pumping mechanism. This model (12) could explain a number of observations in intact acinar cells in which the apical pole Ca\(^{2+}\) stores display higher apparent Ins(1,4,5)P_3 and Ca\(^{2+}\) sensitivity (5, 6). In the present study, we have examined directly whether Ca\(^{2+}\) stores were heterogeneously distributed in individual permeabilized cells. However, no subcellular differences in Ins(1,4,5)P_3 sensitivity could be detected. One possible interpretation of this result is that subcellular regional differences in Ins(1,4,5)P_3 sensitivity depend critically on some aspects of cellular or cytoskeletal or endoplasmic reticulum architecture, which is disrupted by permeabilization. However, as discussed above, we feel it is unlikely that cell permeabilization results in a major redistribution of Ca\(^{2+}\) stores. If our results can be extrapolated to the intact acinar cell, an alternative explanation of why Ca\(^{2+}\) starts to rise in the apical region on agonist stimulation may be the selective presence of an additional Ca\(^{2+}\) release mechanisms in this area of the cell (5, 35). Several studies suggest the existence of cyclic ADP-ribose-induced Ca\(^{2+}\) release which might be mediated by ryanodine receptors (35, 36). Experimental evidence in those studies has been interpreted to suggest that the combined activation of Ins(1,4,5)P_3-sensitive and cyclic ADP-ribose-sensitive mechanisms is required to explain polarized Ca\(^{2+}\) spike generation. However, 5 \muM cyclic ADP-ribose failed to change the Magfura signal in permeabilized pancreatic acinar cells, whereas in the same cells Ins(1,4,5)P_3 induced a normal response. It is possible that cytosolic factors, which are lost during permeabilization and the subsequent extensive perfusion, may be required for the cyclic ADP-ribose response.

Interestingly, other lines of evidence argue against our finding that no regional differences in Ins(1,4,5)P_3 sensitivity exist. Several isoforms of the Ins(1,4,5)P_3 receptor are known to be expressed in pancreatic acinar cells (37, 38). Therefore, multiple isoforms are likely to be translated into functionally operating receptors in pancreatic acinar cells, possibly with non-homogeneous distributions within the cell. So far, immunochemistry with antibodies directed against Ins(1,4,5)P_3 receptors has shown that these receptors are present in the apical pole of pancreatic and airway gland acinar cells (24, 39). In pancreatic acinar cells only type 3 Ins(1,4,5)P_3 receptors were detected, with no evidence being found for the presence of type 1 Ins(1,4,5)P_3 receptors (24). This observation

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