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Norbert Roos
University of Oslo

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A POSSIBLE SITE OF CALCIUM REGULATION IN RAT EXOCRINE PANCREAS CELLS: AN X-RAY MICROANALYTICAL STUDY

Norbert Roos

Electron Microscopical Unit for Biological Sciences
University of Oslo, P. O. Box 1062
N-0316 Oslo 2, Norway

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Abstract

We analysed four subcellular compartments in rat exocrine pancreas cells, zymogen granules, cytoplasm surrounding the zymogen granules, mitochondria and cytoplasm in the basal part of the cells for sodium, magnesium, phosphorus, sulfur, chlorine, potassium and calcium content, using ultrathin frozen-dried cryosections. The highest concentrations of calcium were measured in the zymogen granules and the surrounding apical part of the cell containing Golgi apparatus, smooth endoplasmic reticulum and condensing vacuoles.

Calcium concentrations in the basal part of the cells (mostly rough endoplasmic reticulum) were 60% lower than in the apical part of the cells. The lowest calcium concentrations were measured in mitochondria. The results suggest that other subcellular compartments than the rough endoplasmic reticulum and mitochondria might be involved in the intracellular Ca^{2+} regulation.

KEY WORDS: Quantitative X-ray microanalysis, Exocrine pancreas, Ca^{2+} regulation.

*Address for correspondence:
Norbert Roos
Electron Microsc. Unit for Biol. Sciences
University of Oslo, Postbox 1062
N-0316 Oslo 3
NORWAY Phone No. (02) 454725

Introduction

The role of calcium in secretion

The control of a variety of cellular processes, e.g., the secretion of neurotransmitters, hormones or digestive enzymes, that are packed within intracellular vesicles, was thought to be exercised solely by a rise in cytosolic free Ca^{2+} (Rubin, 1980), since exocytosis involves fusion between membranes and rearrangement of cytoskeletal components. However, the introduction of the fluorescent dye Quin 2 made it possible to measure intracellular free Ca^{2+} directly, (Rink et al., 1985) revealing that there is a variety of calcium responses to secretory stimuli. Whereas mast cells (White et al., 1984) show a large rise in free Ca^{2+} in response to secretagogues, other systems, such as human neutrophils (Virgilio et al., 1984), can be stimulated by some secretagogues (e.g., phorbol esters) with little or no change in free Ca^{2+}. It seems to be clearly established however, that Ca^{2+} serves as the second messenger in the enzyme secretion of exocrine pancreas when stimulated by cholinergic stimuli, gastrin and caerulin and amphibian peptides (e.g., bombesin ands eloidisin) (Gardner, 1979, Gardner and Jensen, 1981, Schulz, 1980). These agents cause Ca^{2+} release from an intracellular store, followed by an increased influx of extracellular calcium which is required for continuous secretion. Various reports from attempts to identify the intracellular store arrived at different conclusions. Chandler and Williams (1978) and Sampson et al. (1983) identified the mitochondria, Wakasugi et al. (1982) and Haase et al. (1984) the cell membrane and Richardson and Dormer (1984) the microsomal fraction as the intracellular stores.

The cytosolic free Ca^{2+} concentration is very low $\leq 10^{-7}$ mol/l. The maintenance of such a low calcium level and its restoration after a transient rise during stimulation requires an active transport mechanism. Plasma membranes, isolated from rat exocrine pancreas cells, contain a very active Mg^{2+} - dependent Ca^{2+} - ATPase (Lambert and Christophe, 1978) as do the membranes of the zymogen granules (Harper et

al., 1978), whereas the enzyme could not be demonstrated in rough surfaced microsomal fractions (Schulz et al., 1981, Martin and Senior, 1980). In subfractions of isolated microsomes from guinea pig parotid glands (Immelmann and Soeling, 1983) and rat submandibular glands (Terman and Gunter, 1983), the fraction with the maximum calcium uptake was not identical with the fraction with the highest RNA content (rough endoplasmic reticulum). However, calcium uptake into the (smooth?) endoplasmic reticulum from guinea pig hepatocytes occurs even at very low Ca^{2+} concentrations at which mitochondrial calcium uptake is inhibited (Burgess et al., 1983).

In order to regulate free Ca^{2+}, the intracellular store should be able to release Ca^{2+}. Such a calcium release, induced by an intracellular messenger, inositol triphosphate, from intracellular non/mitochondrial source, has recently been demonstrated in pancreatic acinar cells (Streb et al., 1983). There is evidence that the affinity of mitochondria for Ca^{2+} in the normal cell is too low to account for significant calcium sequestration (Scarpa and Graziotti, 1973, Somlyo et al., 1982, Martonosi 1983). Only when exposed to unphysiologically high calcium concentrations will mitochondria display a large capacity to accumulate calcium (Burgess et al., 1983, review Somlyo, 1984, Somlyo, 1985). One of the major difficulties in assessing calcium contents of isolated mitochondria, or for that matter of any isolated subcellular compartment, is the possibility of calcium translocation and redistribution during the isolation procedure. Electron probe X-ray microanalysis has been shown to be a valuable tool for the quantitative study of the elemental composition of cells and subcellular compartments in exocrine pancreas (Roomans and Wei, 1985, Roos and Barnard, 1986). We report here on the subcellular elemental distribution in rat pancreatic acinar cells.

Materials and Methods

Freezing and cryosectioning

Male rats (Spraque/Dawley, 200g) were starved overnight, anaesthesized with Nembutal (10mg/100g), dissected and perfused via the aorta descendens for 10 minutes with Krebs/Ringer phosphate buffer (KRPB) containing 4% Dextran pH 7.4. The peristaltic pump delivered 20ml of perfusate per minute. A water bath was used to heat the perfusate to 40°C in order to have a perfusion temperature at the animal of 37°C. The pancreas was excised as a whole and the intact organ suspended over a frame in a petri-dish containing KRPB solution kept at 37°C, thus avoiding mechanical damage. The suspended organ was taken out of the bathing solution, excess buffer was blotted away and impact/frozen against a helium-cooled copper block (Escaig, 1984). On average less than 2 minutes elapsed between excision and freezing of the organ. The frozen samples were quickly transferred to, and stored in, liquid nitrogen. Slamming of the sample against the copper block resulted in a sample of 0.1mm thickness and two flat parallel surfaces, the surface facing the block being the well frozen one (referred to as "freezing front"). For sectioning, small pieces of the sample were either mounted on an aluminium or silver pin using butyl-benzene as a "cryo-glue" or clamped in a vise-type chuck (over liquid nitrogen with 120K the highest temperature recorded). Cryosections were cut on a Sorvall MT5000 microtome equipped with an FS1000 cryoattachment at 200-250nm nominal thickness using glass knives. The glass knives were broken on a modified LKB knifemaker 7801 A (G. Griffiths, personal communication) to obtain very sharp knives. Suitable knives were coated with a very thin layer (2nm) of tungsten in an Edwards coating unit (Stang and Johansen, personal communication) to improve their sectioning qualities and life time. Sectioning temperatures were kept at 138-128K on the knife stage and the specimen holder and at 126-116K in the cryochamber. Sections were manipulated with an eyelash and transferred to 75 mesh copper grids previously coated with Formvar and a thin carbon layer to improve the stability of the Formvar film. The grids were moved to a grid holder and a second Formvar film was used to sandwich the sections. The grid holder assembly fits into the GATAN cold transfer stage. The transfer was done at 120K. A sliding lid protected the sections against frosting. In the column, the stage temperature was gradually increased to 193K. The first signs of freeze-drying (improving contrast) could be seen at temperatures as low as 173K. Freeze-drying was completed after 30 minutes.

X-ray microanalysis and quantitation

X-ray microanalysis was performed with a LINK 860 series 2 energy-dispersive spectrometer. The detector was mounted horizontally on a JEOL 100CX (S)TEM electron microscope and had a resolution of 148eV. The STEM unit was slightly modified in order to overcome fixed spot sizes. The condenser lens 1 could be controlled independently ("free condenser lens 1 control"). The sections were viewed in the scanning-transmission mode and analysed at 80kV with a raster speed of 10s/frame. When using the stationary spot one loses visual control over the area analysed. The goniometer was tilted by 35° towards the detector (Roos and Barnard, 1985). Quantitation was carried out with the QUANTEM/FLS program using the continuum method of Hall (1971) and the corrections for extraneous continuum as described by Gupta and Hall (1979). Standards were prepared according to Hagler et al. (1983) and in later studies as described by Roos and Barnard (1984) and Roos and Morgan (1985). Four animals were used, five cells per animal were chosen and per cell three spectra of each subcellular compartment were taken. The spectra from each animal were averaged and used to calculate the mean value. The number of animals was used to determine the S.E.M. The compartments analysed were (i) zymogen granules (ii) mitochondria (iii) basal cytosol and rough endoplasmic reticulum (iv) apical cytosol excluding zymogen granules.

Results

Freezing and sectioning quality

Since we did not use any cryoprotectants, the quality of the freezing procedure is crucial to subsequent quantitative analysis. We checked all our frozen samples routinely for morphological preservation using a rapid freeze substitution schedule (Roos and Barnard, 1986). Most ($\geq$ 90%) of the samples were very well frozen up to at least 60 µm deep into the tissue. Cryosections of the tissue (Fig. 1) showed that the morphological integrity of the tissue and the cells can be maintained without chemical fixation. Contrast in those sections was very good, even though no stain was present. All the subcellular compartments could be identified easily. Sectioning artefacts such as chatter and compression are due to sectioning on a dry knife and cannot be avoided (Chang et al., 1983). The sections display the occasional holes and cracks which are probably due to the handling of the brittle sections with the eyelash.

Analytical results

The analysis of acinar cells revealed significant differences in elemental composition of the four subcellular compartments (Table 1). Sodium, magnesium, phosphorus and potassium concentrations were highest in the basal part of the cell excluding the mitochondria. The mitochondria had the same low calcium content as the rest of the basal part of the cell but a significantly lower sodium, magnesium, phosphorus, chlorine and potassium content. Only the sulphur concentration was slightly higher. The magnesium, phosphorus and potassium concentrations measured in the apical part of the cell excluding zymogen granules were lower than in the basal part of the cell, whereas sulphur and calcium concentrations were significantly higher. In the zymogen granules sodium, magnesium, phosphorus, chlorine and potassium concentrations were extremely low, but the sulphur and calcium concentrations the highest of all the compartments measured. Whereas the sodium-to-potassium ratio was almost 1:1 in the zymogen granules it varied from 1:3.4 to 1:4.3 in the other compartments. The ratio of the two elements calcium and magnesium was slightly above 1:1 in the zymogen granules, 1:4 in the apical part and 1:14 in the basal part of the cell.

Discussion

The role of calcium as intracellular messenger in the physiological function of different cells requires intracellular sites that can release and take up calcium in order to increase and decrease the cytosolic free Ca^{2+} concentration, which is usually kept at very low levels ($\leq 10^{-7}$ mol/l). In pancreatic acinar cells, there are basically five sites at which a redistribution of calcium could occur, the plasma membrane, the membranes of the endoplasmic reticulum, the limiting membrane of the zymogen granules, the mitochondrial membrane(s) and the Golgi membranes. Uptake of calcium from the cytosol into intracellular

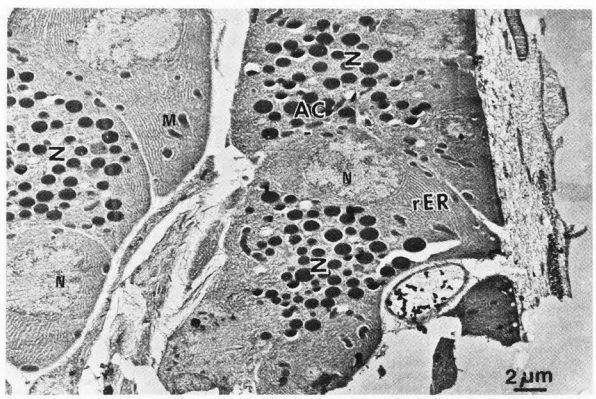

Fig 1: Frozen-dried cryosection of quench-frozen rat exocrine pancreas. AC = apical cytoplasm, rER = rough endoplasmic reticulum, N = nuclei, Z = zymogen granules, M = mitochondria.

Table 1

Elemental distribution in non-stimulated pancreatic acinar cells. Means and S.E.M. of four animals. Values in mmol/kg dry weight.
ZG = zymogen granules, AC = apical part of cell without zymogen granules, ER = basal part of cell without mitochondria, M = mitochondria.

	COMPARTMENT			
	ZG	AC	ER	M
Na	105 ± 9	344 ± 29	358 ± 18	200 ± 8
Mg	39 ± 2	157 ± 14	217 ± 16	102 ± 9
P	126 ± 14	1844 ± 126	2760 ± 147	1188 ± 29
S	674 ± 21	383 ± 20	215 ± 15	404 ± 19
Cl	96 ± 8	318 ± 28	357 ± 23	156 ± 6
K	121 ± 6	1161 ± 98	1554 ± 72	801 ± 24
Ca	54 ± 2	37 ± 1	15 ± 3	14 ± 2
Na/K	0.86 ±0.05	0.29 ±0.01	0.23 ±0.008	0.25 ±0.007
Ca/Mg	1.39 ±0.03	0.24 ±0.02	0.07 ±0.02	0.14 ±0.02

stores and extrusion out of the cell are against its electrochemical gradient. Therefore the process has to be active. Active translocation of calcium across membranes can be performed by Mg^{2+}-dependent Ca^{2+}-ATPases. The existence of this transport system in one or more subcellular compartments would imply that it possibly plays a role in the calcium homeostasis of the cell. Such ATPases have indeed been identified in the plasma membrane of rate exocrine pancreas cells (Lambert and Christophe, 1978, Martin and Senior, 1980) in mitochondria (Martin and Senior, 1980) a membrane fraction derived from zymogen granules (Harper et al., 1978) and microsomes isolated from guinea pig pancreas (Lucas et al., 1983).

Another approach to test the participation of intracellular structures in the regulation of cytosolic calcium is to measure their calcium content. Clemente and Meldolesi (1975a) measured the calcium distribution in subcellular fractions of guinea pig pancreatic acinar cells, others in mitochondria from different sources (review Hansford, 1985). Obviously, during homogenization and cell fractionation there is a high risk of redistribution of mobile electrolytes (leakage, unspecific adsorption). Histochemical methods using precipitating agents to immobilize Ca^{2+} in situ (Schulz et al., 1981), Haase et al., 1984) employ chemical fixatives with the high risk of significant ion redistribution and unspecific precipitation (Barnard et al., 1984a).

Electron probe X-ray microanalysis is a technique that allows the measurement of total calcium with a high spatial resolution (Hall and Gupta, 1983). The risk of elemental redistribution during the specimen preparation can be drastically reduced by using non-cryoprotected cryosections that are transferred to the microscope in the fully-hydrated state and analysed after freeze-drying in the column (Zierold, 1984, Roos and Barnard, 1986, Hagler and Buja, 1986). The use of high concentrations of high-molecular-weight cryoprotectants has an influence on the fluid secretion of rabbit pancreas (Kuypers and Roomans, 1983) and Calliphora salivary glands (Barnard et al., 1984b), can lead to cell shrinkage (Barnard, 1982) and was therefore omitted.

We found the highest calcium and sulphur content in the zymogen granules. The high sulphur values are probably due to the presence of sulphated macromolecules such as lipids, proteoglycans and proteins (Reggio and Palade, 1978, Berg, 1978). The calcium and magnesium concentrations measured in the basal secretion of rabbit pancreas are about 25 mmol/kg protein for both cations (Schreurs et al., 1976). This suggests that they might be present in about equal amounts in the zymogen granules as well, where they are loosely bound to the enzyme proteins but are not exchangeable as long as the enzymes are packed inside the zymogen granules (Schreurs et al., 1976). Our measurements (Ca-to-Mg ratio is 1;1.39) would rather agree with the measurements by Schreurs et al. (1976) than with the data of Clemente and Meldolesi (1975b) who report calcium concentrations four times as high as the magnesium concentrations in isolated zymogen granules. A recent electron probe X-ray microanalytical study (Roomans and Wei, 1985) confirms the data from Schreurs et al. (1976) as well.

The calcium content of mitochondria is the lowest of all compartments analysed. Mitochondria have a relatively low affinity for calcium but a large capacity for accumulating calcium (Vallieres et al., 1975). By measuring the calcium content of isolated mitochondria with the normal "bulk" methods, a small fraction of abnormally calcium loaded mitochondria might compromise the results. Electron probe X-ray microanalytical studies found low mitochondrial calcium levels consistently in exocrine cells (Roomans and Wei, 1985) and muscle (Somlyo et al., 1979, Somlyo, 1985) supporting the idea that mitochondria from muscles and from other tissues do not regulate the cytosolic free Ca^{2+} but are themselves regulated by small changes in free Ca^{2+} (Denton and McCormack, 1980, Hansford, 1985).

The asymmetric structure of pancreatic acinar cells made it possible to analyse precisely the basal part of the cell with rough endoplasmic reticulum tightly stacked. Measurements in this area revealed very high potassium, phosphorus and magnesium values. These high phosphorus and magnesium concentrations reflect the presence of membrane phospholipids and ribosomes, which are known to be rich in phosphorus and magnesium (Siekevitz and Palade, 1960). Surprisingly, the amount of calcium detected was as low as in the mitochondria, whereas it was significantly higher in the cytoplasm surrounding the zymogen granules. This is in conflict with a study using cryosections from pancreatic acinar cells that have been treated with the high-molecular-weight cryoprotectant dextran (Roomans and Wei, 1985). Superficially, it is also in conflict with studies that report active Ca^{2+} transport systems in microsomal fractions of guinea pig pancreatic acinar cells (Lucas et al., 1983). However, in some studies the term microsomal fraction is used without discrimination between rough surfaced and smooth surfaced microsomes (Burgess et al., 1983, Black et al., 1981) or contamination of the preparation with plasma membrane could not be excluded (Lucas et al., 1983) Immelmann and Soeling (1983) could demonstrate that the maximum calcium uptake capacity in subfractions of microsomes derived from guinea pig parotid glands did not comigrate with the fraction containing the highest RNA content (possibly rough endoplasmic reticulum). Terman and Gunter (1983) report that membrane vesicles prepared from rat submandibular glands containing a calcium transport system show different purification properties than the membrane vesicles which contain the standard enzyme markers for total and rough endoplasmic reticulum, Golgi apparatus, plasma membranes and lysosomes, which confirms measurements from Clemente and Meldolesi (1975b) who found that

the calcium content of smooth surfaced vesicles from guinea pig pancreatic acinar cells was highest. Schulz et al. (1981) failed to demonstrate a Mg^{2+}-dependent Ca^{2+}-ATPase in rough endoplasmic reticulum isolated from pancreatic acinar cells and Martin and Senior (1980) found ATPase activity only in mitochondria, the light fraction derived from zymogen granule membranes and plasma membranes, and Meldolesi et al. (1971) did not find ATPase activity in the rough endoplasmic reticulum of guinea pig pancreatic acinar cells. Our calcium measurement could support the findings that smooth surfaced vesicles located in the apical part of the pancreatic acinar cells rather than the rough surfaced (endoplasmic reticulum) membrane network located in the basal part of these cells are the site of calcium storage and release.

There are probably two major calcium fluxes from pancreatic acinar cells into the secreted fluid, a secretory flux associated with the secretion of proteins and a stimulatory flux as a result of calcium extrusion from the cell after a transient rise in cytosolic calcium following stimulation (Schreurs et al., 1976, Cecarelli et al., 1975, Pandol et al., 1985). When analysing the cytoplasm in the apical part of the cell we were sure that we did not analyse underlying zymogen granules since (i) the sections were very thin and (ii) the morphological preservation was so good that any subcellular compartment could be identified unambiguously. An indication that we might have analysed Golgi complexes is the relatively high sulphur concentration which could be the result of a high protein content. However, with our experimental set up, we are not able to demonstrate whether this site with high calcium concentrations is involved in stimulus-secretion coupling (stimulatory calcium flux) or in the secretory calcium flux. One could speculate that in the course of protein sorting in the Golgi apparatus and packing of the proteins into vesicles, calcium is added successively by active transport across the Golgi and zymogen granule membranes. Calcium could either be necessary for packing of the secretory products or simply be disposed of via the secretory pathway to keep intracellular calcium concentrations low.

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References

Barnard T (1982). Thin frozen-dried cryosections and biological X-ray microanalysis. J. Microsc 126, 317-332.

Barnard T, Roos N, Stenmark ST (1984a). X-ray microanalytical cytochemistry of calcium in secretory cells. Proc. VIIth Intern. Congress Histochem. Cytochem. Lunkka, Helsinki p28.

Barnard T, Gupta BL, Hall TA (1984b). Effects of the high-molecular-weight cryoprotectant dextran on fluid secretion by Calliphora salivary glands. Cryobiology 21, 559-569.

Berg NB (1978). Sulphate metabolism in the exocrine pancreas, II. The production of sulphated macromolecules by the mouse exocrine pancreas. J Cell Sci 31, 199-211.

Black BL, Jarett L, McDonald JM (1981). The regulation of endoplasmic reticulum calcium uptake of adipocytes by cytoplasmic calcium. J Biol Chem 256, 332-339.

Burgess GM, McKiney JS, Fabiato A, Leslie BA, Putney JW (1983). Calcium pools in saponin-permeabilized guinea pig hepatocytes. J Biol Chem 258, 15336-15345.

Cecarelli B, Clemente F, Meldolesi J (1975). Secretion of calcium in pancreatic juice. J Physiol 245, 617-638.

Chandler DE, Williams JA (1978). Intracellular divalent cation release in pancreatic acinar cells during stimulus-secretion coupling, II. Subcellular localization of the fluorescent probe chloratetracycline. J Cell Biol 76, 371-385.

Chang I-I, McDowell AW, Lepault J, Freeman R, Walter CA, Dubochet J (1983). Freezing, sectioning and observation artefacts of frozen-hydrated sections for electron microscopy. J Microsc 132, 109-132.

Clemente F, Meldolesi J (1975a). Calcium and pancreatic secretion, I. Subcellular distribution of calcium and magnesium in the exocrine pancreas of guinea pig. J Cell Biol 65, 88-102.

Clemente F, Meldolesi J (1975b). Calcium and pancreatic secretion-dynamics of subcellular calcium pools in resting and stimulated acinar cells. Br J Pharmac 55, 369-379.

Denton RM, McCormack JG (1980). On the role of the calcium transport cycle in heart and other mammalian mitochondria. FEBS Lett 119, 1-8.

Escaig J (1984). Control of different parameters for optimal freezing conditions. In: The Science of biological Specimen Preparation. (eds) JP Revel, T Barnard, GH Haggis. Scanning Electron Microsc Inc. AMF O Hare, Chicago, IL, 6O666, 117-122.

Gardner JD (1979). Regulation of pancreatic exocrine function in vitro: Initial steps in the actions of secretagogues. Am J Physiol 41, 55-66.

Gardner JD, Jensen RT (1981). Regulation of pancreatic exocrine secretion in vitro: the action of secretagogues. Phil Trans R Soc Lond B296, 17-26.

Gupta BL, Hall TA (1979). Quantitative electron probe microanalysis of electrolyte elements within epithelial tissue compartments. Fed Proc 38, 144-153.

Haase W, Friese W, Heitmann A (1984). Electron-microscopic demonstration of the distribution of calcium deposits in the exocrine pancreas of the rat after application of carbachol, atropin, cholecystokinin and procaine. Cell Tissue Res 235, 683-690.

Hagler HK, Lopez LE, Flores JS, Lundswick RJ, Buja LM (1983). Standards for quantitative energy-dispersive X-ray microanalysis of biological cryosections: Validation and application to studies of myocardium. J Microsc 131, 221-234.

Hagler HK, Buja LM (1986). Effect of specimen preparation and section transfer techniques on the preservation of ultrastructure, lipids and elements in cryosections. J Microsc 141, 311-317.

Hall TA (1971). The microprobe assay of chemical elements. In: Physical Techniques in Biochemical Research. (ed) G Oster. Academic Press NY, Vol 1A, 157-275.

Hall TA, Gupta BL (1983). The localization and assay of chemical elements by microprobe methods. Quart Rev Biophys 16, 279-339.

Hansford RG (1985). Relation between mitochondrial calcium transport and control of energy metabolism. Rev Physiol Biochem Pharmacol 102, 1-72.

Harper F, Lamy F, Calvert R (1978). Some properties of a Ca^{2+} and (or) Mg^{2+} requiring nucleoside di-and tri-phosphatase(s) associated with the membranes of rat pancreatic zymogen granules. Can J Biochem 56, 565-576.

Immelmann A, Soeling H-D (1983). ATP-dependent calcium sequestration and calcium/ATP stoichiometry in isolated microsomes from guinea pig parotid glands. FEBS Lett 162, 406-410.

Kuypers GAJ, Roomans GM (1983). Effect of the polymeric cryoprotectant dextran on fluid secretion in the isolated rabbit pancreas. J Microsc 129, 141-146.

Lambert M, Christophe J (1978). Characterization of (Mg, Ca)-ATPase activity in rat pancreatic plasma membranes. Eur J Biochem 91, 485-492.

Lucas M, Galvan A, Solano P, Goberna R (1983). Compartmentation of calcium in digitonin-disrupted guinea pig pancreatic acinar cells. Biochim Biophys Acta 731, 129-136.

Martin SS, Senior AE (1980). Membrane adenosine triphosphate activities in rat pancreas. Biochim Biophys Acta 602, 401-418.

Martonosi AN (1983). The regulation of cytoplasmic Ca^{2+} concentration in muscle and nonmuscle cells. In: Muscle and Nonmuscle Motility. (ed) A Stracher, Academic Press NY, Vol 1 233-357.

Melodelsi J, Jamieson JD, Palade GE (1971). Composition of cellular membranes in the pancreas of the guinea pig. III. Enzymatic activities. J Cell Biol 49, 150-158.

Pandol SJ, Schoeffield MS, Sachs G, Muallem S (1985). Role of free cytosolic calcium in secretagogue - stimulated amylase release from dispersed acini from guinea pig pancreas. J Biol Chem 260 10081-10086.

Reggio HA, Palade GE (1978). Sulfated compounds in the zymogen granules of the guinea pig pancreas. J Cell Biol 77, 288-314.

Richardson AE, Dormer RL (1984). Calcium-ion-transporting activity in two microsomal subfractions from rat pancreatic acini. Biochem J 219, 679-685.

Rink TJ, Pozzan T (1985). Using quin in cell suspensions. Cell calcium 6 133-144.

Roomans GM, Wei X (1985). X-ray microanalysis of resting and stimulated rat pancreas. Acta Physiol Scand 124, 353-359.

Roos N, Barnard T (1984). Aminoplastic standards for quantitative X-ray microanalysis of thin sections of plastic-embedded biological material. Ultramicroscopy 15, 277-286.

Roos N, Morgan JA (1985). Aminoplastic standards for electron probe X-ray microanalysis (EPXMA) of ultrathin, frozen-dried cryosections. J Microsc 140, RP3-RP4.

Roos N, Barnard T (1985). A comparison of subcellular element concentrations in frozen-dried, plastic-embedded, dry-cut sections and frozen-dried cryosections. Ultramicroscopy 17, 335-344.

Roos N, Barnard T (1986). Preparation methods for quantitative electron probe X-ray microanalysis of rat exocrine pancreas: A review. Scanning Electron Microsc. 1986; II: 703-711.

Rubin RP (1980). Calcium and cellular secretion. Plenum Press, NY.

Sampson HW, Kiessel DJ, MacKenzie-Graham L, Piscopo I (1983). A cytochemical study of the effect of cholinergic and adrenergic stimulation on calcium fluxes of rat parotid gland. Histochem 79, 193-203.

Scarpa A, Graziotti P (1973). Mechanisms for intracellular calcium regulation in heart. I. Stopped-flow measurements of Ca^{2+} uptake by cardiac mitochondria. J Gen Physiol 62, 756-772.

Schreurs VVAM, Swarts HGP, DePont JJHHM, Bonting SL (1976). Role of calcium in exocrine pancreatic secretion. III. Comparison of calcium and magnesium movements in rabbit pancreas. Biochim Biophys Acta 436, 664-674.

Schulz I (1980). Messenger role of calcium in function of pancreatic acinar cells. Am J Physiol 239, G335-G347.

Schulz I, Dimura T, Wakasugi H, Haase W, Kribben A (1981). Analysis of Ca^{2+} fluxes and Ca^{2+} pools in pancreatic acini. Phil Trans R Soc Lond B296, 105-113.

Siekevitz P, Palade GE (1960). A cytochemical study of the pancreas of the guinea pig. VI. Release of enzymes and ribonucleic acid from ribonucleoprotein particles. J Biophysic Biochemic Cytol 7, 631-649.

Somlyo AP, Somlyo AV, Shuman H (1979). Electron probe analysis of vascular smooth muscle. composition of mitochondria, nuclei and cytoplasm. J Cell Biol 81, 316-335.

Somlyo AP, Somlyo AV, Shuman H, Endo M (1982). Calcium and monovalent ions in smooth muscle. Fed Proc 41, 2883-2890.

Somlyo AP (1984). Cellular site of calcium regulation. Nature 309, 516-517.

Somlyo AP (1985). Excitation-contraction coupling and the ultrastructure of smooth muscle. Circulation Res 57, 497-507.

Streb H, Irvine RF, Berridge MJ, Schulz I (1983). Release of Ca^{2+} from a nonmitochondrial intracellular store in

pancreatic acinar cells by inositol-1, 4, 5-triphosphate. Nature 306, 67-68.

Terman BJ, Gunter TE (1983). Characterization of the submandibular gland microsomal calcium transport system. Biochim Biophys Acta 730, 151-160.

Vallieres J, Scarpa A, Somlyo AP (1975). Subcellular fractions of smooth muscle: Isolation, substrate utilization and Ca^{2+} transport by main pulmonary and artery and mesenteric vein mitochondria. Arch Biochem Biophys 170, 659-669.

Virgilio FDi, Lew DP, Pozzan T (1984). Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic Ca^{2+} levels. Nature 310, 691-693.

Wakasugi H, Kimura T, Haase W, Kribben A, Kaufmann R, Schulz I (1982). Calcium uptake into acini from rat pancreas: Evidence for intracellular ATP-dependent calcium sequestration. J Membrane Biol 65, 205-220.

White JR, Ishizaka T, Ishizaka K, Sha'afi RJ (1984). Direct demonstration of increased intracellular concentration of free calcium as measured by Quin-2 in stimulated rat peritoneal mast cell. Proc Natl Acad Sci USA 81, 3978-3982.

Zierold K (1984). Experiences in X-ray microanalysis of freeze-dried cryosections. Scanning Electron Microsc. 1984; IV: 1867-1874.

Discussion with Reviewers

G. M. Roomans: In Fig 1, both zymogen granules and mitochondria are surrounded by a "halo", suggesting differential shrinkage. What is your opinion about this phenomenon? Zierold (Scanning Electron Microsc.; 1986; II: 713-724) noted that mitochondria have a lower water content than cytoplasm. Could that in part be due to the loss of water during freezing or differential shrinkage during freeze-drying?
Author: Mitochondria and most probably the zymogen granules have a lower water content than cytoplasm. We assume that during freeze-drying the surrounding cytoplasm shrinks more than the components with lower water content, thus forming a "halo" around them.

H. Y. Elder: What was the thickness of your Formvar films and of the carbon coatings of the films? Was the second Formvar of the sandwich also coated? And what was the approximate total thickness of the supporting film sandwich?
Author: Our Formvar films are usually 100 mm thick and the carbon coating between 5-10 mm. The second Formvar film had the same thickness as the first and was not carbon coated, i.e. the whole sandwich is approximately 210 mm thick.

H. Y. Elder: What elements are present in your Formvar support films? In our laboratory S usually proves to be the major element present in the Formvar. Have your data been corrected for such as this?
Author: We usually find a Si peak in the film spectra and traces of Cl. We do not find evidence of the presence of S in our supporting film.

H. Y. Elder: In your excellent freeze-dried cryosection could you comment on the small electron-opaque spherical contaminants overlying the capillary(?) in the bottom right of the field?
Author: We are not sure what these particles represent. Since they are confined to the capillary, it is relatively unlikely that they are contaminants. The perfusion buffer (KRPB) contained 4% dextran to substitute for blood proteins. The particles might be precipitates of dextran and solutes.

H. Y. Elder: One way of testing whether the greatest concentrations of Ca^{2+} in your findings were in the zymogen granules and apical SER-rich cytoplasm would be to see if any changes are detectable after activation of the pancreas. Although it is of course the ionised concentration which is physiologically important, the total stored Ca^{2+} reveals meaningful changes in distribution and/or concentration in some cell types after secretagogue treatment. Have you tried experiments of this nature?
Author: Yes, we have measured the elemental distribution in a few pancreatic acini after sustained stimulation. However, we do not have a complete set of results yet.

R. L. Dormer: To what extent do you think these measurements of total Ca in subcellular organelles will necessarily reflect its ability to contribute: a) to the regulation of cytoplasmic free Ca^{2+} ; b) to accumulating a releasable Ca^{2+} store. For example, if the plasma membrane is active in extruding Ca^{2+} from the cell this would not be shown by the present technique. In addition, the amount of Ca^{2+} needed to be released from an intracellular store may not be as large as in skeletal muscle (possibly as low as 0.1 mmol/kg dry weight).
Author: Quite right. Further experiments as suggested above by H. Y. Elder and already under way in our laboratory will show whether the technique is capable of detecting the expected calcium changes or not.