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PROTON-INDUCED AND ELECTRON-INDUCED X-RAY MICROANALYSIS OF INSULIN-SECRETING CELLS

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Abstract

Elemental redistribution induced by insulin secretion, was investigated by electron and proton probe X-ray microanalysis. In particular, ion fluxes following immediately upon stimulation were studied. As the sensitivity of the electron probe was insufficient, the proton microprobe was employed. In order to see whether the cell is asymmetric with respect to Ca^{2+} influx, the cells were stimulated in the presence of Sr^{2+} (as a Ca^{2+} analog). Insulin-secreting cells (RINm5F cells and isolated mouse β-cells) were cultured on grids and shock-frozen at 2-30 seconds after stimulation. In a large number of cells, the major elements and and large fluxes were analyzed by the electron microprobe. In the proton microprobe, selected cells were analyzed and elemental maps were compared with electron micrographs of the same cells. The proton microprobe, but not the electron microprobe, could detect an influx of Sr in response to K^+-stimulation for 2 seconds, in RINm5F-cells. No polarization of Sr^{2+} uptake in RINm5F-cells could be detected, and the β-cells did not respond to high K^+ by uptake of Sr. Momentary stimulation of β-cells also resulted in a significant increase in Na, detected by the electron probe. Spreading of the β-cells on the substrate appears to influence the subcellular elemental distribution. Thus, the proton probe has potential to detect small changes in elements such as those occurring after short-time stimulation.

Key Words: insulin-secreting cells, calcium, strontium, proton probe, electron probe, cell culture.

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Introduction

Ion fluxes are of fundamental importance in cell signalling. In living cells these fluxes can be studied with light microscopy, using ion specific fluorescent dyes (Tsien, 1989). However, this technique does not provide information concerning the relationship between ions and the cellular ultrastructure. Elemental distribution can also be investigated employing cryo-techniques followed by electron microprobe or proton microprobe X-ray microanalysis. Because of the lower background radiation produced by protons interacting with matter, the sensitivity obtained is about 1-2 orders of magnitudes better with a proton beam than with an electron beam. In the present study we were interested in the mechanism of insulin secretion from the pancreatic β-cell. *In vivo*, secretion of insulin is mainly stimulated by elevation of the blood glucose level and is modulated by various hormones and neurotransmitters. The metabolism of glucose induces insulin release by depolarizing the β-cell plasma-membrane. This depolarization leads to opening of voltage activated Ca^{2+}-channels (L-type Ca^{2+}-channels), allowing influx of Ca^{2+} and a rise in the cytoplasmic concentration of free Ca^{2+}, $[Ca^{2+}]_i$ (Prentki and Matchinsky, 1987). The increase in $[Ca^{2+}]_i$ stimulates the process of exocytosis by, so far, unknown mechanisms. The β-cell glucose transporters have been localized mainly to parts of the cell membrane rich in microvilli and facing adjacent cells. This co-localization of glucose transporters and microvilli suggests some interaction between the transporters and the cytoskeleton, implying that the β-cell may be morphologically asymmetric (Orci *et al.*, 1989). In view of the important role of Ca^{2+} in the secretory process it is of interest to clarify whether an uptake of Ca^{2+} is localized to a specific area of the cell-membrane. Perhaps the membrane is connected with intracellular structures like the insulin-containing secretory granules, which can be revealed by the spatial resolution of the electron microscope.

In the present investigation insulin-secreting cells from a rat insulinoma cell-line (RINm5F) (Oie *et al.*, 1983) and β-cells isolated from mice (*ob/ob*) (Lernmark, 1974) were used. The cells were stimulated for various times and shock-frozen, allowing studies of elemental distribution as function of time. Since RINm5F-cells are insensitive to glucose-stimulation we induced opening of the Ca^{2+}-channels by depolarization with high concentrations of K^+ (Arkhammar *et al.*, 1987).

Analysis of characteristic X-rays does not give any information on whether the element analyzed is a free ion or bound to a macromolecule. To discriminate between Ca that is already present in the cell and Ca^{2+} entering as a result of stimulation, Sr was used as a tracer. Sr^{2+} is known to enter the Ca^{2+} channels and to mimic the role of Ca^{2+} in the secretory process (Somlyo and Somlyo, 1971; Wroblewski *et al.*, 1989; Ozaki *et al.*, 1992) and endogenous levels of the element have not been detected with the present techniques (Tipton *et al.*, 1965; Juntti-Berggren *et al.*, 1991). Thus, the presence of Sr in a stimulated cell represents Ca^{2+} that has entered through L-type Ca^{2+}-channels. To determine whether the Sr^{2+} uptake is polarized, the stimulation time must be minimized since the ions diffuse rapidly in the cytoplasm. This implies that small concentrations of Sr have to be detected, which provided an additional motive to use the proton microprobe.

Materials and Methods

Cells

A cloned insulin-secreting cell line, RINm5F, derived from a transplantable rat islet tumor (Oie *et al.*, 1983) was used. Pancreatic islets were isolated from adult non-inbred obese hyperglycemic mice, *ob/ob* (Hellman, 1965) which were starved overnight. The islets were isolated by a collagenase technique and disrupted into a single cell suspension, comprising more than 90% β-cells, by vigorous shaking in a Ca^{2+}- and Mg^{2+}-deficient medium (Lernmark, 1974; Arkhammar *et al.*, 1986).

Cell Culture

The RINm5F-cells were cultured in RPMI 1640 culture medium (Gibco Ltd., Paisley, Scotland) supplemented with 8% fetal bovine serum, 2% newborn bovine serum (Flow), 100 IU/ml of penicillin, 100 mg/ml of streptomycin, 2 mM of L-glutamine and 20 mM HEPES. The cells were grown in culture flasks (Nunc, Roskilde, Denmark), at 37°C in an incubator with 5% CO_2 in humidified air (Andersson, 1978). Before the experiments the RINm5F-cells were detached from the flasks by trypsination with a solution of 0.05% trypsin in 0.02% EDTA (Sigma, St. Louis, MO, USA). The flasks were flushed with culture medium that was collected in a sterile centrifugation tube and pelleted at low speed. The cell pellet was suspended in fresh medium and seeded onto Formvar-coated titanium grids (75 mesh, Agar Aids). The grids, which were attached to a coverslip by the Formvar, were coated with a thin layer of carbon and placed into Petri-dishes (Nunc), before the cells were seeded.

The β-cell-suspension was seeded as drops onto the grids and when the cells had attached, after 3-4 hours, more medium was added. When the RINm5F-cells were seeded onto the grids, culture medium was already present in the Petri-dishes, covering the grids. The difference in the seeding procedure between RINm5F cells and β-cells is because of the abundance of the former cells and the fact that the fraction of β-cells able to attach was much smaller than that of the RINm5F cells. The cells were grown on the grids for 1-3 days.

Preparation for Analysis

The Ca^{2+}-medium used in the experiments was (in mM) 125 NaCl, 5.9 KCl, 1.28 $CaCl_2$, 1.2 $MgCl_2$ and 25 HEPES, pH adjusted to 7.4 with NaOH (Hellman, 1975). The medium was supplemented with 1 mg/ml bovine serum albumin and 3 mM glucose. In the experiments where Sr^{2+} was used instead of Ca^{2+}, 1.28 mM $CaCl_2$ was replaced by 2.56 mM $SrCl_2$. The cells were stimulated by increasing the concentration of KCl with 25 mM. Grids were moved, with pliers (1), from the culture medium to the experimental solution (described above) for the time chosen; (2) from the experimental solution to ice-cold distilled water for rinsing during five seconds; (3) the grids were blotted on a filter paper and (4) shock-frozen in liquid propane cooled by liquid nitrogen. RINm5F-cells were stimulated for two to 30 seconds, whereas β-cells were only stimulated for two seconds. The grids were freeze-dried overnight at -80°C and 10^{-5} torr and before analysis they were covered with a thin carbon layer to avoid charging by the beam.

Analysis

Electron probe X-ray microanalysis was carried out at 100 kV in a Philips 400 transmission electron microscope (TEM), equipped with a LINK QX 200 energy-dispersive spectrometer system or in a JEOL 1200 TEMSCAN, equipped with a Tracor 5500 spectrometer system. Analysis was carried out for 100 seconds (live time). The size of the spot, while impinging on a sample, was about 1 μm. Quantitative analysis was based on the ratio of characteristic intensity to the background intensity in the same energy region

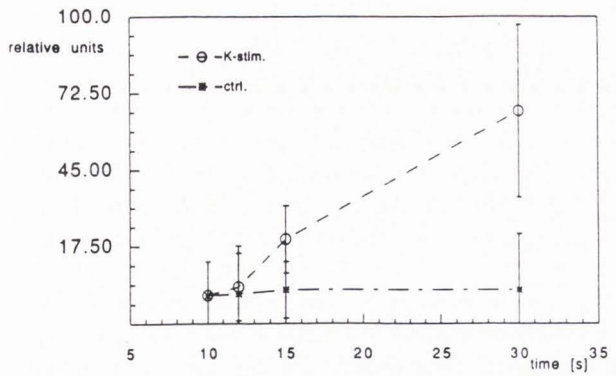

Fig. 1. Electron probe analysis of RINm5F cells showing uptake of Sr after depolarization by 25 mM KCl. Means and standard errors are shown (174 cells were analyzed).

(peak-to-background (P/B) ratio) (Hall *et al.*, 1973; Roomans, 1981). P/B ratios or peak-to continuum ratios obtained in the cells were compared to those obtained from standards, consisting of gelatin containing known amounts of metal and mineral salts (Roomans, 1988).

To identify cells in the proton microprobe, the specimens were first examined in the transmission electron microscope and photographed. In the proton microprobe analyzing chamber, orientation of the proton beam onto the sample was simplified by using imaging by scanning transmission ion microscopy (STIM) (Watt *et al.*, 1992). Selected cells were analyzed with the proton microprobe and both elemental maps (time for analysis: 1-2 h) and point-spectra (time for analysis: 10 minutes) were acquired. Elemental maps were obtained for 37 cells (15 β-cells and 22 RINm5F-cells), point analysis was performed on 11 cells, on the nuclear area and other selected points (5 β-cells and 6 RINm5F-cells). Because of the small number of analyzed cells, no statistical analysis could be carried out. For proton microprobe analysis, the Scanning Proton Microprobe at Oxford was used (Grime and Watt, 1990), with a proton energy of 3.0 MeV and a spot size of 1 μm before impinging on the target. For a beam current of 200 pA, 1% of the beam current falls outside a region of 3×3 μm (Breese *et al.*, 1993). The resolution in the elemental-maps (Na, K, Cl and Ca), is 256×256 pixels per map and in the Zn- and Sr-maps (and in the Ca map of Fig. 1b) 64×64 pixels per map, because of the lower amounts of these elements. The X-ray signals, the signals from the backscattered protons and maps of the proton energy loss (STIM) in the sample, were collected. From the backscattered proton signal (Rutherford Back Scattering, RBS) the sample contents of carbon, nitrogen, oxygen and the sample thickness were calculated. The elemental concentrations were

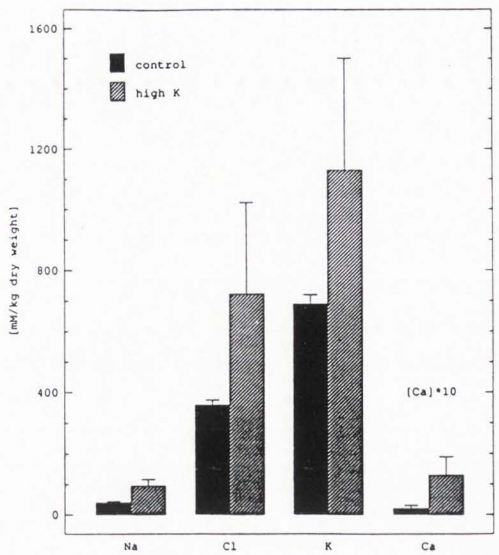

Fig. 2. Effect of K^+ depolarization during 20 seconds on elemental composition of RINm5F cells analyzed by electron probe microanalysis. Means and standard-errors are shown. The mean K/Na ratios were 18 in the controls (number of cells; 68) and in the stimulated (number of cells; 43) it was 6 .

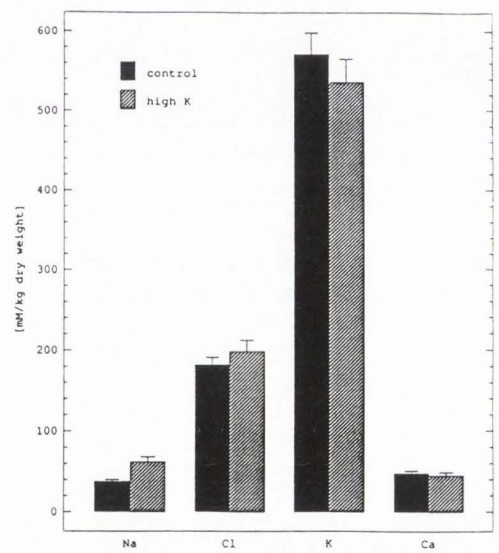

Fig. 3. Effect of K^+ depolarization on elemental composition of β-cells analyzed by electron probe microanalysis. Means and standard-errors are shown. The mean K/Na ratio was 15 in the controls (number of cells; 81) and in the stimulated cells (number of cells; 47) it was 10.

calculated from the X-ray intensities and the RBS signal (Chu *et al.*, 1978), using a standardless quantitative method (Grime and Dawson, 1994).

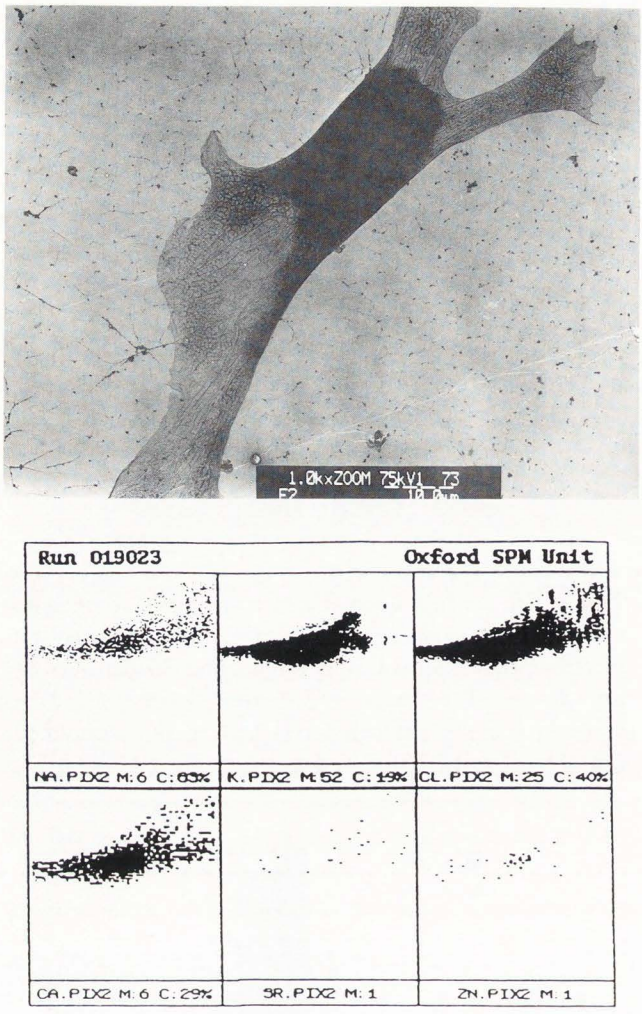

Fig. 4. a. Transmission electron micrograph (bar 10 μm) and **b.** Elemental maps obtained by proton probe microanalysis of a spread β-cell. Size of scan, 100 × 100 μm. The mean K/Na ratio in this cell was 6.2. Color map transposed to grey-scale map.

Results

To detect a significant Sr uptake in RINm5F-cells, using the electron microprobe, a stimulation time of 30 seconds was necessary (Fig. 1). The effect of K^+ stimulation on other elements (after about 20 seconds of stimulation) is shown in Fig. 2. The most prominent change was an increase of Na ($p < 0.05$). We then studied pancreatic β-cells, which were exposed to high K^+ for two seconds. The electron probe showed that all elements except Ca, were found in lower concentrations in β-cells compared to RINm5F-cells (Figs. 2 and 3). As in RINm5F-cells, a significantly elevated Na concentration ($p < 0.01$) was found in stimulated cells compared to controls. No Sr was detected in the β-cells. High concentrations of Ca in β-cells have previously been shown by Berggren *et al.* (1978), using flameless atomic absorption spectrometry and Wolters *et al.* (1979), employing staining techniques and X-ray microanalysis. A transmission electron micrograph of a spread control β-cell is shown in Fig. 4a. The elemental distribution of the same cell is given in the maps (Fig. 4b). In this spread β-cell, the area of high levels of Ca and Zn corresponds to the area of high K and the nucleus, which is similar to the RINm5F-cells but differs from the distribution in the less spread β-cells. In Fig. 4b, the results of combining the high spatial resolution of the electron probe and the higher elemental sensitivity of the proton probe are shown.

Using the proton microprobe, we could detect an uptake of Sr after only two seconds of K^+ stimulation in the RINm5F-cells. This was shown both by point analysis (Fig. 5) and elemental maps, shown in Fig. 6a (control cells) and Fig. 6b (stimulated cells). It was only in stimulated RINm5F-cells (3 cells) that point-analysis showed Sr-content and no Sr was detected in the controls nor in the β-cells. The elemental maps show the distribution of Na, K, Cl, Ca and Zn. These maps show that Ca and Zn were found in the nuclear area, co-localized with high amounts of K. Because RINm5F-cells spread out on the substrate the nucleus can be easily discerned.

In the experiments with mouse pancreatic β-cells, the short stimulation time (two seconds) was applied. Elemental maps obtained by proton microprobe analysis of control β-cells (Fig. 7a) and stimulated β-cells (Fig. 7b), showed the distribution of Na, K, Cl, Ca, Sr and Zn. In contrast to RINm5F-cells, no Sr was detected in β-cells. Most β-cells did not spread out on the Formvar film as readily as RINm5F-cells. The elemental distribution in β-cells that had flattened out differed from that in cells that remained somewhat spherical. Ca and Zn were still co-localized in these spherical cells, but they were not co-localized with K in the perinuclear area (Figs 7a and 7b). On the other hand, in the spread β-cells (such as the cell shown in Fig. 1), the area of high levels of Ca and Zn corresponds to the area of high K and the nucleus, which is similar to the RINm5F-cells.

Discussion

This work was undertaken to study elemental distribution in insulin-secreting cells under conditions stimulating exocytosis and particularly, to explore whether these cells are polarized regarding Ca^{2+} uptake. However, because a stimulation time of at least 30 seconds had to be applied to obtain a significant Sr uptake (Sr as a Ca analogue) in the RINm5F-cells when

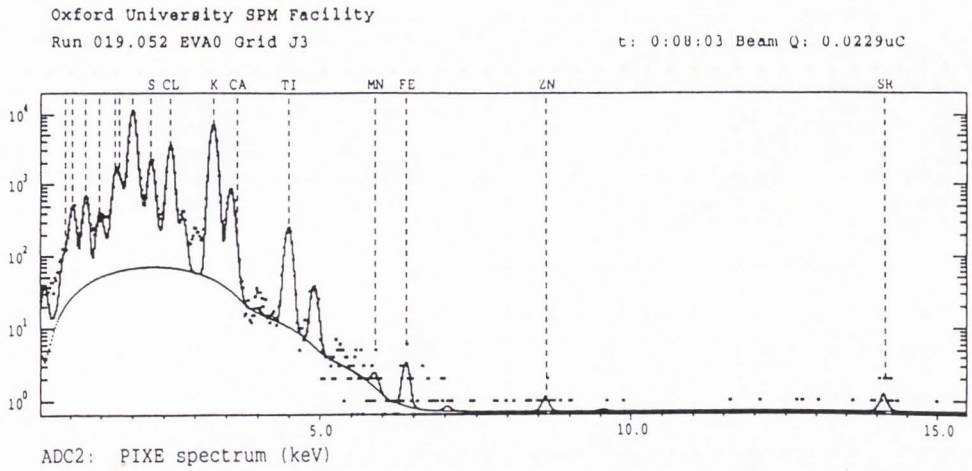

Fig. 5. X-ray spectrum obtained by the proton microprobe from a RINm5F-cell that was stimulated to secrete insulin in the presence of Sr (stimulated with high K^+ for 2 s and analyzed for 8 minutes).

whether these cells are polarized regarding Ca^{2+} uptake. However, because a stimulation time of at least 30 seconds had to be applied to obtain a significant Sr uptake (Sr as a Ca analogue) in the RINm5F-cells when the cells were analyzed by the electron probe, we could not expect to be able to detect polarization in the cells. Therefore, the potential of the proton probe in subcellular analysis was investigated. Among the other elements analyzed (Fig. 2), Na showed a significant increase in response to K^+ stimulation. Broadening in the data obtained from the stimulated cells is probably an effect of heterogeneity in the capability of the cells to respond to stimulation. Electron probe microanalysis of the mouse pancreatic β-cell, stimulated for only two seconds showed an increase in Na that was even more pronounced than that of the RINm5F-cells. An increased uptake of Na^+ in β-cells has been shown previously (Richardson *et al.*, 1980). After different pretreatment, however, glucose stimulation (by increasing the concentration from 3 to 20 mM) of β-cells from obese hyperglycemic mice, may lead to a decrease both in total Na and in Na^+, measured by integrated flame photometry and dual wavelength fluorometry, respectively (Wesslén *et al.*, 1989).

In RINm5F-cells, increased Na may be caused by an influx of the ion through voltage-dependent Na^+-channels (Rorsman, 1986). Such Na^+-channels have not been found to operate in mouse β-cells under the present experimental conditions (Rorsman *et al.*, 1986). In mouse pancreatic β-cells, an increase in Na subsequent to K^+-depolarization may be explained by inhibition of the Na^+/K^+-ATPase and/or an increased activation of the Ca^{2+}-Na^+-exchange. However, in the absence of experimental support, this notion has to be considered as speculative.

In order to detect the small amounts of Sr that enter immediately when the Ca^{2+}-channels open, a more sensitive method is required. The proton microprobe could both detect Sr in RINm5F-cells that had been stimulated for two seconds and provide intracellular distributions of biologically important elements such as Ca, Fe and Zn. However, no polarization of the Sr content was observed in the RINm5F-cells, indicating that the RINm5F-cells are not polarized, or that the techniques used were unable to detect a polarized Sr distribution. A polarization in RINm5F-cells is less likely than in β-cells, since RINm5F-cells represent a cloned insulin-secreting tumor cell line that has been kept in culture for several generations and may have lost its ability to polarize. There was a difference between the more and less spread β-cells in their distribution of Ca, Zn and K, which suggests a redistribution in ion content while cells spread onto a substrate. In thin cryosections of pancreas (Norlund *et al.*, 1987), the elemental distribution more resembled that of the more spherical β-cells, with Ca and Zn in the secretory granules with a relatively low K concentration, but not in the nuclear area with high K concentration. This may suggest a loss of the *in vivo* organization in cultured cells. There were no significant differences in overall elemental concentrations, but the K/Na ratios (a sign of successful preparation) of the more spherical β-cells were lower than the K/Na of the flattened β-cells. This could be an indication of somewhat spherical β-cells not being in a optimal condition and thus unable to respond to depolarization by K^+ by a Sr-uptake.

The distribution of Zn is of particular interest in insulin-producing cells, because in the secretory granules

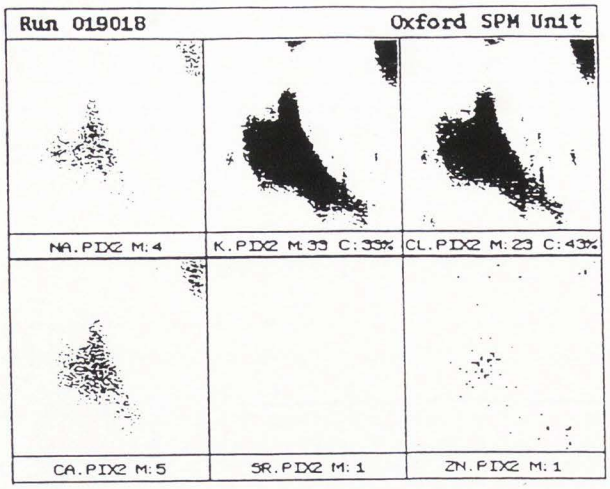

Fig. 6a. Elemental map obtained by the proton probe of control RINm5F cells showing Na, K, Cl, Ca, Sr and Zn distribution. The mean K/Na ratio in these cells was 5.6. Size of scan, 60 × 60 μm. Color map transposed to grey-scale map.

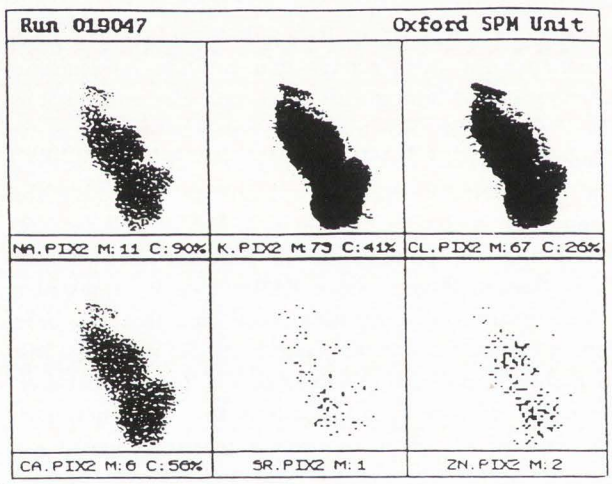

Fig. 6b. Elemental map obtained by the proton probe of stimulated RINm5F cells showing Na, K, Cl, Ca, Sr and Zn distribution. The mean K/Na ratio in these cells was 3.0. Size of scan, 50 × 50 μm. Color map transposed to grey-scale map.

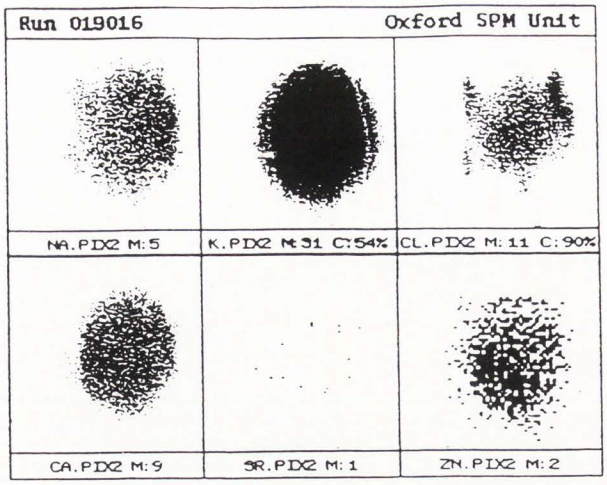

Fig. 7a. Elemental map obtained by the proton probe of an control β-cell showing Na, K, Cl, Ca, Sr and Zn distribution. The K/Na ratio was 3.5. Size of scan, 25 × 25 μm. Color map transposed to grey-scale map.

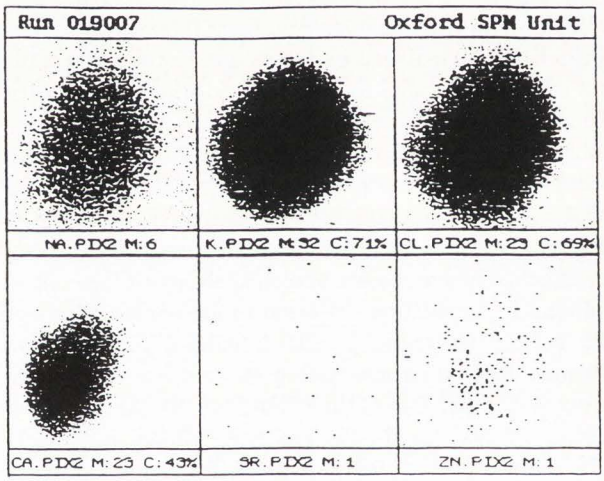

Fig 7b. Elemental map obtained by the proton probe of a stimulated β-cell showing Na, K, Cl, Ca, Sr and Zn distribution. The K/Na ratio was 3.0. Size of scan, 13 × 13 μm. Color map transposed to grey-scale map.

the hormone is crystallized with two Zn atoms per insulin hexamer (Frank and Veros, 1970). This co-existence of Zn and insulin in the granules might therefore offer a way to localize the granules without actually visualizing them (Foster *et al.*, 1993). In particular, this may be useful when the ultrastructure is obscure as it is in the more spherical β-cells. Insulin granules contain significant amounts of Ca (Falkmer *et al.*, 1985; Foster *et al.*, 1993) and thus the co-localization of Zn and Ca in the pancreatic β-cells may be an indicator of insulin granules. According to this finding the distribution of insulin-containing granules is altered when the β-cells spread onto the substrate. From being concentrated to a hemisphere in the somewhat spherical β-cell the granules in the flattened cells were found in the nuclear area, i.e., the thickest part of the cell. These findings indicate that significant changes occur in the distribution of granules and perhaps also in that of other organelles when cells spread which is a limitation of the use of cultured cells.

This study confirms that the combined use of the electron probe and the proton probe provides complementary information (Forslind *et al.*, 1985). Use of the same samples and sample preparation method makes comparisons more reliable. An advantage of the electron probe is its accessibility making it possible to analyze a larger number of cells to provide better statistical precision. However, low concentrations of elements cannot be detected by the electron probe, which reduces its applicability and thus requires the use of the proton probe (Forslind *et al.*, 1985). Because of limited access to the proton probe, the electron probe remains the instrument of choice for elements occurring in sufficiently high concentrations such as Na, K and Cl. It is advantageous first to analyze the cells in the electron probe, e.g., to see whether they have been damaged in the preparation process. Damage can be inferred from the ultrastructure and a low K/Na ratio. Such a procedure ensures that only correctly prepared samples are analyzed by the proton microprobe. The proton microprobe can then provide more thorough information on the same specimen or a specimen from the same series as the one checked by the electron microprobe. In this study, where only a small number of cells were analyzed by the proton probe, the fact that Sr was detected in stimulated cells only, is most encouraging for future analysis with the proton probe. The need for standards and the procedure of background subtraction, necessary in the electron probe studies, also introduce a source of error. The possibility to use RBS is an advantage of the proton probe analysis that should result in more accurate concentrations.

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Discussion with Reviewers

K. Malmqvist: Do you know of any alternative technique to the proton microprobe for localized quantitative trace element analysis to complement the electron microprobe?

Authors: Both laser microprobe microanalysis and ion microprobe microanalysis are more sensitive than electron probe microanalysis. Their resolution is inferior to electron probe microanalysis, but in the same order as proton probe microanalysis. Also, for these techniques, the number of laboratories involved in analysis of

biological specimens is very small, which makes these techniques difficult to access in a similar way as the proton microprobe.

K. Malmqvist: Would the approach used in this work be of more general use in other in vitro investigations?
Authors: Yes, this approach can be used for all types of experiments involving ion fluxes in cultured cells.

B. Forslind: You state in the discussion that the electron probe and proton probe methods as you have employed them give complementary information. How well do the data on potassium (K) and chlorine (Cl) overlap with your two methods?
Authors: The values for K and Cl concentrations did not differ significantly between the two methods. While the same grid was analyzed with both methods significant differences were obtained for the Na and the Ca concentrations. The background subtraction performed by the program PIXAN, in proton microprobe analysis, may underestimate the background. Thus the proton microprobe yielded higher Na concentrations than the electron microprobe. The electron probe but not the proton probe detected Ca in the RINm5F-cells, which was surprising in view of the fact that the proton probe is more sensitive. However, the software used in the the proton probe may have difficulties with the K K_{β}/Ca K_{α} overlap.

B. Forslind: The content of Zn, Sr and Ca measured by PIXE analysis is given as a density distribution for each element in your pixel mappings. What are the absolute amounts of those elements? Could you comment on cells *in situ* and in culture?
Authors: Literature values measured in cryosections or islets from ob/ob mice (all concentrations given in [mmol/kg dry weight]), analyzed with [1] proton probe microanalysis (Juntti-Berggren *et al.*, 1991) are: Ca: 35, Zn: 4, Fe: 4.5, [2] flameless atomic absorption spectrometry (Berggren *et al.*, 1977): Ca: 30-60, [3] X-ray microanalysis (Norlund *et al.*, 1987): Ca: 18-155, Zn: 1-37. Our results from proton-probe analysis (means and standard errors of means) in the cultured β-cells were for Ca: 200 $\pm$ 136, Zn: 25 $\pm$ 10 Fe: 19 $\pm$ 7 and in RINm5F-cells for Ca: 47 $\pm$ 23, Zn: 120 $\pm$ 31 and Fe: 28 $\pm$ 7. Stimulated RINm5F-cells did also take up Sr and the average amount found in those that had detectable amounts of the element was 174 $\pm$ 110 mmol/kg dry weight.