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BARIUM ACCUMULATION IN RAT PANCREATIC B CELLS

S. L. HOWELL AND MARGARET TYHURST
School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, Sussex, England

SUMMARY

Barium has been used as an electron-opaque substitute for calcium in a study of the distribution of divalent cations between organelles in homogenates or intact rat islets of Langerhans. These were incubated in the presence of barium acetate. Accumulation of electron-opaque deposits was stimulated during incubation of islets in the presence of high glucose concentrations and was diminished in conditions in which intracellular cyclic AMP levels were raised. Mitochondria were found to be the principal sites of accumulation of electron-opaque deposits. Addition of dinitrophenol to homogenates or intact islets abolished mitochondrial barium accumulation. X-ray microanalysis of the deposits in frozen sections showed them to consist predominantly of barium and phosphate.

These experiments serve to emphasize further the critical role of mitochondria in the regulation of divalent cation accumulation in B cells, and to confirm that a direct effect on intracellular distribution of divalent cations may represent one important mechanism of action of cyclic AMP in regulating insulin secretion.

INTRODUCTION

Extracellular calcium is essential for the maintenance of insulin secretion from pancreatic B cells, and there is much current interest in defining the role of this ion in the secretory process. Recent studies have involved estimation of either ^{45}Ca fluxes into (Malaisse-Lagae & Malaisse, 1971) or out of (Malaisse, Brisson & Baird, 1973) intact islets, or net accumulation of ^{45}Ca in homogenates or partially purified subcellular fractions (Howell & Montague, 1975; Howell, Montague & Tyhurst, 1975). These approaches have yielded valuable information, but neither has permitted exact identification of the intracellular sites of calcium accumulation and storage within individual cells.

Direct visualization of intracellular sites of calcium accumulation by conventional electron microscopy is rather difficult because of the low atomic number of calcium; however, barium, another alkaline earth cation which can substitute for calcium in a variety of secretory processes including insulin release (Milner & Hales, 1969), will form deposits which can be readily visualized in the electron microscope. The purpose of this study was to attempt localization of the sites of barium accumulation in islet cells in various states of secretion; in this way it was hoped to determine which sites might be responsible for secretion-related calcium accumulation, since tissue handling of these 2 cations in islets seems likely to be similar.

MATERIALS AND METHODS

Incubation of homogenates

Islets of Langerhans were isolated from pancreas of 200–250 g female rats by a collagenase procedure (Howell & Taylor, 1966) and were then washed with bicarbonate-buffered medium (Gey & Gey, 1936) from which calcium was omitted. In the studies using broken cell preparations the islets were homogenized in 0·25 M sucrose in 10 mM Tris-HCl (pH 7·2) and incubated for 20 min in a buffer containing 0·125 M sucrose, 35 mM KCl, 10 mM Tris-HCl, 4 mM $MgCl_2$, 4 mM K_2HPO_4, 10 mM sodium succinate and 2 mM barium acetate in conditions described in detail by Howell & Montague (1975). The particulate material was then sedimented by centrifugation (24000 g, 10 min) and fixed for electron microscopy.

Incubation of intact islets

In these studies isolated islets were incubated in medium containing 2·5, 5 or 10 mM barium acetate, together with 110 mM NaCl, 4·7 mM KCl, 1·2 mM KH_2PO_4, 18·7 mM $NaHCO_3$ and 1·2 mM $MgCl_2$, pH 7·4 (Somlyo, Somlyo, Devine, Peters & Hall, 1974). Glucose, 3-isobutyl-1-methylxanthine or dinitrophenol were added at the concentrations indicated in Results. In experiments in which insulin secretion was studied, samples of incubation medium were removed after 60 min incubation at 37 °C and their insulin content determined by radioimmunoassay (Hales & Randle, 1963).

Electron microscopy

After brief washing, islets or pellets which had been incubated in barium-containing solutions were fixed in 3% glutaraldehyde in phosphate buffer pH 7·4, post-fixed in 2% OsO_4, dehydrated and embedded in epoxy resin by a conventional procedure. Thin sections were examined in an AEI EM6B electron microscope without subsequent staining, or in a few cases after staining with a saturated solution of uranyl acetate in 50% ethanol. For quantitative estimation of the number of barium deposits per mitochondrion, a standard morphometry grid was used, and only mitochondria under intersections of the grid were assessed. In these experiments several islets obtained from each of four experiments performed on separate occasions were examined.

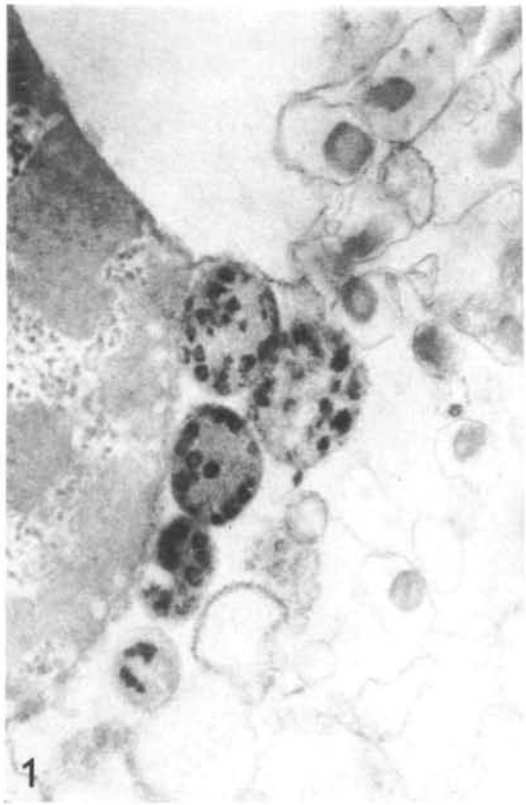
Specimens for X-ray microanalysis were incubated with 10 mM barium acetate for 45 min, rinsed briefly and then immersed in liquid nitrogen. Frozen sections of the unfixed tissue were prepared using an LKB Cryokit and procedures which have already been described (Howell & Tyhurst, 1974). Sections (~ 120 nm thick) were placed on Formvar-coated nickel grids and brought slowly to room temperature. A thin carbon film was placed over the sections which were analysed in a Jeol 100C microscope fitted with an energy-dispersive X-ray microanalysis system (Link Systems Ltd, High Wycombe), at Jeol Ltd, Colindale, London NW9.

Figs. 1, 2. Thin sections of pelleted homogenates which had been incubated in a buffer containing 0·125 M sucrose, 35 mM KCl, 10 mM Tris-HCl, 4 mM K_2HPO_4, 10 mM sodium succinate and 2 mM barium acetate for 20 min in the presence of 1·25 mM ATP (Fig. 1) or 1·25 mM ATP + 0·25 mM 2,4-dinitrophenol (Fig. 2). Fig. 1, × 24000; Fig. 2, × 21000.

Figs. 3, 4. Thin sections from B cells of intact islets which were incubated with 10 mM barium acetate for 45 min in the presence of 20 mM glucose.

Fig. 3. Deposits are restricted to the mitochondria and to a fine precipitate which is unevenly dispersed along the plasma membrane (arrows). Unstained, × 14000 approx.

Fig. 4. The mitochondria are seen to contain 1–6 deposits each. Two of the storage granules contain single deposits within the sac (arrows), the others contain no precipitate. Stained with 50% ethanolic uranyl acetate, × 24000 approx.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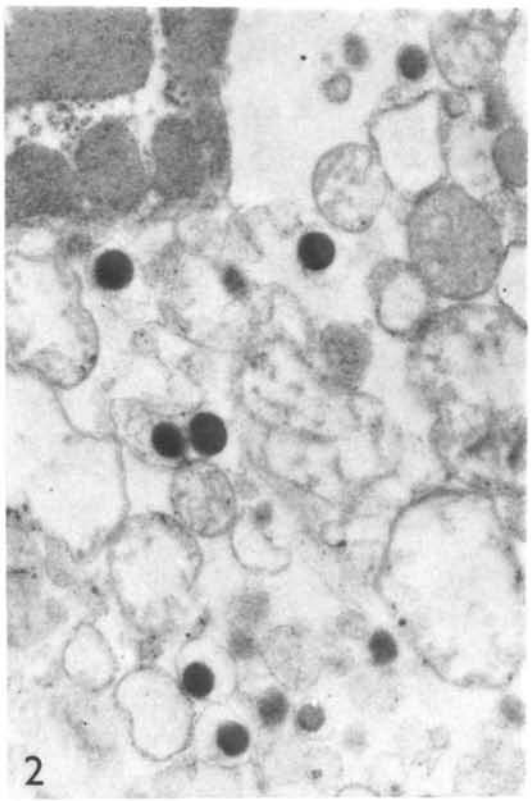
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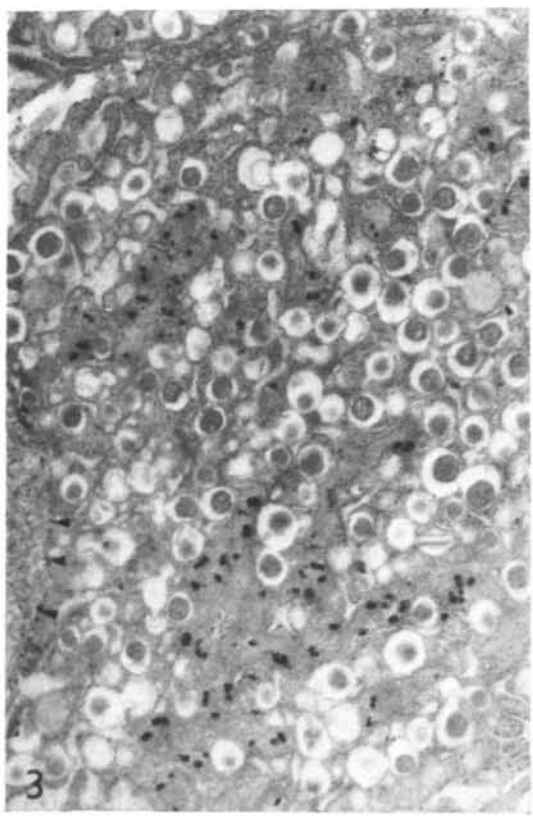
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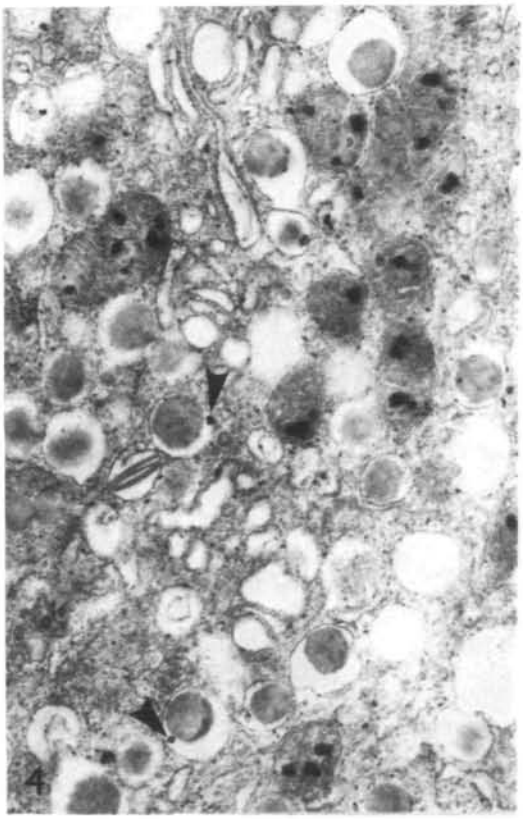
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RESULTS

Barium uptake by subcellular fractions

Examination of thin sections of pelleted homogenates containing nuclei, storage granules, microsomes and mitochondria showed the mitochondria to be the principal sites of barium accumulation. Uptake was strongly stimulated by addition of 1·25 mM ATP (Fig. 1) and was completely abolished in the presence of 0·2 mM dinitrophenol (Fig. 2). Storage granules and microsomes failed to exhibit electron-opaque deposits, either in the presence or absence of ATP.

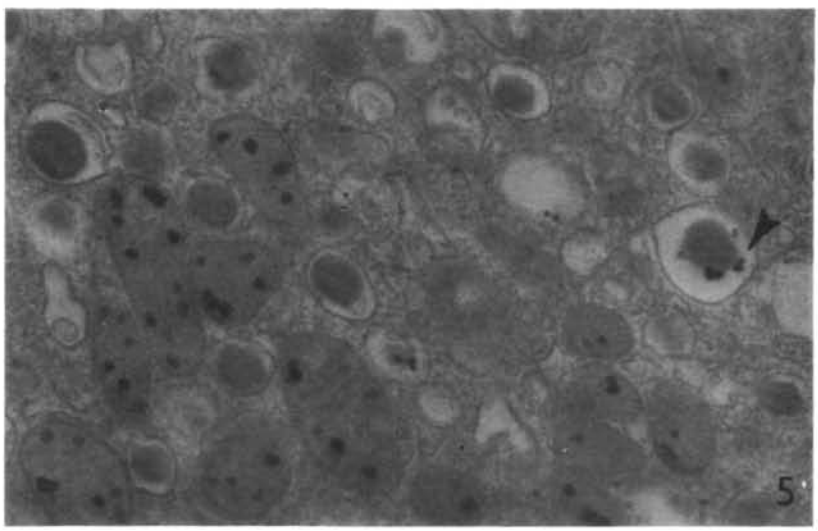


Fig. 5. Section treated as for Figs. 3, 4. In the area shown, each mitochondrion contains electron-opaque precipitates. Unusually, one of the granules also contains several deposits (arrow), which are apparently located on the surface of the granule core. Unstained, × 35000 approx.

Uptake in intact cells

Examination of electron micrographs of islets which had been incubated for 45 min in basal medium containing 5 or 10 mM barium acetate showed several sites of precipitation: the mitochondria, which contained multiple deposits of diameter in the range 20–40 nm, the plasma membrane where the deposits were smaller, and apparently randomly distributed along the membrane (Fig. 3), and in occasional storage granules which also contained smaller deposits (Figs. 4, 5). Only the mitochondrial deposits, which were by far the most prolific, were considered in the present study. Detailed examination showed them to be present within the matrix of almost every mitochondrion in each cell (Figs. 4, 5); they appeared to be approximately spherical and varied in number between mitochondria in individual cells, within a range of 2–8 deposits per profile.

Preliminary experiments included a study of the time course of barium accumulation by islet mitochondria, the incubation medium containing 10 mM barium acetate

in each case. Islets were withdrawn for fixation 5, 10, 20 and 30 min after the start of incubation and the resultant time course of accumulation is shown in Fig. 6 which represents the mean of 175–200 observations of individual mitochondrial profiles at each time point. Although significant deposits were found after only 5 min of incubation, 20 min were required to obtain the maximum levels of accumulation, which were

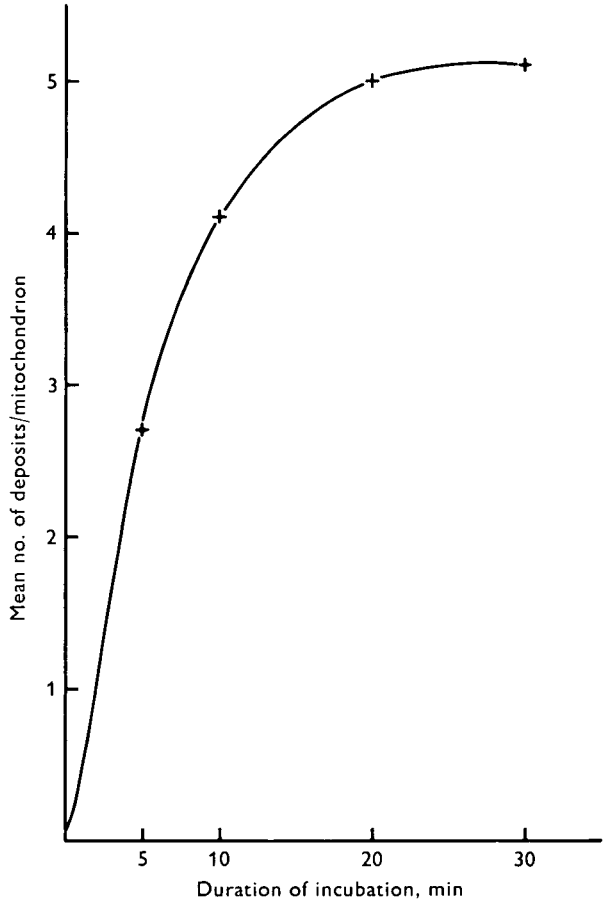


Fig. 6. Time course of barium accumulation by pancreatic B cell mitochondria during incubation with 10 mM barium acetate and 20 mM glucose in the conditions described in the text. Each point is the mean of 175–200 observations of randomly selected mitochondria.

then maintained for at least a further 25 min, the longest time period which was tested. An incubation of 45 min duration was used routinely and further experiments centred on quantitative analysis of the number of deposits which were obtained after incubation of islets in various conditions.

Addition to the basal medium of 5 mM glucose, which maintains insulin secretion in a resting condition, resulted after 45 min incubation in $3{\cdot}8 \pm 0{\cdot}25$ (mean and standard error of mean, 176 observations) deposits per mitochondrial profile. Addition of 20 mM

glucose, which stimulates secretion of insulin, to the incubation medium caused an increase to 5·1 ± 0·21 (211 observations) deposits per profile. Incubation with 20 mM glucose together with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0·5 mM), which raises cyclic AMP levels, resulted in 3·3 ± 0·22 (172 observations) deposits per mitochondrial profile. Finally, addition of 20 mM glucose plus 0·2 mM 2,4-dinitrophenol, an inhibitor of oxidative phosphorylation which dramatically lowers ATP levels in islet cells and inhibits ^{45}Ca accumulation by isolated organelles, almost completely abolished barium deposition by mitochondria in intact cells (Fig. 7).

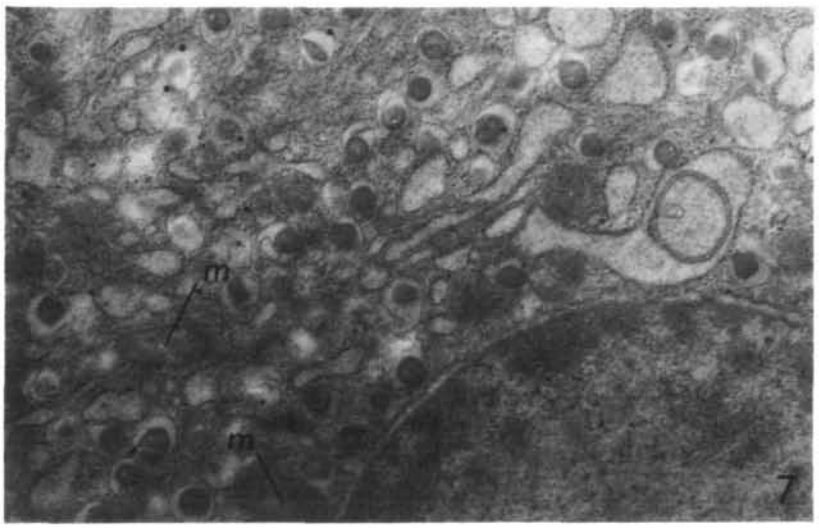


Fig. 7. Addition of 0·25 mM 2,4-dinitrophenol to islets otherwise incubated in conditions identical to those for Figs. 3, 4 resulted in complete abolition of barium uptake by the mitochondria (*m*). Unstained section, × 13000 approx.

Barium deposits in frozen sections

Examination of unfixed frozen sections of islets which had previously been incubated with 5 mM barium acetate for 45 min showed a pattern of electron-opaque deposits which was similar to that seen with conventional sections. There was no evidence of precipitation in the other organelles which could be identified in the sections.

X-ray microanalysis

Surprisingly, and in apparent contrast to the results of Somlyo *et al.* (1974), X-ray microanalysis of the electron-opaque deposits in conventional sections failed to show the presence of barium within the deposits. However, analysis of exactly similar deposits in frozen sections of unfixed islets (Howell & Tyhurst, 1974) which had been incubated in 10 mM barium acetate before freezing readily showed the presence of large quantitites of barium, evidently in association with phosphate (Fig. 8A). The apparent ratio of barium : phosphate varied in different deposits between extremes of 1 : 2 and 2 : 1, making a direct estimate of the concentrations of the 2 ions very difficult.

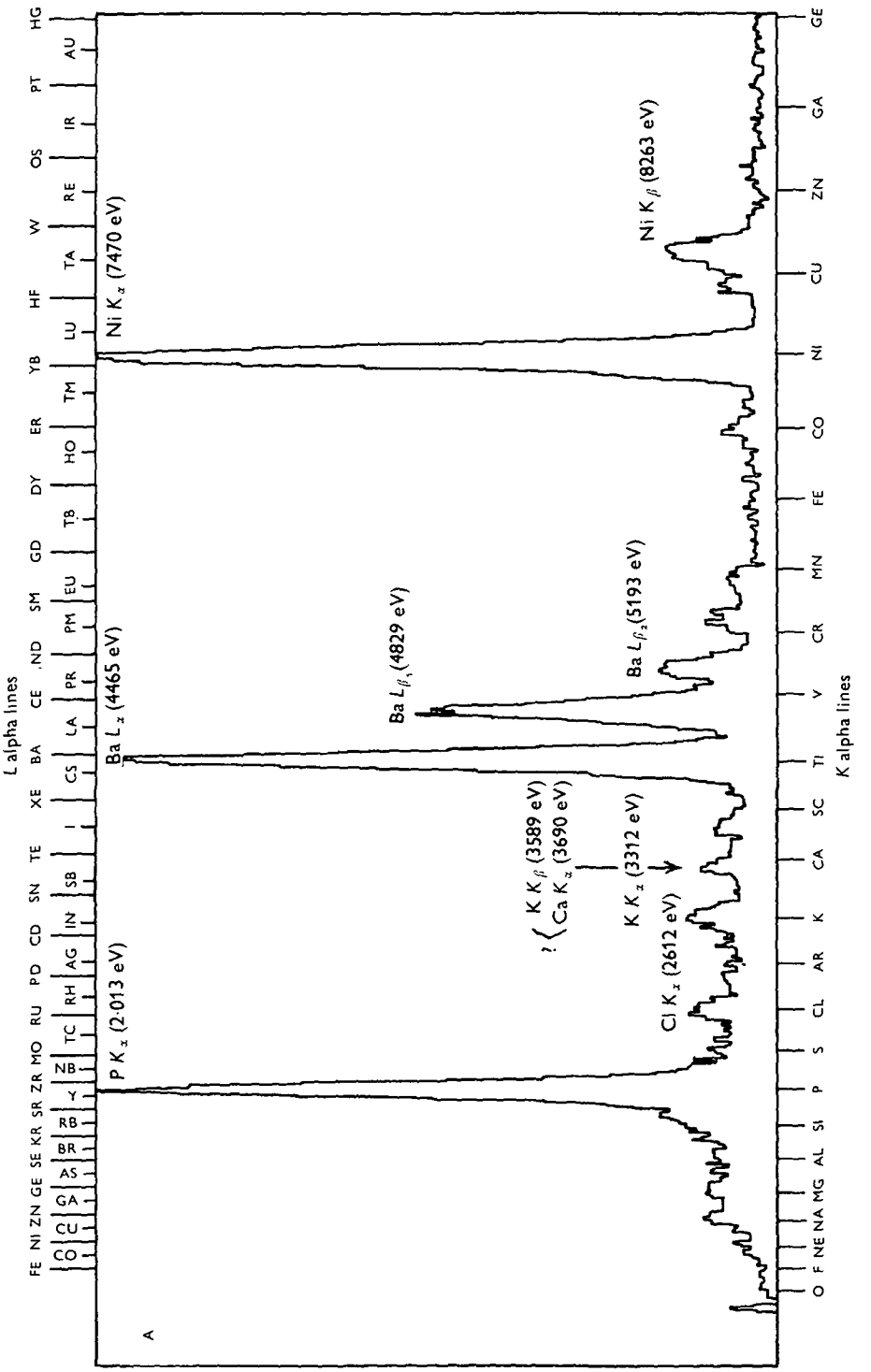
In any case the efficiency of detection of the analyser for barium and phosphorus may be very different, so that it is impossible to compare concentrations directly from relative peak heights without the use of suitable standards for comparison. Analysis was possible using a spot diameter of 20 nm by using a STEM attachment, and the patterns obtained were compared with an area 1 μm displaced from the mitochondrion in question. These latter areas were devoid of detectable barium or phosphate (Fig. 8B). The nickel peaks present in both scans originate from the nickel supporting grids.

DISCUSSION

Barium can apparently provide an effective substitute for calcium in a number of secretory cells, including adrenal medulla (Douglas & Rubin, 1964), neurohypophysis (Dicker, 1966), mast cells (Foreman & Mongar, 1972) or platelets (Sneddon, 1972), and in smooth muscle (Somlyo *et al.* 1974). In the case of pancreatic B cells, Milner & Hales (1969) and Hales (1970) showed that at low concentrations (0·1–0·2 mM) barium was able to substitute for calcium in permitting stimulation of secretion from rabbit pancreas slices in response to a variety of agents, while at higher concentrations barium itself could stimulate secretion in the absence of other stimuli. Maximal barium-induced stimulations were obtained in the concentration range of 5–20 mM and further increases in secretion rate could be observed after the simultaneous addition of theophylline, glucagon or cyclic AMP. Glucose, leucine or tolbutamide on the other hand provoked no further stimulation of secretion when they were added in the presence of 5–20 mM barium (Hales, 1970). These results seem to be true also for isolated rat pancreatic islets (Howell & Tyhurst, unpublished observations). Further experiments also showed that protein biosynthesis is unaffected by the addition of barium concentrations up to 10 mM (data not shown), again suggesting that addition of this ion has no adverse effect on islet function, and this impression was confirmed by ultrastructural observations (Figs. 3–5).

Barium uptake by subcellular fractions

The first series of experiments was performed in order to determine the sites of divalent cation accumulation in homogenates or subcellular fractions. Earlier experiments studying ^{45}Ca accumulation in these conditions showed that uptake of the isotope was rapid, ATP-dependent and required the presence of a permeant anion (oxalate or phosphate). The characteristics appeared to be those of mitochondrial accumulation of calcium, but it could not be completely excluded that other organelles, and particularly the storage granules which are already known to be sites of calcium storage (Howell *et al.* 1975), could also be responsible for part of the uptake which was observed. Experiments were therefore performed in conditions identical to those utilized in calcium uptake studies with subcellular fractions, but with substitution of 2 mM barium for 20 μM ^{45}Ca and these showed conclusively that mitochondria were the only important sites of accumulation of barium in any of the organelles (nuclei, mitochondria, granules, rough and smooth microsomes) which were regularly observed in the pellets.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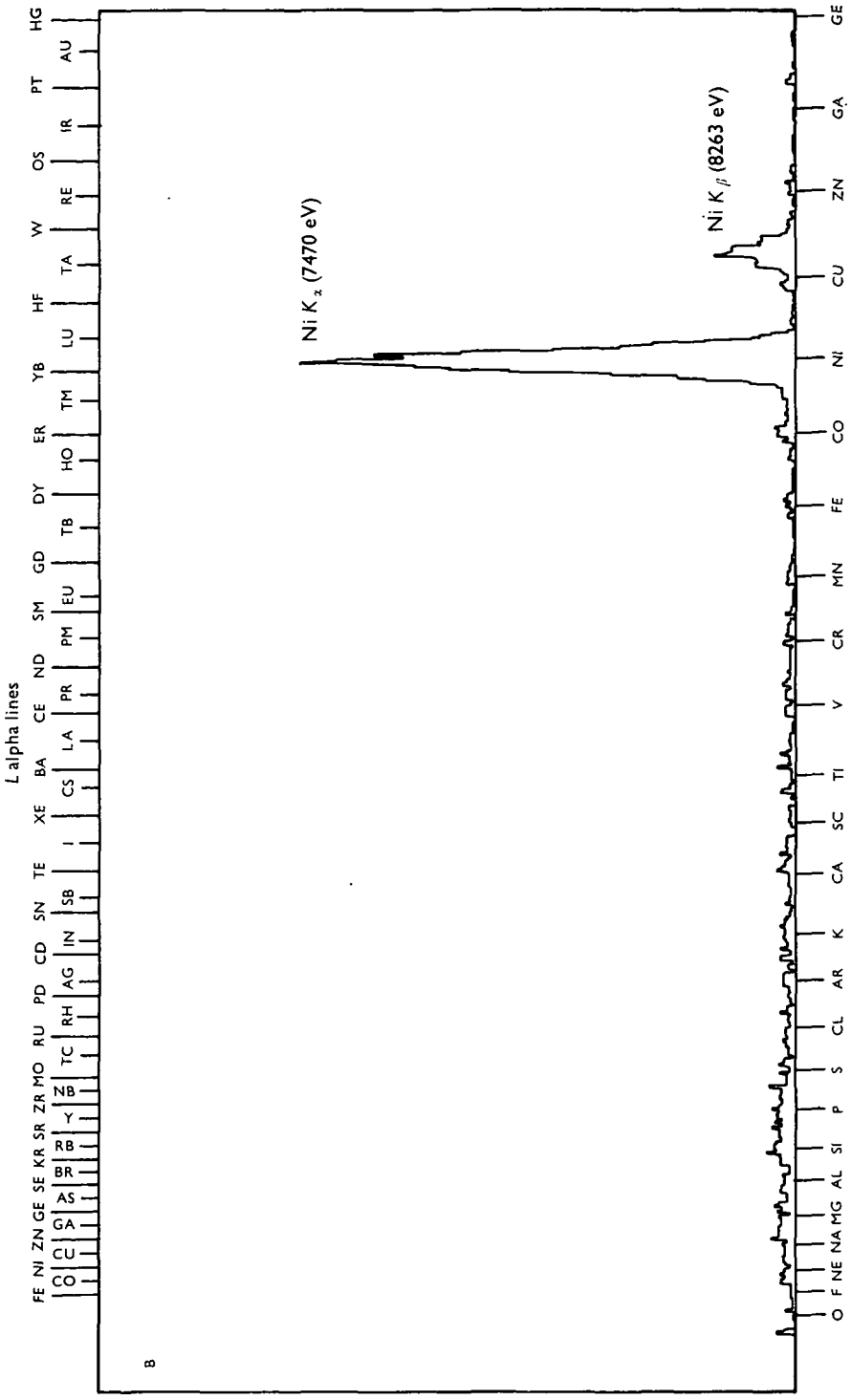

Fig. 8. A, electron-microscope X-ray microanalysis of a mitochondrial barium deposit in an unfixed frozen section of a rat islet which had been incubated with 10 mM barium acetate and 20 mM glucose for 45 min. The major peaks (left to right) are phosphorus K_α, barium L_α and L_β and nickel K_α and K_β from the grid. B, analysis of the same grid square with the spot displaced from the precapitate by 1 μm. The major peaks are nickel K_α and K_β from the grid.

These characteristics of barium accumulation by isolated organelles are similar to those of ^{45}Ca uptake reported previously and strongly suggest that in these conditions mitochondria are the major sites of uptake of both cations. However, the incubation conditions used, which were designed as optimal for studies of calcium accumulation by mitochondria (Borle, 1974), might be quite unsuitable for demonstrating uptake by other organelles. We therefore performed further studies which involved incubation of intact islets in a medium containing 5–10 mM barium acetate in place of calcium chloride, and assessment of the pattern of barium accumulation by the intact B cells.

Barium uptake by intact cells

In these experiments a similar pattern of uptake was observed, the mitochondria being the principal sites of accumulation. The effect of glucose in increasing storage of barium in intact B cell mitochondria is consistent with previous observations of a net increase in ^{45}Ca uptake by isolated islets as studied by direct washout (Malaisse-Lagae & Malaisse, 1971) or lanthanum membrane-sealing (Hellman, Sehlin & Taljedal, 1976) procedures. Hellman *et al* (1976) in fact suggested from these data that an intracellular site with high affinity for calcium might be involved in mediating its accumulation and the present experiments suggest that mitochondria might provide such a site. Glucose itself had no effect on calcium accumulation by isolated mitochondria, which may indicate that its metabolism, perhaps reflected in an increased ATP flux, is required to stimulate divalent cation accumulation and storage in intact B cell mitochondria.

In most cells which were examined every mitochondrion showed the presence of barium deposits, and in some but not all sections a very small proportion of the storage granules showed the presence of deposits within the granule sac, although a quantitative estimate of their distribution and number has not so far been attempted. This suggests, as had previous autoradiographic studies (Howell & Tyhurst, 1976), that although mitochondria and granules both contained high calcium concentrations as determined by microanalysis, only the mitochondria represent a readily exchangeable pool in these conditions.

It was also shown that the effects of cyclic AMP in diminishing calcium accumulation by isolated subcellular particles (Howell & Montague, 1975; Sehlin, 1976) can be reproduced in experiments involving barium uptake in intact B cells. This shows that such a mechanism is not restricted to the artificial conditions of isolated mitochondrial incubations, and again suggests that alteration of intracellular divalent cation distribution, and in particular raising of cytosolic calcium levels by inducing efflux of calcium from mitochondrial stores, may be an important mechanism of action of cyclic AMP in the regulation of insulin secretion.

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