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Autometallography allows ultrastructural monitoring of zinc in the endocrine pancreas

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Abstract Zinc is intimately involved in insulin metabolism, its major known role being the binding of insulin in osmotically stable hexamers in β-cell granules. To investigate the anatomical distribution of zinc ions necessary for insulin binding we examined the rat pancreas by autometallography (AMG). AMG demonstrates chelatable zinc and is a sensitive marker for zinc in vesicles and also a surrogate marker for recently described zinc pumps regulating intravesicular zinc metabolism. Zinc ions were found in α- and β-cell granules, primarily in the periphery of the granules. Only occasionally was zinc seen in other islet cell types. AMG allows the study of the microscopic and ultrastructural localisation of free zinc ions in the pancreas. The applicability of the method at the ultrastructural level in particular makes AMG a very sensitive tool in future studies on the role of zinc ions in the pancreas.

Keywords Alpha-cells · Beta-cells · Pancreas · Ultrastructure · Zinc

Introduction

The storage of insulin ready for excretion on metabolic demands in β-cells of the pancreas requires crystallisation of insulin molecules. This is obtained by complexing six insulin molecules with two zinc atoms via histidine binding sites. This binding results in osmotically stable complexes suitable for storage until required for secretion (Hill et al. 1991). Zinc seems to be important for storage alone since insulin production is normal in zinc-free environments (Howell et al. 1978). It has been hypothesised that the pancreas could act as a zinc reservoir (MacDonald et al. 1994; Shisheva et al. 1992; Zimny et al. 1993). Zinc within β-cells is primarily found in secretory granules (Gold and Grodsky 1984; Norlund et al. 1987). Also α-cells have been found to contain zinc in up to 10–15% of their granules. This has been suggested to represent co-production of insulin along with traditional α-cell products (Epand 1982; Foster et al. 1993). Chelation of zinc from the pancreas results in overt diabetes (Epand et al. 1985), and the zinc content of β-cells in insulin-dependent diabetes is reduced by 50% (Kiilerich et al. 1990). Recently, it was suggested by Kim et al. (2000) that release of zinc from secretory vesicles might contribute to islet cell death, particularly in hyperinsulinaemic states. As shown by Palmiter et al. (1996), zinc ions demonstrated by autometallography (AMG) may be a sensitive marker for one or more zinc transporting proteins, which actively regulate zinc concentrations in vesicles. Thus zinc plays an active role in both α- and β-cells, and studies of its anatomy and physiology seem warranted. Zinc in the pancreas has previously been demonstrated by a variety of fluorescent dyes, by autoradiography, and by the use of dithizone, and in 1968 Pihl demonstrated "heavy metals", primarily zinc, in pancreatic islets by a silver sulphide technique (Jindal et al. 1993; Pihl 1968; Westmoreland and Hoekstra 1969). Since AMG improves sensitivity compared to the above methods and is particularly suited for ultrastructural studies of vesicular zinc (Danscher 1996; Danscher et al. 1985), we have employed an AMG method for the demonstration of zinc ions in the rat pancreas.

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Materials and methods

Experimental animals

Male Wistar rats, with an average weight at killing of 190 g, were raised and housed under standard laboratory conditions. A total of 40 rats was analysed. Institutional ethical guidelines were obeyed.

Control animals were sham-injected with saline and perfused with glutaraldehyde and otherwise processed as described below.

Treatment

The autometallographic procedure requires that free zinc ions are captured by sulphides or selenium prior to silver development. During pentobarbiturate anaesthesia a number of administration routes for sulphides were tested in order to optimise the staining of the pancreas.

Intravenous injection of sulphide followed by transcardial fixation resulted in highly reproducible and constant staining patterns and was easy to administer. The following procedure proved superior: intravenous sulphide (200 mg/kg body weight sodium sulphide; 40 mg/ml solution) was injected through the inferior caval vein and allowed to circulate for 3 min. This was followed by transcardial perfusion with glutaraldehyde (1%, 100 mM phosphate-buffered aqueous solution) for 6 min at 150 mmHg.

Tissue samples

The part of the pancreas closest to the spleen was isolated, removed and immersed in glutaraldehyde until further use. Following dehydration small randomly chosen tissue blocks were embedded in Epon, and 3-µm-thick sections were cut.

Autometallography

Mounted Epon sections on glass slides were dipped in 0.5% gelatine and dried. Development took place at 27°C in a dark room by immersing gelatinised sections in the developer comprising 60 ml gum arabic (33% aqueous solution), 10 ml citrate buffer (pH 3.4), 15 ml hydroquinone (aqueous solution containing 0.85 g) and 15 ml silver lactate (aqueous solution containing 0.12 g). The development was continued for 70 min, whereafter sections were immersed in water and subsequently in Farmer's solution for 10 s, rinsed in water and finally counterstained with 1% toluidine blue.

Light microscopy was performed with 3-µm-thick Epon sections. Electron microscopy was performed after re-embedding Epon sections onto a block by cutting 50-nm-thick sections which were counterstained by uranyl acetate (10 min) and lead citrate (1 min). Sections were examined by routine transmission electron microscopy.

Result of the experimental procedure

AMG results in dark brown or black silver deposits easily identified in light microscopic sections. In the electron microscope, silver deposits appear as electron-dense dots.

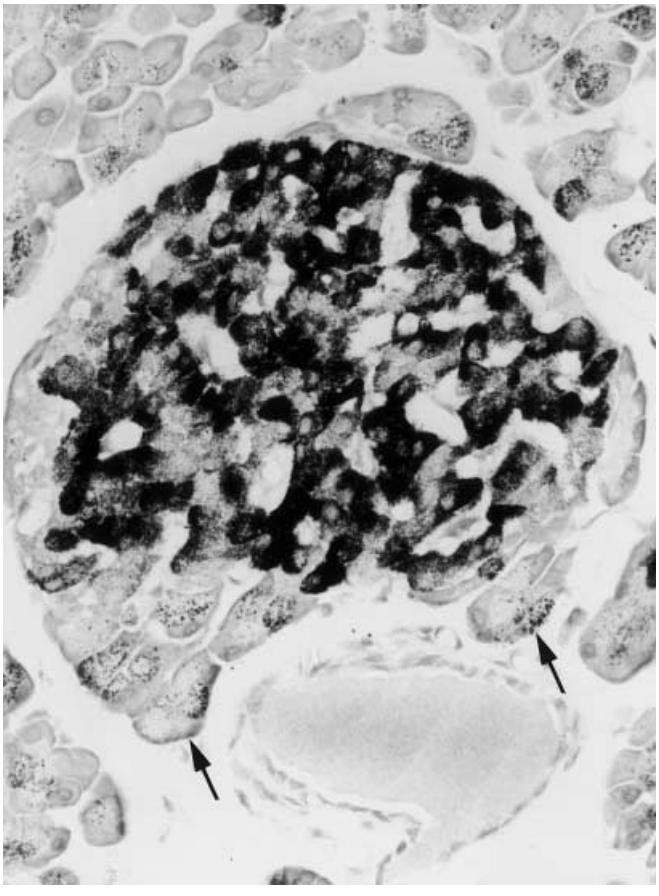

Fig. 1 Rat islet of Langerhans with intensely stained cells in most areas. In some peripheral areas (*arrows*) less intensely stained cells with silver grains scattered in the cytoplasm are seen. Silver grains represent silver sulphide complexes developed by autometallography (AMG) and appear black or dark brown in the light microscope. Also in some exocrine cells the cytoplasm contains zinc; this is not unspecific background staining, but represents chelatable zinc in zymogen granules, a phenomenon seen also in other exocrine glands, for example, salivary glands. Light micrograph, original magnification ×100

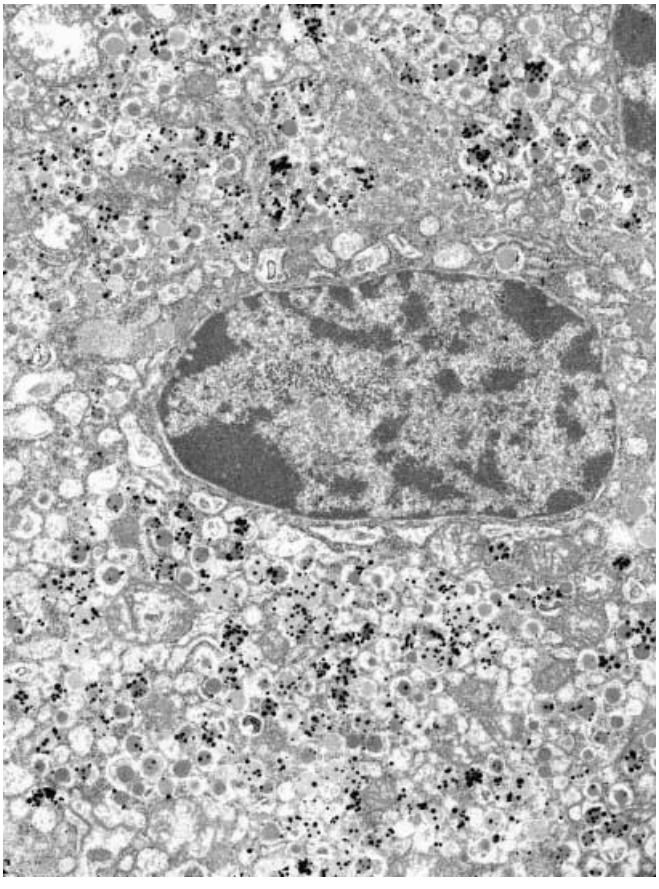

Fig. 2 β-cell densely packed with secretory granules of which a large number are zinc-positive to varying degrees. Numerous granules are, however, left unstained. It might be hypothesised that this obvious difference reflects some specific metabolic activity in the granules, for example, the activity of zinc pumps being zinc-positive when the zinc-dependent packaging of insulin precursors is active. Electron micrograph, original magnification ×4,200

Controls

Sections from sham-injected animals not treated with sulphide were devoid of silver grains.

Results

As seen in Fig. 1, islets of Langerhans appeared densely stained contrasting the surrounding tissue in the rat pancreas. At low magnifications, islets of Langerhans were easily defined by the presence or non-presence of silver grains. There was little variation in the intensity of the staining among different islets and no islet was left unstained. In the part of the pancreas examined here (the left hemipancreas) all islets thus appeared with a similar intense stain. An estimated 80% of cells in each islet contained several granules which seemed dispersed uniformly in the cytoplasm. Occasionally, silver stained tissue was seen in the exocrine part of the pancreas where the staining was much less intense and much more dispersed.

By routine electron microscopy we identified β-cells and α-cells. Zinc in β-cells was invariably found in close association with secretory vesicles characterised by a relatively small size, varying electron density and characteristic halos, i.e. secretory granules containing insulin products (Fig. 2). Occasionally crystalline structures, suggesting the presence of insulin, were found in zinc-positive granules (Fig. 3). Very heavily stained granules were present along with more sparsely stained or non-stained granules. The size, localisation or electron density of stained granules did not differ from that of sparsely stained granules. Within granules zinc was found anywhere. In some granules, however, the staining was most intense in the periphery of granules in contrast to more central parts (Fig. 4).

In α-cells the staining was less intense. As in the β-cells, zinc granules were located in secretory granules. In some areas of the pancreas only a minority of granules per cell was stained. However, all α-cells examined in the electron microscope were stained to some extent (Fig. 5). Within granules zinc was located primarily in the periphery (Fig. 6). We did not identify insulin-like crystals in zinc-positive α-cell granules. Without the combination of AMG and immunohistochemistry we were unable to safely identify cell types other than α- and β-cells. However, no cell examined in the electron

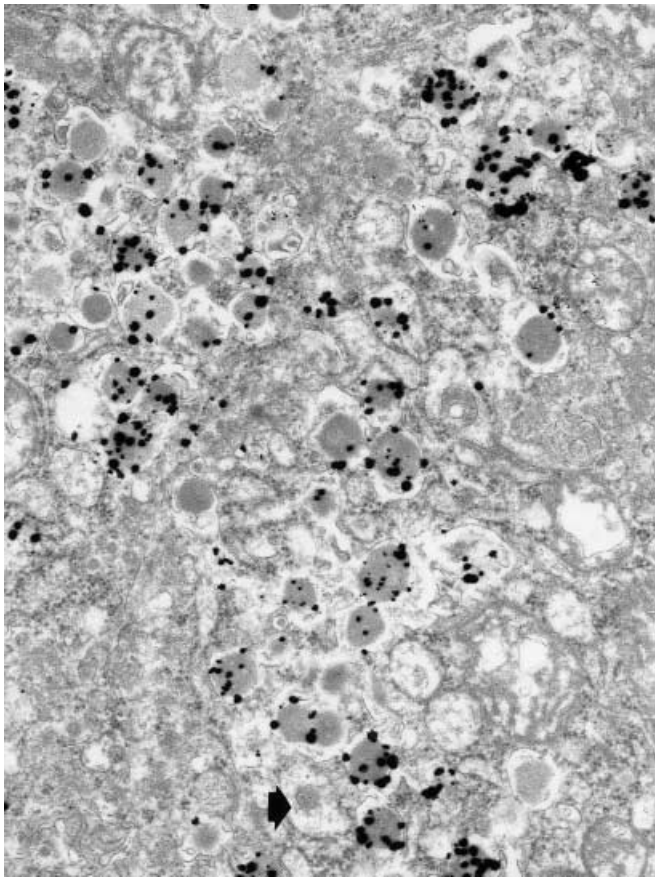

Fig. 3 A closer look at a β-cell similar to that shown in Fig. 2. The distribution of zinc is precisely monitored after AMG. Both zinc-positive and zinc-negative granules are present in this section. *Arrow* indicates crystalline-like intravesicular structure. Electron micrograph, original magnification ×10,000

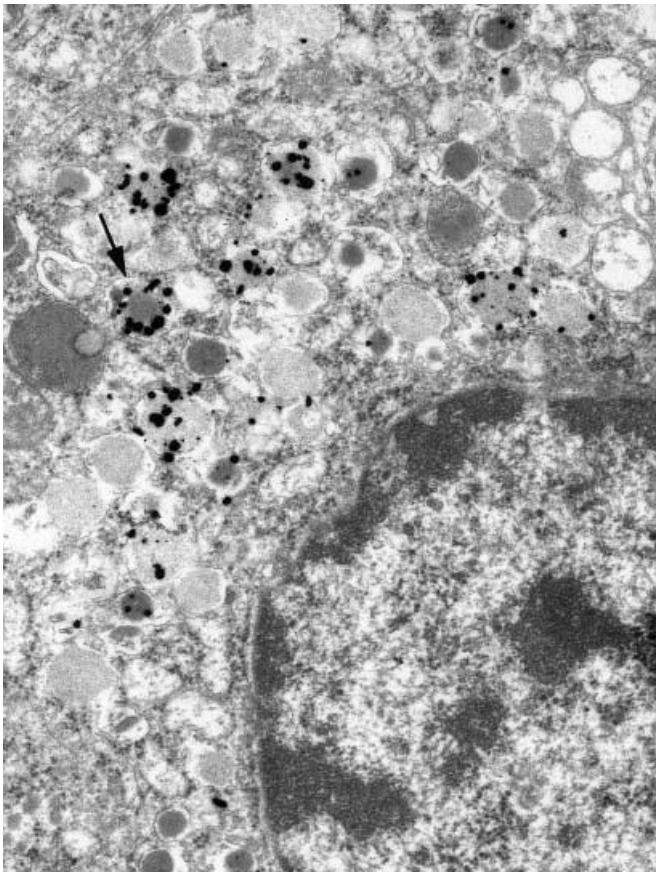

Fig. 4 With AMG chelatable zinc can be found in the periphery of some β-cell vesicles (*arrow*), but in others more evenly distributed throughout the vesicle. Electron micrograph, original magnification ×10,000

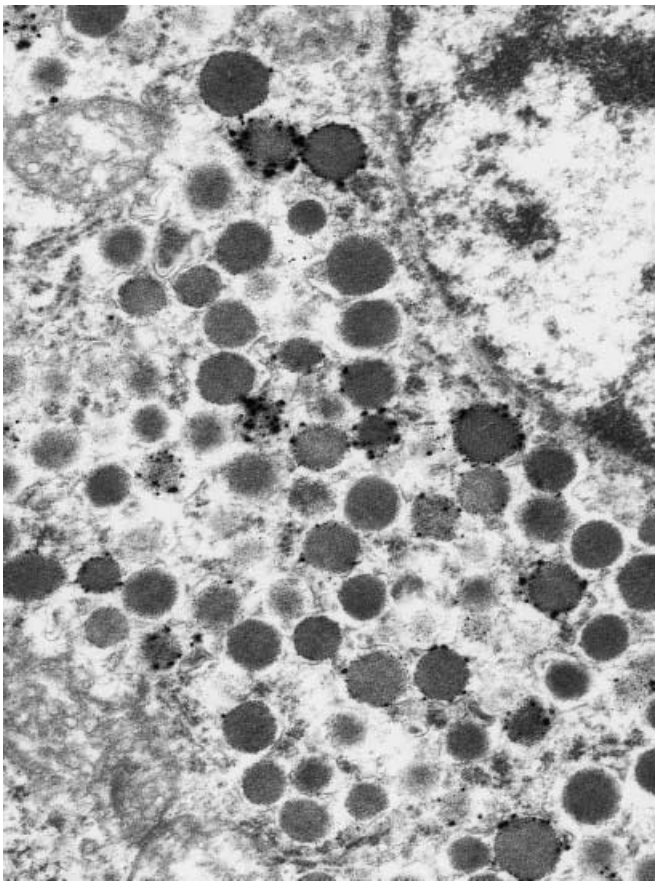

Fig. 5 In α-cells zinc-positive granules are much rarer, and if present the staining appears much more scattered. Granules are easily distinguished from those of β-cells. Zinc is almost exclusively located in the granule periphery. Electron micrograph, original magnification ×10,000

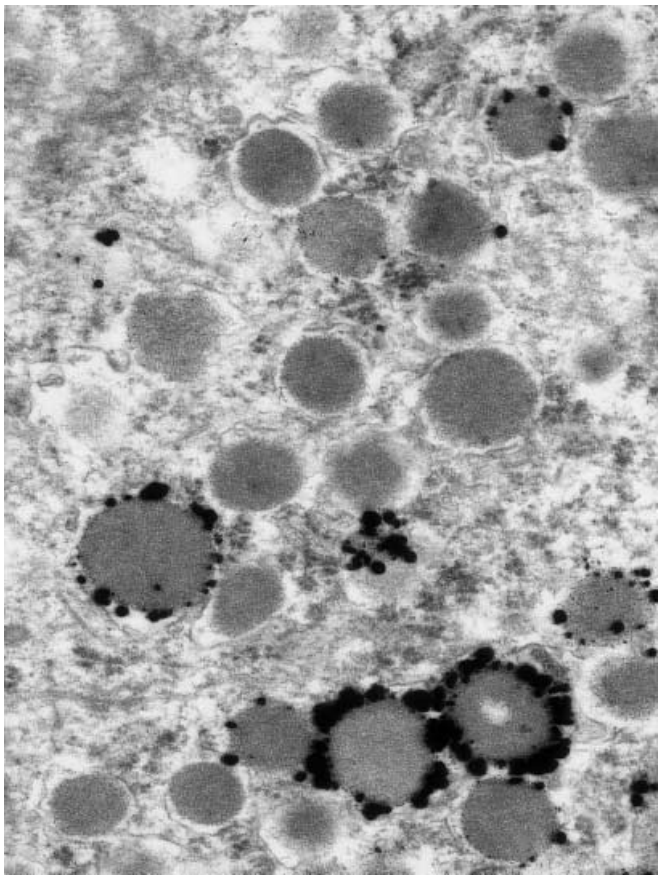

Fig. 6 Close-up of granules from an α-cell. Zinc is found in close association with secretory granule membranes only. Electron micrograph, original magnification ×18,000

microscope was left unstained, with the α- and particularly β-cells remaining the most intensely stained. Zinc was thus probably present also in pancreatic endocrine cells secreting hormones other than insulin and glucagon. Exocrine cells of the pancreas were only examined sporadically. However, exocrine zymogen granules contained zinc scattered within them.

Discussion

Sensitivity and specificity

AMG per se may demonstrate a number of heavy metals bound as sulphides or selenides in biological tissue. Metallic gold will also trigger the autometallographic development procedure. Metals most likely to create false-positive patterns are gold, silver or mercury (Danscher 1996). These metals will ignite autometallographic development with or without prior treatment of tissue with sulphide as used in this experiment. Although histochemical methods for the differentiation among AMG-positive metals exist, their presence is best controlled for by the use of parallel-treated control animals. In this case we found no autometallographic deposits in control animals. The specificity for zinc by the present sulphide approach has been demonstrated for vesicles in the central nervous system by proton-induced X-ray emission analysis, showing that zinc is the only substrate responsible for autometallographic grains after sulphide perfusion (Danscher et al. 1985). The sensitivity of the methodology is probably at the level of a few zinc atoms if two or more are located close (within nanometres) to one another (Danscher 1996).

Substrate for AMG in endocrine pancreatic cells

Enzyme-bound zinc, abundant in all cells, is not demonstrated by AMG. Most likely, in proteins, single zinc atoms are bound too tightly within tertiary structures to be trapped by infused sulphide molecules. The present results and previous studies on the localisation of AMG grains in other organ systems suggest that we demonstrate chelatable zinc ions representing free zinc ions fluxing in and out of insulin hexamers (and, for the α-

cells, unknown structures). As shown recently, AMG-positive zinc ions are the substrates for active zinc transporters (Palmiter et al. 1996) which may also be present in the pancreas. It is likely that these zinc pools, insulin-related zinc in secretory vesicles and zinc pump-related zinc in vesicle membranes are the chemical basis for the staining seen here. The physiological correlate of the AMG staining is thus suggested to be zinc, which is transported in a controlled fashion into β-cell granules whereafter it is trapped within insulin hexamers. Using a somewhat similar approach Yoshinaga and Ogawa (1975) were able to describe zinc in β-cells of the pancreas only. The finding of silver grains located in the core of β-cell granules differs somewhat from the results described here. The authors elaborated their study by treating rats with glucose, resulting in a more diffuse silver staining of β-cell granules. They also treated rats with sulphonyl urea, resulting in silver grains concentrating on vesicle membranes. The presence of zinc in glucagon-containing granules might be the result of an active role of zinc in glucagon processing, as suggested by Epand et al. (1985), or the result of an α-cell co-production of insulin (Epand 1982; Foster et al. 1993). However, we could not identify insulin crystals associated with α-cell zinc, and there seems to be a distinct α-cell staining pattern differing from that seen in β-cells; thus the substrate for the α-cell stain awaits further examination. In conclusion, we describe here a specific and very sensitive method for the ultrastructural demonstration of physiologically relevant zinc pools in the pancreas. This method can provide the morphological basis for further studies of zinc in physiological and pathological β- and/or α-cell conditions, in which the metal may be intimately involved.

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