Zinc-Enriched Endocrine Cells in the Rat Pituitary —A Combined Autometallographic and Immunohistochemical Study

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Zinc ions have been demonstrated in the anterior pituitary of rat by autometallography (AMG) which involves *in vivo* exposure to sodium selenite leading to creation of zinc selenide crystal lattices that are later silver enhanced by AMG in the sections. In this study, autometallography is combined with immunohistochemistry in order to present the coexistence of zinc ions and hormones in endocrine cells of the rat pituitary. The results showed that the majority of zinc-enriched (ZEN) cells are somatotrophs. All

the somatotrophs observed were loaded with AMG grains. Zinc ions were also found to be present in gonadotrophs, corticotrophs and thyrotrophs. A low density of AMG silver grains was found in about 60% of gonadotrophs, 8% of corticotrophs and less than 10% of thyrotrophs. Lactotrophs did not appear to contain zinc ions. The present observations may provide a clue to the physiological function of zinc ions in the rat pituitary.

Key words: Endocrine cells, Rat, Pituitary, Zinc ions, Autometallography, Silver enhancement, Immunohistochemistry

I. Introduction

By way of zinc-specific autometallographic techniques, i.e. the selenium method [2] and the Timm method [1, 4, 22], zinc ions have been demonstrated in the synaptic vesicles of zinc-enriched (ZEN) neurones in the mammalian, avian and fish brains [5, 16, 17]. The ZEN neurones, believed to be a subgroup of glutaminergic neurones, are present throughout the central nervous system, but are most abundant in the telencephalon [1, 6, 8, 9, 11, 12, 19]. Secretory granules containing zinc ions have been demonstrated in both endocrine and exocrine cells of many other organs and cells, e.g. in the testis, prostate, epididymis, pancreas, submandibular glands, Paneth cells and bone [4, 7, 10, 20, 21]. Recently, membrane proteins that behave as zinc pumps have been described [15]. The immunohistochemical patterns of these pumps, named ZnT-3 in the mouse brain, are almost identical to the histochemical staining patterns found by the autometallographic zinc methods [1, 2, 4, 15].

The rat pituitary gland is very rich in zinc. Particle induced X-ray emission (PIXE) measurement showed the

total amount of zinc to be 74 ng/mg dry weight in males and 100 ng/mg dry weight in females. However, the largest zinc pool (>95%) is most likely incorporated in the proteins and cannot be detected by autometallography. Only a small zinc pool (<5%) is present as zinc ions that can be demonstrated by AMG [24]. At the electron microscopic level, zinc ions have been localised mainly in the secretory granules of endocrine cells [23, 24].

The present combination of immunohistochemistry and zinc ion histochemistry was applied in order to identify the types of endocrine cells in the rat pituitary that contain zinc ions and to see if the AMG distribution pattern indicates a function of the metal in the preservation of the vesicular content or hormone release in these endocrine cells.

II. Materials and Methods

Ten female Wistar rats with a mean weight of 200 g were used in this study.

Tissue preparations

The animals were anaesthetised and injected with 20 mg/kg sodium selenite intraperitoneally. After a survival period of 2 hr the animals were killed by transcardial perfusion with 400 ml of 2% glutaraldehyde in 0.1 M

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phosphate buffer (pH 7.4). The pituitary glands were removed, immersed in the same fixative for 4 hr and then embedded in Epon, and polymerised at a temperature below 45°C for 50–70 hr. Coronal semithin sections, 3 µm thick, were cut and placed on glass slides and dried for one hour on a heating plate kept at 45°C. The sections were coated with 0.5% gelatine and allowed to air-dry for 30 min prior to development.

Autometallography

The slides were immersed in an autometallographic developer containing 60 ml protecting colloid (stock solution: 50% w/v crude gum arabic in deionised water), 10 ml citrate buffer (stock solution: 25.5 g citric acid monohydrate + 23.5 g sodium citrate dihydrate in 100 ml deionised water) and 0.85 g hydroquinone in 15 ml deionised water. Immediately before development, 0.12 g silver lactate, dissolved in 15 ml deionised water, was added to the developer. The slides were placed in jars and covered with the AMG developer. The set-up was then covered by a light-tight hood in order to reduce the tendency of self-induced creation of AMG grains in the developer. Development took place in a water bath at 26°C.

At the end of development (60 min), the AMG developer was replaced by a 5% thiosulphate solution for 5 min. The sections were then rinsed in running tap water (40°C) for 20 min in order to remove the gelatine film on the surface of the sections and finally dipped twice in deionised water before being processed for immunostaining.

Immunohistochemistry

After treatment with saturated alcoholic sodium hydroxide for 45 to 60 min to remove resin, the sections were washed in absolute ethanol (10 min × 2), rehydrated in 95% and 70% ethanol, distilled water, and 0.01 M phosphate buffered saline (PBS, pH 7.1). Immunostaining was performed by the avidin-biotin peroxidase complex method using reagents purchased from Vector Laboratories (Burlingame, CA, USA). The sections were treated with 0.5% hydrogen peroxide in methanol for 1 hr at 37°C, followed by 1:30 normal goat serum or 1% bovine serum albumin in PBS for 1 hr at 4°C to block non-specific bindings. The sections were then incubated with

the primary antibody (Table 1) for 20 hr at 4°C. After washing in PBS, the sections were incubated for 1 hr at room temperature with either biotin-conjugated goat anti-rabbit IgG (1:200) or biotin-conjugated goat anti-guinea pig IgG (1:200), according to donor species of the primary antibody. This was followed by thorough rinsing and incubation with peroxidase- or alkaline phosphatase-labelled avidin-biotin complex (1:100) for 45 min at room temperature. The enzyme labels were visualised by incubation with Vector SG substrate (for peroxidase) to obtain a blue-grey reaction product, or with Vector Blue substrate (for alkaline phosphatase) to give a blue colour. Finally, the sections were quickly dehydrated in a graded series of alcohols, cleared in xylene, and coverslipped using Permount (Fisher Scientific).

Controls

Brain tissue from a sodium selenite treated rat, known to contain ZEN neurones, was used as positive control. The negative controls included (1) the omission of sodium selenite injection and (2) intraperitoneal injection with 1 g/kg body weight diethyldithiocarbamate (DEDTC), a chelating agent, one hour before the injection of sodium selenite. In addition, pituitary sections from the negative controls were immunostained in the same manner as the AMG sections to see if the AMG process might affect immunostaining.

III. Results

Under the light microscope, the presence of zinc ions was seen as deposits of dark brown AMG silver grains. The AMG grains were found in the cytoplasm of a number of pituitary endocrine cells. The positive cells were confined to the pars distalis of the rat pituitary. The majority of the stained endocrine cells were small and ovoid in shape, and were often arranged in clusters in the periphery of cell cords, adjacent to the sinusoids (Fig. 1). They were less abundant in the periphery of the pituitary gland. This distribution pattern was similar to that of the somatotrophs, as revealed by double-staining with anti-serum against growth hormone (GH). In fact, most, if not all, of the somatotrophs contained grains but not all the AMG grains were localised to somatotrophs (Fig. 2A).

Table 1. Primary antibodies used in this study*

Antibodies	Product codes	Working dilution
Rabbit anti-human GH	AFP-C11981A	1:2,500
Rabbit anti-human ACTH	AFP-39013082	1:10,000
Rabbit anti-rat beta-TSH	AFP-1274789	1:10,000
Rabbit anti-rat PRL	AFP-425-10-91	1:25,000
Guinea-pig anti rat beta-FSH	AFP-85GP9691BFSHB	1:5,000
Guinea-pig anti rat beta-LH	AFP-22238790GPOLHB	1:2,000

*Antibodies were donated by National Hormone Pituitary Program.

Fig. 1. Distribution of zinc-enriched cells in the rat anterior pituitary. Zinc ions were visualised by autometallography (AMG) which generated a deposition of silver grains in the cytoplasm of zinc-containing cells. Cells with a high density of grains were often arranged in clusters, in the periphery of cell cords, adjacent to the sinusoids. Magnifications: **A**=600 $\times$; **B**=1500 $\times$. Bars: **A**=20 μ m; **B**=10 μ m.

Fig. 2

Immunohistochemical studies using antisera to the other pituitary hormones showed partial overlap between zinc-containing cells and a number of cell types, but the intensity of AMG staining was far most pronounced for the somatotrophs.

The gonadotrophs, immunoreactive to follicle-stimulating hormone (FSH) and luteinising hormone (LH), varied from oval to slightly angular in shape. About 60% of these cells showed a low density of AMG grains (Fig. 2B, C). In sections double-stained for zinc and gonadotrophs, the majority of zinc-containing cells were not immunoreactive to FSH or LH antibodies.

Only a small proportion (about 8%) of corticotrophs, immunoreactive to adrenocorticotrophic hormone (ACTH), appeared to be zinc-positive (Fig. 2D). Since corticotrophs are known to have a similar distribution as the somatotrophs in the rat pituitary, apart from the fact that they are more concentrated in the centre of the cell cords, the cytoplasmic processes of the stellate corticotrophs are usually in juxtaposition to somatotrophs. In our double-stained preparation it was accordingly observed that the corticotrophs were often found adjacent to, and in close association with, zinc-containing cells, as would be expected if the majority of the zinc-containing cells were somatotrophs.

The thyrotrophs, identified by antibody to thyroid-stimulating hormone (TSH), constituted the smallest population of hormone-producing cells in the pituitary gland studied. They appeared as large, polygonal cells distributed in the centre of cell cords. Less than 10% of the thyrotrophs exhibited AMG grains, and the density of grains in these ZEN cells was relatively low (Fig. 2E).

The analysed pituitary sections contained a large population of prolactin (PRL)-immunoreactive lactotrophs, but none of them contained AMG grains (Fig. 2F).

The immunostaining results were similar whether or not the preparations had been processed for AMG. Omission of sodium selenite injection gave a negative result, while DEDTC treatment prior to injection of sodium selenite, a treatment that has been proved to block the AMG staining in brains [5] resulted in blank sections.

IV. Discussion

The present study demonstrates that autometallography and immunohistochemistry can be combined on sections from rat pituitary for co-localising zinc ions and bioactive proteins or peptides. In 1982, the selenium method was invented [2] and for the first time it became possible to chemically bind zinc ions *in vivo*. Approximately one hour after an intraperitoneal injection of sodium selenite, zinc ions in ZEN cells in the entire organism are bound *in situ* as zinc selenide crystal lattices i.e. nano-sized clusters of zinc selenide molecules. When sections from such animals are exposed to a photographic developer containing silver ions, i.e. an autometallographic developer, the zinc selenide crystallites grow bigger because the silver ions adhering to their surface are reduced to silver atoms. In this way the zinc selenide crystals are located precisely where they were created, and can be observed at both light and electron microscopic levels. Because of high sensibility, specificity and quality of morphological preservation, the *in vivo* selenite method is often used for detecting zinc ions in tissue sections. The selenite method is the only technique available for tracing ZEN neurones by retrograde axonal transport of zinc selenide crystals created by local or systemic treatment with selenium [3].

Immunostaining on both AMG and non-AMG preparations resulted in the same quality and at light microscopic levels the dark-brown AMG grains were easily distinguishable from the blue or grey-blue immunoreaction product. The combined technique therefore seems to provide a useful tool to investigate the coexistence of zinc and any bioactive substances. If the antigens are sensitive to glutaraldehyde, an alternative fixative such as a mixture of glutaraldehyde and paraformaldehyde can be used [13].

Thorlacius-Ussing demonstrated the presence of zinc in the secretory granules of three pituitary cell types (somatotrophs, corticotrophs, and thyrotrophs) identified by ultrastructural morphology [23]. The use of ultrastructural characteristics to identify pituitary cell types has its limitations e.g. Nakane mentioned that while somatotrophs and lactotrophs may be identified with a high degree of certainty under the electron microscope, corticotrophs and thyrotrophs are sometimes difficult to distinguish without the aid of immunocytochemistry [14]. The use of

Fig. 2. Semithin sections of rat anterior pituitary. AMG and immunohistochemistry were performed on the same sections to demonstrate zinc ions and endocrine cells, respectively. Deposits of dark brown AMG silver grains were found in zinc-enriched cells. Hormone-containing cells were stained blue (after alkaline phosphatase staining with Vector Blue substrate) or blue-grey (after peroxidase staining with Vector SG substrate). Magnification = 875 $\times$. Bars = 20 μ m. **A:** A high density AMG grains was co-localised with GH (blue) in the majority of somatotrophs (arrows). AMG grains were also found in a small number of unstained cells (arrowheads). **B:** A low density of fine AMG grains was present in a proportion of gonadotrophs immunoreactive to anti-FSH (blue-grey) (arrow). Arrowhead indicates a FSH-positive cell which lacks AMG grains. **C:** LH-immunoreactive gonadotrophs (blue-grey) also showed partial overlap with fine AMG grains (arrows). **D:** A small proportion of corticotrophs, identified with anti-ACTH (blue), contained AMG silver grains (arrows). **E:** Very little overlap was observed between AMG grains and TSH-immunoreactive thyrotrophs (blue). **F:** No co-localisation between AMG grains and PRL (blue) was detected.

specific antibodies in our investigation enabled us to identify pituitary cell types according to the hormone contained. Our results confirmed the earlier report that most AMG deposits are found in the somatotrophs, and lesser amounts in the corticotrophs and thyrotrophs [23]. The observation that about 60% of gonadotrophs contain AMG precipitation and that the lactotrophs are completely devoid of zinc ions is new.

Presently, we cannot explain why zinc ions coexist with some pituitary hormones, but not with prolactin, or why somatotrophs contain more and larger AMG grains compared to gonadotrophs or thyrotrophs. The presence of zinc ions in synaptic, secretory or lysosomal vesicles suggests that ionic zinc is essential for some common biological function. It has been suggested that zinc ions in the beta-cells of pancreatic islets of Langerhans have the function of clustering proinsulin in the vesicles in order to make it osmotically invisible until it is released by exocytosis [18]. It is possible that zinc ions have a similar function in some of the pituitary cells and that future studies will reveal zinc pumps in the membranes of the secretory vesicle.

The present study suggests that zinc ions may exert a function in the synthesis, storage or release of growth hormones. The presence of zinc ions in a sub-pool of secretory granules in gonadotrophs, corticotrophs and thyrotrophs, and the fact that many of these cells did not contain detectable zinc may be due to different states of maturity of the vesicles. However, the exact function of the zinc ions in the pituitary still remains to be clarified.

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