Plurihormonality in the secretory granules of the normal human pituitary. An immunoelectron microscopic study

M. Slater

Electron Microscope Unit, Prince Henry Hospital, Little Bay, Sydney, NSW 2036 (Australia)
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Summary. Normal human autopsy anterior pituitary tissue from 5 cases was embedded in LR White resin and immunolabelled using silver-enhanced 5-nm protein A gold probes. Follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), luteinizing hormone (LH), adrenocorticotrophic hormone (ACTH), growth hormone (GH) and prolactin (PRL) were immunolocalised to the level of secretory granule.
A two-sided double-labelling method was used to cross-react two hormones at a time with respect to their corresponding antibodies. All possible combinations of the six pituitary hormones were tested. Plurihormonal granules were found that contained LH + FSH, LH + TSH, and FSH + TSH. Each hormone was also found in monohormonal granules. Granule diameter was significantly larger in the pluri as opposed to monohormonal granules.

Pituitary cell types have often been identified on the basis of size, shape and distribution of the cells and their granules. This method is often inaccurate and may be complicated further by the maturity, plane of section and normal wide variation observed in these cells [1–4]. Immunoelectron microscopy, however, allows positive identification of a particular cell by demonstrating the hormone/s it contains.
To date, multihormonal pituitary cell identification has usually been performed on normal animal and human adenoma tissue. Studies on the normal human pituitary have been rare. The extrapolation of findings from one species to another can introduce error [2]. It was generally assumed that one cell and its granules produced one hormone. Immunoelectron microscopy (IEM) has demonstrated that one cell type can contain the granules of many hormones [5–7]. Multiple hormones have been found in the same granule [8–11] and in monohormonal granules alongside them [12–14]. This introduced the notion of plurihormonality in pituitary cells. These studies used rat, cow or human adenoma cells [5, 13, 15–18]. Otherwise, the concept of plurihormonality of the secretory granules was rarely considered [5–7, 21].

Materials and methods

Five normal human anterior pituitary samples were removed at autopsy within 24 h of death. The patients (three females and two males) were all aged 60–77 and died of causes unrelated to the pituitary. Portions of the five normal human pituitaries were fixed in 1% glutaraldehyde in cacodylate buffer, 0.1 M, pH 7.4 for one hour (to facilitate immunolabelling). Some tissue was also fixed in 2.5% glutaraldehyde to maximise preservation. The tissue was then placed in cacodylate buffer for three changes of 10 min each. Dehydration was carried out in 15-min changes of 30%, 50%, 70% and 2 changes of 100% ethanol (30 min each). A mixture of 1:1 ethanol/LR White resin was prepared and the tissue transferred to it for 1 h, followed by 100% LR White resin changed twice over 48 h. Curing was carried out using gelatin capsules in a 50 °C oven, overnight.

In order to minimise the variation of granule size due to maturity, plane of section and the secretory state of the cell, 100 granules per section and 6 blocks from each case were randomly selected. Sections from each block were both single and double-labelled. The granules of each hormonal type were measured relative to the known size of the (non silver-enhanced) gold probe, as the grating replica could not be used at such high magnifications. The data were then pooled for statistical analysis and the mean and standard error calculated. Results were given to the nearest 5 nm, in keeping with other reports in the literature.

Immunostaining

Following the use of a semi-thin (1 μm) section for tissue orientation, gold sections were cut with a diamond knife, on to nickel grids (300 mesh). Once dry, sections were floated on 10% foetal calf serum (FCS) in 0.1 molar phosphate-buffered saline (PBS) made with ion-free water. This was to block non-specific binding. The grids were then washed in 1% bovine serum albumin (BSA) in PBS, by placing them on the surface of drops of PBS (3 changes of 10 min each). The grids were then placed on the surface of a drop of primary antibody. The antisera used for each of the pituitary hormones were from the 'Histogen PAP' pituitary hormone kit manufactured by BioGenex Laboratories (USA). They were diluted 1:10 with 1% bovine serum albumin (BSA) in 0.1 M phosphate-buffered saline (PBS) before use. The dilution of the antisera as supplied in the kit was not supplied. The grids were then washed on 3 successive drops of BSA/PBS and placed on a drop of protein A conjugated to 5-nm gold particles (from Sigma) for 45 min. Following labelling with the gold probe, the grids were washed on 3 successive drops of BSA/PBS, followed by a water rinse. At this point the grids were either silver-enhanced or stained routinely in uranyl acetate and Reynold's lead citrate. Silver enhancement was not used in the double-labelling studies, as the relative size difference between the probes was important.

Using the two-sided protein A/gold method of Holm and Nesland [22], unstained sections were labelled for each hormone separately and also double-labelled with each of the other pituitary hormones, in order to detect any granule plurihormonality. All possible combinations were tested. Each side of a 300 mesh nickel grid was immunolabelled with a different pituitary antibody and gold probe size e.g. 5 nm gold / anti-FSH on one side and 10 nm gold / anti TSH on the other. Care was taken not to allow immunoreagents to wet both sides of a grid. After routine staining with uranyl acetate and Reynold's lead citrate, the sections were viewed using a Phillips 300 electron microscope at 80 KV.

Controls

Portions of each pituitary were also routinely processed in paraffin and examined for the presence of pituitary hormones using immunoperoxidase light microscopy techniques. In this way positive controls were established by demonstrating the presence of each hormone. The specificity of the antibodies was measured by radioimmunoassay. Specificity for human growth hormone was 100% for GH, 1% PRL, < 1% for TSH, < 1% FSH. TSH was 100% for TSH, 4% for LH, 1% FSH, < 1% GH. FSH was 100% for FSH, LH 12%, TSH 13%. The specificity of the other hormones was measured by positive immunostaining of the relevant cells. This indicated a 12% cross-reactivity of LH with FSH, and 1–4% reactivity for TSH with LH and FSH, which could account for 1–12% of the observed plurihormonal granule labelling. However, these granules demonstrated strong plurihormonality with virtually no non-specific labelling (fig. 3). This indicated a result that was not artifactual or due to the cross-reactivity of alpha subunits. Negative controls were established by the replacement of primary antibodies with either non-immune rabbit serum or PBS. This resulted in the presence of only occasional particles of gold across the entire ultrathin section and no labelling in the target granules. The effectiveness of the method was further supported by the specificity and intensity of the immunogold labelling. None of the monohormonal granules exhibited plurihormonal labelling. The low level of non-specific labelling both in the background and in non-reactive granules, confirmed this view (fig. 2).

Results

Control studies, where the primary antibody was omitted, showed no labelling. The preservation of antigenicity necessitates the use of 1% glutaraldehyde which resulted in incomplete fixation. An optimally preserved reference was provided by parallel processing of a portion of the same tissue in 2.5% glutaraldehyde (fig. 1). Minimal autolysis was noted (fig. 1) and the granule sizes did not vary significantly from case to case. The presence of each pituitary hormone was revealed by a strong positive immunolabelling reaction in the granules (fig. 2). Non-specific labelling was minimal and the negative controls showed no reaction for each of the pituitary hormones.

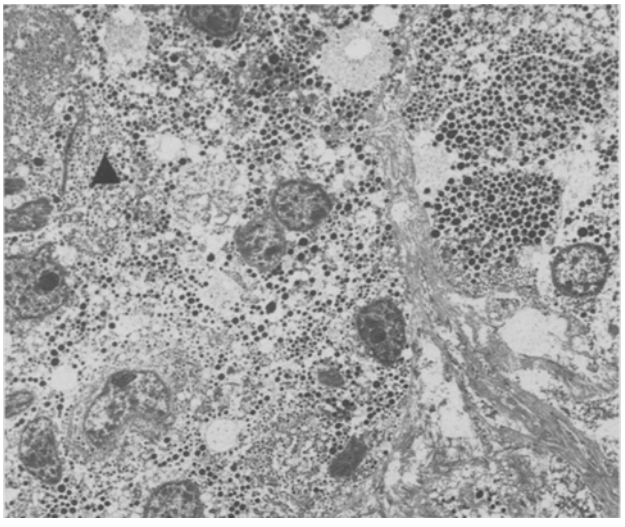

Figure 1. 1200 × 2.5% Glutaraldehyde. Electron micrograph showing anterior pituitary cells with numerous hormone granules. Uranyl and lead staining. The area in which multihormonal granules were found is indicated (arrowhead).

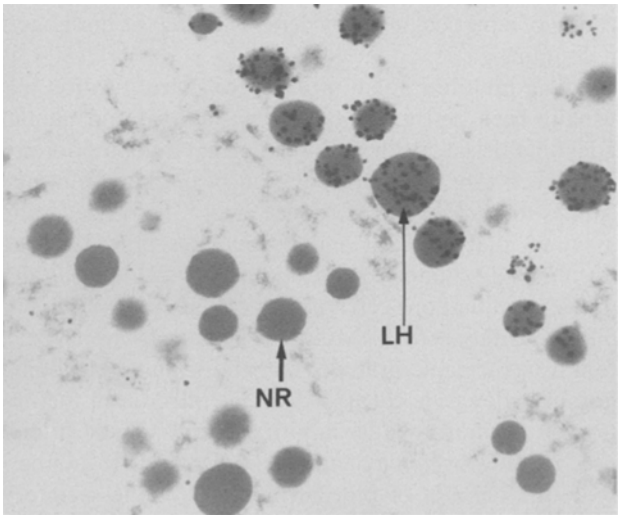

Figure 2. 70,000 × 1% Glutaraldehyde. Monohormonal granules labelled for luteinizing hormone. 5-nm protein A gold, silver-enhanced (long arrow). Adjacent granules were non-reactive (short-arrow – NR). The non-specific background labelling was low.

Granules exhibiting a strong immunoreaction were often adjacent to granules exhibiting no reaction (fig. 2).

The monohormonal granules in these areas measured as follows: growth hormone (GH) granules averaged 190 +/− 15 nm (mean +/− standard error), adrenocorticotrophic hormone (ACTH) granules: 180 +/− 16 nm, follicle stimulating hormone (FSH) granules: 80 +/− 17 nm, luteinizing hormone (LH) granules: 150 +/− 22 nm, thyroid stimulating hormone (TSH) granules: 160 +/− 10 nm, and prolactin (PRL) granules: 70 +/− 13 nm.

Double-labelling for each hormone with the other hormones unexpectedly revealed LH with TSH (fig. 3), LH

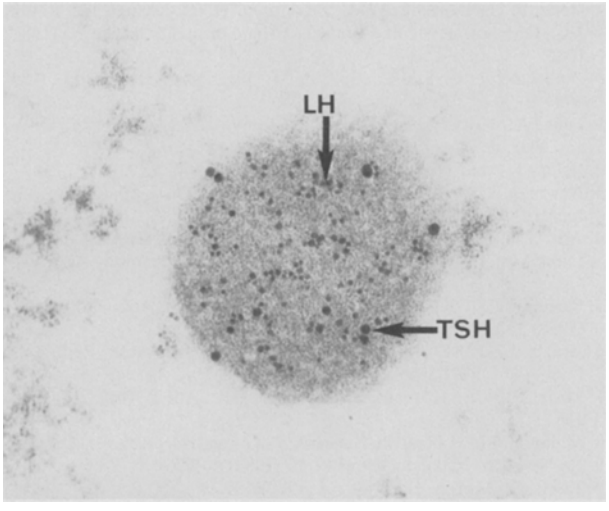

Figure 3. 200,000 × 1% Glutaraldehyde. Plurihormonal granule. Double-labelled for luteinizing hormone (5-nm gold probe) and thyroid stimulating hormone (10-nm gold probe). Not silver-enhanced.

with FSH, and FSH with TSH in the same granule. These granules were larger, on the average, than the monolabelled granules, i.e. LH/FSH: 260 +/− 20 nm, FSH/TSH: 90 +/− 12 nm, LH/TSH: 270 +/− 15 nm. They represented about 35% of the total labelled granules in these areas. The monohormonal granules tended to be PRL, LH or FSH, and did not favour a cluster formation or a position adjacent to the nuclei (fig. 1).

Discussion

Pituitary cells can only be unequivocally identified by their hormones, which are contained in cytoplasmic granules. In this study it was noted that each hormone granule type had a similar size, position and cluster formation tendency, in all the specimens. Double-labelling on normal human pituitary tissue demonstrated LH with FSH, LH with TSH (fig. 3), and FSH with TSH, in the same granule. GH, PRL, TSH, and ACTH occupied separate granules exclusively. ACTH and GH, while appearing to occupy a similar type of cell and granule, were not found together in the same granule or group of granules. Some gonadotrophic granules double-labelling for FSH and LH, and others were monohormonal (fig. 2). An overview of a typical area in which the multihormonal granules were located is shown in figure 1 (arrowhead). These granules were larger in size and exhibited less electron density (fig. 3), than the monohormonal granules (fig. 2). This was possibly an effect of maturation.

Apart from the work of Newman et al.[23], Asa et al.[24], Heitz et al.[19], and Zurschmiede and Landolt[20] on normal human pituitary, the few papers that have been published using human tissue have dealt with growth hormone adenomas and/or prolactinomas[5–7,21,25,26]. GH and PRL have been found together in the same cell in animal and adenoma tissue[5–7,16,27]. A few papers discuss hormone combinations other than GH and PRL such as TSH and GH in the same granule in the frog *Rana ridibunda*[28], and FSH and ACTH in the same granules in the rat[11]. In a light microscopic (immunoperoxidase) study on human adenomas, Tasca[29] reported various cellular hormone combinations, including LH with FSH, FSH with TSH, and FSH with LH and TSH in the same cells. No mention of plurihormonality within the granules was made. In studies using rat pituitary, monohormonal cells each secreting LH, FSH and ACTH were found to coexist with multihormonal cells that produced combinations of LH, FSH and ACTH[11,30–32]. In a study on a case of normal human pituitary adjacent to prolactinoma tissue, Newman et al.[23] found FSH, LH and PRL together in the same granule. This finding was similar to that of Childs et al.[30–32] in the rat. In a recent paper Childs et al.[33] reported ACTH and TSH in the same rat pituitary cells, while Asa et al.[24] found GH and PRL in the same granule in human foetal mammosomatotrophs. Newman et al.[34] had previously found multihormonal granules with ACTH and PRL coexisting in the same human endocrine granule. The granules as described in Newman's[23] study were larger than the granules in the current study, but the size ratios between each hormonal granule type were similar. Both studies used LR White embedding medium which has been observed to result in a 14% shrinkage in the embedded tissue[35]. Despite this it is interesting to note that the granule sizes in this study were still larger than those reported in animal and adenoma material embedded in conventional media.

A possible explanation for the presence of multihormonal granules is offered by Newman et al.[23] and Ishibashi and Shino[17]. They suggest that a fusion between two monohormonal cells might result in a multihormonal cell. They also suggest that different hormones are produced in the same Golgi cisternae and that a failure in the mechanism that separates them would result in a multihormonal cell. Childs[36] proposed that pluripotential stem cells exist in the pituitary, and that these can respond to varying demands by changing their secretory functions. It would appear that, at least to some extent, plurihormonality may be a common feature of the normal human pituitary.

Silver enhancement allowed the use of 5-nm gold probes to maximise labelling, and facilitated visualisation of the probe while scanning the section at low magnification (fig. 2). It could not, however, be used in the double-labelling studies where the probe size differential was of paramount importance (fig. 3).

Ideally, autopsy material should not be used if possible. Fresh normal human pituitary is, however, difficult to obtain. Absorbtion of each antibody against its corresponding human anterior pituitary hormone would be preferable to radioimmunoassay, but this is difficult to achieve. However, the low non-specific background labelling, lack of plurihormonal labelling in the monohormonal granules and lack of labelling in granules adjacent

to reactive granules (mono and pluri) were indicators that the antibodies were of adequate specificity.

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Effect of hyperkalemia on insulin secretion

R. Martinez, B. Rietberg, J. Skyler, J. R. Oster and G. O. Perez*

Medical and Research Services, Veterans Administration Medical Center and the Department of Medicine, University of Miami School of Medicine, Miami (Florida 33125, USA)
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Summary. The effect of hyperkalemia on insulin secretion remains undefined. We evaluated portal and peripheral insulin levels in anesthetized dogs after infusions of KCl. The mean maximal increase in peripheral plasma potassium at infusion rates of 0.2 mEq/kg/h was 0.68 ± 0.20 mEq/l. There were no significant increases in either portal or peripheral insulin levels. In contrast, in six dogs whose plasma potassium concentration increased in each case by more than 2.0 mEq/l (infusion rate of 0.5 mEq/kg/h), portal insulin levels increased fivefold ($p < 0.05$). We conclude that only marked increases in plasma potassium concentration stimulate pancreatic insulin secretion.
Key words. Insulin; potassium; hyperkalemia; portal vein; glucose.

It is well established that insulin promotes cellular potassium uptake and lowers blood potassium concentration [1–5] and that a basal blood level is necessary to allow normal potassium tolerance [6]. The effect of hyperkalemia on insulin secretion remains undefined, however. The results of several studies [4, 5, 7, 8] suggest that stimulation of insulin production is only observed in the presence of marked increases in serum potassium concentration (e.g., greater than 1–2 mEq/l). Unfortunately, in these studies, sampling from the portal circulation was not performed. Thus, it is possible that smaller increments in plasma potassium enhanced insulin secretion, which was masked because of the capacity of the liver to extract insulin. In the present study, we evaluated the pancreatic insulin response to graded degrees of hyperkalemia in anesthetized dogs with intact kidneys. The results validate the