Identification, cellular and subcellular distribution of 21 and 72 kDa proteins (tuberalins?) secreted by specific cells of the pars tuberalis

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Abstract

The cell types of the pars tuberalis (PT) are the follicular cells, the pars distalis cells and the so-called PT-specific cells. The latter are distinct endocrine cells displaying melatonin receptors. Although the nature of the secretory product(s) of the PT-specific cells has not yet been clarified, the function of these cells has started to be unfolded. For practical reasons, previous authors have designated the, as yet, unidentified PT hormone(s) as tuberalin(s). PT-specific cells synthesise the common α subunit of the pars distalis glycoprotein hormones, and it has been suggested that tuberalin would correspond to the β chain of a specific glycoprotein secreted by these cells.

The aims of the present investigation were to identify the compounds secreted by the specific cells of bovine PT, and to establish their cellular and subcellular distribution. For this purpose, proteins secreted into the culture medium of PT explants were separated by electrophoresis and used to raise antibodies. Two of these proteins, with an apparent molecular mass of 21 and 72, generated antibodies (Ab-21 and Ab-72) that differentially immunoreacted with PT-specific cells. These two antibodies were used for immunoblotting of conditioned medium and of PT explants, and for light and electron microscopy

immunocytochemistry. In immunoblots, Ab-21 reacted with compounds of 21, 22, 47 and 52 kDa, whereas Ab-72 revealed a compound of 72 kDa only. Ab-72 immunoreactive material corresponded to a protein, here designated as tuberalin I, secreted by a small population of PT-specific cells (type 2 cells), and stored in 140 nm secretory granules. Immunoreactive tuberalin I was missing from bovine pars distalis and from rat PT. The predominant population of PT-specific cells (type 3 cells) secreted and stored, within 280 nm secretory granules, an Ab-21 immunoreactive protein, here designated as tuberalin II. All cells of rat PT immunoreacted with Ab-21. In the cells of bovine and rat PT, immunoreactive tuberalin II was mostly confined to a paranuclear spot; this spot also bound wheat germ agglutinin and reacted with an antibody against the α chain of glycoprotein pars distalis hormones.

It is suggested that tuberalin II would correspond to the β chain of a specific glycoprotein secreted by type 3 PT-specific cells. In bovine PT, the cells displaying immunoreactive tuberalins I and II did not react with any of the antibodies against pars distalis hormones.

Journal of Endocrinology (2001) **168**, 363–379

Introduction

The pars tuberalis (PT) is a distinct region of the adeno-hypophysis. Although it undergoes certain phylogenetic variations of its cellular organisation, the PT is present in most tetrapods and missing from fish (Wingstrand 1966, Fitzgerald 1979). In mammals, it consists of strands of a bilayered to multilayered glandular epithelium surrounding the hypophyseal stalk and extending along the ventral surface of the median eminence (Gross 1984). The PT consists of three clearly distinguishable cell populations; namely, PT-specific cells, follicular cells and secretory cells identical to those of the pars distalis (PD) cells (Wittkowski *et al.* 1999). The PT-specific cells are distinctive endocrine cells that differentiate very early in ontogenesis and that remain active throughout the life-span (Cameron & Foster 1972, Stoeckel *et al.* 1973, 1979, Dellmann *et al.* 1974,

Fitzgerald 1979). After Morgan and his coworkers first described the presence of melatonin receptors on the PT cells (Williams & Morgan 1988, Morgan *et al.* 1989, 1992) the challenge has been to unfold the functional role of the PT, and to identify and establish the nature of the product(s) secreted by the PT-specific cells. Meeting the former challenge has been started. Primary cultures of sheep PT release into the medium a series of proteins; the secretion of some of these proteins was stimulated by forskolin and inhibited by melatonin (Morgan *et al.* 1992, 1994). Conditioned medium from ovine (Morgan *et al.* 1996) and bovine (Lafarque *et al.* 1998) PT cells increases prolactin (PRL) secretion by cultured PD cells. There is also evidence that a PT factor regulates the secretory activity of a second non-lactotropic cell type of the PD (Morgan *et al.* 1996). Although the PT-specific secretory product(s) has not been identified, the evidence

supporting its existence is strong enough to have led Stoeckel *et al.* (1994) and Morgan *et al.* (1996) to designate this factor as 'tuberalin'. We shall follow this nomenclature. Some findings point to the existence of more than one tuberalin (Morgan *et al.* 1996).

Several attempts have been made to identify and characterise the nature of tuberalin(s). In most species studied, PT-specific cells do not react with conventional hypophyseal stain or with any antiserum directed towards hormones of the PD (Baker & Yu 1975, Baker 1977, Osamura & Watanabe 1978, Gross 1984). Attempts to alter the cell morphology by gonadectomy, thyroidectomy or hypophysectomy have also failed in this cell type (Young *et al.* 1966, Gross 1978, 1983, Böckers *et al.* 1990). The α subunit, but not the β subunits, of PD glycoprotein hormones have been immunocytochemically demonstrated in the PT-specific cells of some mammals (Stoeckel *et al.* 1993, 1994, Böckers *et al.* 1994, Wittkowski *et al.* 1992, 1999). The biological activity of the common α chain of PD secretory glycoproteins does not mimic that of tuberalin(s) (P J Morgan, unpublished observation cited in Morgan *et al.* 1996). Thus, it has been suggested that tuberalin corresponds to the β chain of a specific glycoprotein secreted by PT-specific cells (Stoeckel *et al.* 1993, 1994, Wittkowski *et al.* 1999).

Studies carried out in cell cultures of ovine PT strongly indicate that tuberalin(s) is secreted constitutively, with regulation occurring at the level of synthesis (Morgan *et al.* 1994). This results in the accumulation of tuberalin in the culture medium from unstimulated PT cells (Morgan *et al.* 1996). This also implies a very low concentration of tuberalin(s) in the PT cells proper. Thus, it appears that the best experimental strategy to isolate tuberalin(s) is to use conditioned medium of PT as a source, rather than using extracts of PT, a traditional approach to purifying new hormones.

The aim of the present investigation was to identify the compounds secreted by the PT-specific cells, and to establish their cellular and subcellular distribution. The conditioned medium of bovine PT explants was used as the source of PT secretory products.

Materials and Methods

Collection and organ culture of PT

The neural stalk, containing the median eminence and PT, and the PD of cattle of both sexes were obtained at the local (Valdivia) slaughterhouse from 0900–1200 h, throughout the year. After dissection from the skull, the neural stalk was opened by inserting one arm of fine scissors through the infundibular recess, and this was immersed in cold Hanks' solution (Sigma, St Louis, MO, USA), and transported to the tissue culture laboratory. The time that elapsed between death and immersion in Hanks' solution was about 10–20 min. Under a dissecting micro-

scope, the PT was divided into two halves. The rostral half, close to the hypothalamus, was separated from the median eminence by careful dissection under the microscope. PT explants of about 0.5 mm each side were prepared; each PT rendered about 15 explants. The explants were cultured for 8 days in nutrient mixture F-12 (Ham's; Sigma) mixed with Dulbecco's modified Eagle's medium (Sigma) at a ratio of 1:1, containing an antibiotic/antimycotic cocktail. Groups of 45 explants each were cultured in 10 cm Petri dishes containing 15 ml culture medium. The explants were incubated at 37 °C in an atmosphere of 95% air and 5% CO₂, for 8 days. The conditioned medium was collected at days 2, 5 and 8 of culture, transferred to a polypropylene centrifuge tube and spun at 10 000 *g* for 1 min. The supernatant was collected and lyophilised in a speed-vac (Virtis, New York, USA) device until a protein concentrate of about 1 mg/ml was obtained; this roughly implied concentrating the 10–12 ml supernatant obtained from each Petri dish to about 1.5 ml. The samples were dialysed against distilled water for 48 h and further concentrated. An aliquot of the concentrate was used for protein determination according to Bradford (1976). Aliquots were lyophilised and stored at –20 °C.

A few explants were collected after 2, 5 and 8 days of culture, and processed for light and electron microscopy (see below).

Tissue extracts

Fresh bovine PT and PD, and PT explants obtained after 8 days of culture were extracted in a medium containing 50 mM ammonium bicarbonate, 0.5 mM phenylmethylsulfonyl fluoride and 1 mM EDTA. Each tissue sample was homogenised in 1 ml extraction medium using a Potter–Eveljheim homogeniser, sonicated for 15 s and cooled on ice for 30 s. This sonication–cooling cycle was repeated three times. The extract was centrifuged at 10 000 *g* for 30 min at 4 °C; the supernatant was spun at 12 000 *g* for 45 min at 4 °C. This second supernatant was regarded as a crude extract of PT or PD. The protein content was determined by Bradford's method. Aliquots were lyophilised and stored at –20 °C.

Electrophoresis and western blot analysis

Samples of 60 μ g proteins of conditioned medium and 90 μ g proteins of extracts of PT explants were subjected to SDS–polyacrylamide gel electrophoresis (Laemmli 1970), using a 5–15% polyacrylamide linear gradient. Gels were stained with Coomassie blue, or were electrotransferred to nitrocellulose sheets (Towbin *et al.* 1979) using 25 mM Tris–HCl, pH 8.3, containing 192 mM glycine, 0.2% SDS and 20% methanol, at 0.1 A; transfer time was 8 h. Saturation of protein-binding sites with 5% non-fat milk in 0.1 M phosphate-buffered saline (PBS) containing 0.15 mM NaCl, was followed by immunoperoxidase

(PAP) staining, using 4-chloro-1-naphthol (Sigma) as electron donor. The following primary antibodies were used: (1) antibody (Ab)-2 at a 1:250 dilution and (2) Ab-72, at a dilution of 1:250. The primary antibodies were diluted in PBS containing 0.7% lambda carrageenan (Sigma) and 0.01% thimerosal. The blots were sequentially incubated in: (1) the primary antibody, for 18 h; (2) anti-rat IgG, 1:50 dilution, for 2 h; (3) rat-PAP, 1:75 dilution, for 1 h. Peroxidase reaction product was visualised by incubating the blots in a solution containing 0.01% 4-chloro-1-naphthol (Sigma) in methanol, and 0.01% H₂O₂ (Merck, Darmstadt, Germany). All incubations were at room temperature, in a moist chamber and under continuous agitation. Control transfers were incubated with pre-immune serum or processed for the PAP method but omitting incubation in the primary antiserum. All control reactions were negative.

Raising of antibodies

The conditioned media collected at 2, 5 and 8 days of culture from 50 Petri dishes containing 45 PT explants in 15 ml medium per dish (total of about 2250 PT explants corresponding to 150 bovine PTs) were pooled, vacuum concentrated (total protein content 5.5 mg) and used to run seven preparative gel electrophoreses under the same conditions as those described above. The gels were stained with Coomassie blue. Eight bands with an apparent molecular weight of 72, 50, 44, 35, 29, 25, 21 and 17 kDa were selected on the following grounds: (1) presence of these bands in the conditioned media collected at 2, 5 and 8 days of culture; (2) the 21, 29, 44, and especially the 72 kDa bands, were also selected taking into account the findings by Morgan *et al.* (1994) concerning biosynthetically labelled PT proteins released to the culture medium by cultured PT cells. Each band was cut out of the gel according to the procedure of Cozzani & Hartmann (1980). The bands with the same apparent molecular weight obtained from the seven preparative gels were pooled and kept at -20 °C. Each of the eight pools of bands was immersed in 2 ml PBS and ground using a Potter-Eveljheim homogeniser. A total of about 3 ml immunogen was obtained from each pool of bands, and used for the immunisation of two rats according to a protocol previously described (Nualart *et al.* 1991). Three days before the third and fourth immunisation, blood samples of about 4 ml were obtained, under ether anaesthesia, by orbital puncture (Van Herck *et al.* 1997). Seven days after the fourth immunisation, the rats were bled by heart puncture, under ether anaesthesia. Serum obtained from each blood sample was aliquoted and kept frozen. Each of the antisera obtained was used for the immunostaining of sections of bovine PT and PD. The antisera were labelled as Ab-72, Ab-50, Ab-44, Ab-35, Ab-29, Ab-21 and Ab-17. Those that produced a positive immunoreaction (Ab-72 and Ab-21) were used for

immunoblotting of PT-conditioned media and extracts of PT explants collected after 8 days of culture.

Tissue samples for immunocytochemistry

Pituitaries from adult bovines and PT explants 8 days old were fixed by immersion in Bouin's fluid for 2 days, dehydrated and embedded in paraffin. Sagittal and frontal serial sections, 4 µm thick, were mounted on separate slides. Some PT explants and bovine PT were fixed by immersion in Bouin's fluid and fragmented into blocks of about 1 mm thick; these were dehydrated in methanol and acetone and embedded in butyl-methyl-methacrylate (Rodríguez *et al.* 1984). Serial 1 µm methacrylate sections were individually mounted on separate slides. This allowed for several sections through the same cells, which were mounted separately.

The central nervous system of adult Sprague-Dawley rats, of both sexes, was fixed by vascular perfusion with Bouin's fluid. Fixation was under ether anaesthesia and was performed around noon. Embedding was in paraffin. Sagittal and frontal adjacent sections through the hypothalamo-hypophyseal region were mounted on separate slides. The medial basal hypothalamus of hamsters of both sexes was fixed by immersion in Bouin's fluid and embedded in paraffin.

Immunostaining and lectin binding

Bovine hypophysis Sections were processed according to the immunoperoxidase method of Sternberger *et al.* (1970). The sections were sequentially incubated in (1) the primary antiserum (raised in rats or rabbits), for 18 h; (2) the secondary antibody (anti-rat IgG or anti-rabbit IgG) diluted 1:15, for 30 min; (3) rat PAP (Dr H Folch, Instituto de Inmunología, Universidad Austral de Chile, Valdivia, Chile), or rabbit PAP (Sigma), 1:75 dilution, for 30 min. Diaminobenzidine (DAB) was used as electron donor. All antisera and the PAP complex were diluted in Tris buffer, pH 7.8, containing 0.7% non-gelling seaweed lambda carrageenan (Sigma) and 0.5% Triton X-100 (Sigma). Use of preimmune serum and omission of incubation in the primary antiserum in the immunostaining procedure were used as control tests.

A first series of adjacent paraffin-mounted sagittal sections of bovine pituitary containing PT and PD was immunostained using Ab-72, Ab-50, Ab-44, Ab-35, Ab-29, Ab-21 and Ab-17 as primary antibodies. They were all tested at dilutions of 1:250 and 1:500. Only Ab-72 and Ab-21 produced a positive reaction in cells of the PT.

In a second series of sagittal sections of the bovine pituitary, in a series of transversal sections of the stalk (PT plus median eminence), and in a series of sections of PT explants 8 days old, 11 adjacent sections were immunostained using the following primary antibodies (all of them were diluted at 1:1000): (1) Ab-21; (2)

Ab-72; (3) anti-human (h) adrenocorticotrophin (ACTH) (antibody created by A F Parlow (AFP), NIDDK, Torrance, CA, USA; for immunocytochemistry (IC), IC-3, AFP 6141); (4) anti-hPRL (NIDDK, AFP-9042B); (5) anti-h growth hormone (GH) (NIDDK, AFP-C11981A); (6) anti-h β luteinizing hormone (LH) (NIAMDD IC-1, AFP-54372); (7) anti-h β follicle-stimulating hormone (FSH) (NIAMDD IC-1, AFP-3710194); (8) anti-h β thyroid-stimulating hormone (TSH) (NIAMDD); (9) anti- β -endorphin (kindly provided by Dr D Smyth, MRC, London, UK); (10) anti-rat α subunit of glycoprotein PD hormones (NHPP-NIDDK, AFP-66P9986).

The eleventh section was used for wheat germ agglutinin (WGA) binding (see below). Ab-21 and Ab-72 were raised in rats; all others were raised in rabbits. Several immunostaining sessions were performed, the only difference among them was the order in which the antibodies and the lectin were used in the series of 11 sections. This allowed comparison, on adjacent sections, of the distribution of PD cell types and PT-specific cells, as well as the Ab-21-binding sites and the lectin-binding sites. Peroxidase-labelled WGA (affinity: glucosamine, sialic acid; Sigma), dissolved in Tris buffer, pH 7.8, at a concentration of 2.5 μ g/ml, was used. Sections were incubated in the lectin for 1 h, at 22 °C. DAB reaction was used to visualise the binding sites.

In a different experiment, all antibodies against PD hormones (3 to 9 in the list above) were pooled so that all of them reached a dilution of 1:1000. This pool was used to immunostain paraffin sections of PT to detect, in a single section, all PD cells present in the PT and the PT-specific cells. Sections adjacent to those immunostained with the pool of antibodies were immunostained with Ab-72 and Ab-21.

The cellular distribution within the bovine PT of the different compounds reacting with antibodies 1–9 listed above, and the probable coexistence within PT cells of two or more of these compounds, was investigated by staining adjacent 1 μ m thick methacrylate sections with each one of the nine antibodies. Since all cells in the PT have a diameter larger than 20 μ m, sections through the same cells were included in the series of nine adjacent sections and, consequently, the immunoreactivity of each individual cell to each of the nine antibodies could be tested.

Since Ab-21, at a dilution of 1:1000, produced a weak immunoreaction in cells of the bovine PD, the following protocols were applied. (1) Ab-21 was diluted 1:250, 1:500, 1:1000, 1:2000, 1:4000 and 1:8000, and used for the immunostaining of sagittal paraffin sections of the bovine pituitary (PT plus PD). (2) Ab-21 was incubated for 18 h, at room temperature, with an ammonium bicarbonate extract of bovine pituitary gland, at a ratio of 100 μ g/ml antibody diluted 1:1000. The absorbed antiserum was spun at 12 000 g for 10 min. The supernatant was used to immunostain sagittal paraffin sections of the bovine pituitary.

Rat hypophysis Adjacent paraffin sections of the rat hypothalamo–hypophyseal region, containing PT and PD, were immunostained with Ab-72, Ab-21, anti- β TSH, and for lectin binding. Three lectins were used: (1) WGA (see above); (2) *Limax flavus* agglutinin (LFA; affinity exclusively for sialic acid). Unlabelled LFA (Calbiochem, San Diego, CA, USA) at a concentration of 7 μ g/ml in 0.1 M Tris buffer, pH 7.3, was used. The lectin-binding site was revealed by the immunoperoxidase method using an anti-LFA serum (raised in our laboratory); (3) peanut (*Arachis hypogaea*) agglutinin (PNA; affinity terminal residues of galactose). Galactose is the subterminal sugar residue in complex-type glycoproteins (Lis & Sharon 1986). Removal or absence of sialic acid residues leaves galactose as the terminal residue, which in turn results in the binding of PNA (Herrera & Rodríguez 1990). Sections were incubated in peroxidase-labelled PNA (Sigma), 4 μ g/ml in 0.1 M Tris buffer, pH 7.3.

To investigate co-localisation of Ab-21 immunoreactive material with the α chain of glycoprotein PD hormones, several sections were processed according to the following protocol: (1) immunostaining using anti-rat α subunit of glycoprotein PD hormones raised in rabbits, diluted 1:500, and using 3-amino-9-ethylcarbazole as chromogen (red-dish colour); (2) microphotographs of the PT were obtained; (3) antibodies were eluted from the section using the oxidising mixture of Gomori (González & Rodríguez 1980); (4) immunostaining using anti-rabbit IgG as primary antibody; a negative reaction was taken as evidence that the antibodies had been eluted from the section; (5) immunostaining using Ab-21 as primary antibody and DAB as electron donor; (6) microphotographs of the same microscopic fields recorded in (2) were obtained.

Hamster hypophysis Adjacent paraffin sections of the hamster hypothalamo–hypophyseal region, containing PT and PD, were immunostained with Ab-72 and Ab-21.

Conventional transmission electron microscopy

Bovine neural stalks were fixed by immersion in a mixture containing 2% paraformaldehyde, 0.5% glutaraldehyde and 15% of a saturated picric acid solution, buffered to pH 7.2 with 0.1 M monosodic–disodic buffer. After washing in the same buffer for 30 min, the tissue blocks were fixed in 0.5% OsO₄ in 0.1 M phosphate buffer, pH 7.4, for 2 h. Subsequently, the tissues were washed with distilled water for 15 min, dehydrated and embedded in butyl-methyl-methacrylate (Rodríguez *et al.* 1984). Ultrathin sections were stained with uranyl acetate and lead citrate. Semithin sections stained with toluidine blue–borax were used for orientation.

Electron microscopy immunocytochemistry

Ultrathin methacrylate sections, adjacent to those used for conventional transmission electron microscopy, were

Figure 1 SDS-PAGE of conditioned medium of explants of bovine PT and of extracts of PT explants. (A) Preparative gel electrophoresis of conditioned medium stained with Coomassie blue (C.B.). (Lane b) The bands selected to raise antibodies are labelled by their molecular weight (from p14 to p72). (Lane a) Molecular weight markers. (B) Analytical gel electrophoresis of conditioned medium and of extracts of PT explants stained with Coomassie blue (runs 1 and 2), and electrotransferred and immunostained with the antisera raised against the 21 kDa band (Ab-21, runs 3 and 4), and the 72 kDa band (Ab-72, runs 5 and 6) of conditioned medium. Run 7 (c) was immunostained using preimmune serum. Arrows point to the 72 kDa band, arrowheads indicate the bands immunoreactive with Ab-21.

mounted on nickel grids, treated with 1% H_2O_2 for 10 min, and washed in distilled water. This was followed by a few rinses in Tris buffer, pH 7.8. For the immunoperoxidase procedure, sections were run through the following steps: (1) incubation in the primary antibody (Ab-72, diluted 1:1000; Ab-21, diluted 1:1000; anti-h β LH, diluted 1:1000), at room temperature, for 24 h; (2) washes in Tris buffer, pH 7.8; (3) incubation in the second antibody (dilution 1:50), for 5 min; (4) washes in Tris buffer, pH 7.8; (5) incubation in PAP (dilution 1:75), for 5 min; (6) washes in Tris buffer, pH 7.8; (7) incubation in the DAB solution prepared as indicated above, 1 min; (8) washes in distilled water, 15 min. Then, the sections were run through the silver methenamine procedure that allowed visualisation of the antibody-binding site by the presence of fine and densely packed silver grains (Rodríguez *et al.* 1984). Finally, the sections were contrasted with lead citrate. All antibodies were diluted in Tris buffer, pH 7.8, containing 0.1% normal ovine serum. Controls included: (1) immunostaining omitting incubation in the primary antibody; (2) immunostaining, but incubation in non-immune serum instead of the primary antiserum; (3) silver methenamine staining without previous immunoperoxidase staining. Ultrathin sections were

examined under a Hitachi H-700 electron microscopy using 150–200 kV accelerating voltage.

Rats and hamsters used in this investigation were handled according to the guidelines of the National Research Council of Chile.

Results

Protein composition of PT-conditioned medium

Coomassie blue staining of SDS-PAGE gels of conditioned medium of PT explants cultured for 5 or 8 days revealed a large number of bands, with their apparent molecular size ranging between 72 and 10 kDa (Fig. 1A).

The immunoblot of PT-conditioned medium and of extracts of PT explants 8 days old, using Ab-21 as primary antibody, revealed the former four bands of 52, 47, 22 and 21 kDa (Fig. 1B); only the 52 and 22 kDa bands were detected in the PT explants (Fig. 1B). When blots of PT-conditioned medium and of extracts of PT explants were immunostained with Ab-72, only a band of 72 kDa was visualised (Fig. 1B).

Cellular organisation and cell populations of PT explants

At the end of the culture period (8 days) the explants showed a structure resembling that of the PT *in situ*, namely, clusters of cells separated by connective tissue septa; the former blood vessels appeared as cavities of variable shape and size. The cells of most clusters presented a nucleus with abundant euchromatin. In a few clusters, mostly localised in the core of the explants, cell nuclei were small with densely packed chromatin (Fig. 2A). Adjacent serial sections of 8-day-old explants immunostained with (1) Ab-21; (2) Ab-72; (3) anti-h β LH; (4) anti-h β FSH; (5) anti-hPRL; (6) anti-h β TSH; (7) anti-hACTH; (8) anti- β -endorphin; (9) anti-hGH revealed separate populations of cells that immunoreacted with antibodies 1–6. Immunoreactive gonadotrophs (Fig. 2B) and thyrotrophs were more abundant in the core of the explant. Ab-21 revealed numerous cells, most of which were grouped into clusters localised at the periphery of the explant (Fig. 2C). Ab-21 immunoreactivity was distributed evenly throughout the cytoplasm. Ab-72 immunoreacted with isolated cells randomly distributed throughout the explant (Fig. 2D). The study of serial 1 μ m methacrylate sections immunostained with the nine antibodies listed above demonstrated that those cells reacting with Ab-21 or with Ab-72 did not correspond to any of the cells that immunoreacted with anti-LH, anti-FSH, anti-PRL or anti-TSH.

Cellular organisation and cell populations of the bovine PT

All cells of the bovine PT were organised into follicles; in tissue sections the latter appeared spherical or elongated. A thick layer of connective tissue surrounded each follicle (Fig. 3). Blood vessels, especially capillaries, ran through the connective tissue septa. Follicles were constituted of follicular and secretory cells.

Follicular cells These extended from the basal lamina to the core of the follicle, where they joined together delimiting a central cavity (Fig. 3). Near the follicle lumen these cells interdigitated extensively (Fig. 3). The most distinct feature of these cells was the large number of smooth surfaced canaliculi and mitochondria (Fig. 3). The follicle lumen was filled with a material of high electron density (Fig. 3).

Secretory cells Cells containing immunoreactive LH, FSH, PRL, TSH, GH, ACTH and β -endorphin were detected in the bovine PD as well as in the PT. In the PT they were abundant in the caudal half of the gland. Immunostaining of adjacent 1 μ m methacrylate sections of PT showed co-localisation of ACTH and β -endorphin; all other PD hormones were detected in separate cell types. This study also revealed that there was a large population

of PT cells that did not react with any of the antibodies against PD hormones. The existence of a PT-specific cell population also became apparent in paraffin sections of bovine PT immunostained with a pool of antibodies against all PD hormones. In this preparation, the immunoreactive cells that at the caudal end of the PT outnumbered the non-reactive cells, became progressively scarce towards the rostral end of the gland. Here, the PT-specific cells were the predominant cell population.

At the ultrastructural level, the secretory cells were identified by the presence of secretory granules and a well-developed machinery for protein synthesis. All secretory cells were in contact with the basal lamina of the follicle, but none of them reached the follicle lumen (Fig. 3). In the rostral half of the PT, where the PT-specific cells predominated, seven cell types could be distinguished, mostly based on the size, density and cytoplasmic distribution of their secretory granules. Only types 1, 2, 3 and 4, whose granules immunoreacted with Ab-21 or Ab-72, will be described.

Cell types of the bovine PT immunoreacting with Ab-72 and Ab-21

Type 1 cells These cells were round with a diameter of 8–10 μ m and with their cytoplasm evenly immunostained with Ab-72 and Ab-21 (Fig. 4B and E). These cells were more abundant in the rostral half than in the caudal half of the gland. One or two of these cells per follicle were seen in paraffin sections 4 μ m thick; they were all in contact with the basal lamina of the follicle (Fig. 4B and E).

The cytoplasm was restricted to a thin perinuclear ring, containing scarce rough endoplasmic reticulum (RER) cisternae, a few mitochondria and relatively few secretory granules, with a maximal cross sectional diameter of about 90 nm (Fig. 3). The granules were distributed throughout the cell, and many of them concentrated at the vascular cell pole (Fig. 5A). All granules reacted with Ab-21 (Fig. 6), and with Ab-72 (Fig. 5A and Table 1). Adjacent ultrathin sections immunostained with both antibodies demonstrated that both immunoreactivities coexisted in the same granules.

Type 2 cells These had an ovoid or spherical shape, with a major diameter of about 7–10 μ m. They all reacted with Ab-72. The immunoreactive cells appeared to be evenly distributed throughout the gland. Approximately one type 1 cell per follicle was seen in paraffin sections 4 μ m thick (Fig. 2E). In the immunostained cells, immunoreactivity was seen throughout the cytoplasm (Fig. 2F). Type 2 cells did not react with Ab-21 (Table 1).

The ultrastructural study revealed that type 2 cells had a cytoplasm restricted to a thin perinuclear ring, containing scarce cisternae of the RER and a few mitochondria

Figure 2 (A) Paraffin section of an explant of bovine PT cultured for 8 days (haematoxylin–eosin stain). (B) Section adjacent to that of (A), immunostained for β LH. (C) Area similar to that shown framed in (A); immunoperoxidase staining using Ab-21. (D) Section adjacent to that of (A), immunostained using Ab-72; arrows point to immunoreactive cells. (E) Paraffin section of bovine PT (*in situ*) immunostained with Ab-72; arrows show immunoreactive cells (type 1 and 2), arrowheads point to PT median eminence border. (F) Detailed magnification of (E). Scale bars: (A, B and E) 30 μ m; (C, D and F) 7.5 μ m.

Figure 3 Transmission electron microscopy of bovine PT, showing the cellular arrangement of two follicles. FC, follicular cells; F, follicular lumen; 1, 2 and 3 secretory cells, types 1, 2 and 3; asterisk, large amount of intracellular canaliculi in follicular cells; long arrows, images suggesting that secretory granules of type 3 cells become part of cytophagic bodies; shorter thicker arrow, process of a type 3 cell filled with secretory granules. Scale bar: 416 nm.

(Fig. 3). Secretory granules, 140 nm in diameter, were numerous, distributed throughout the cytoplasm (Figs 3 and 5B), and their content selectively reacted with Ab-72 (Fig. 5B).

Type 3 cells These were elongated or pear shaped with a major diameter of about 15 μm (Fig. 4E), and were immunostained by Ab-21, but not by Ab-72 (Table 1). The Ab-21 immunoreactive material was mainly confined to a paranuclear mass, which at low magnification gave this cell population a characteristic spotlike appearance (Fig. 4A and B). At high magnification, the immunoreactive paranuclear spot appeared as densely packed granules (Fig. 4E). A weak reaction could also be seen in other areas of the cytoplasm. The paranuclear mass was strongly and selectively labelled by the antibody against the α chain of the glycoprotein PD hormones. Type 3 cells were numerous throughout the gland, but they were especially abundant in the rostral third of the PT, where they represented the major secretory cell population (Fig. 4B and E). Numerous type 3 cells were present in each follicle (Fig. 4A and B).

Type 3 cells were characterised by abundant cisternae of RER distributed throughout the cytoplasm and by secretory granules with a maximal cross-sectional diameter of 280 nm. These granules were relatively scarce and randomly distributed throughout the cytoplasm; their density increased in discrete cell processes extending over the basal lamina (Fig. 3). In several cell profiles, the granules appeared clustered in a region close to the nucleus. Another distinct characteristic of this cell type was the presence of irregularly shaped, membrane-bound structures filled with an electron-dense material, resembling crynophagic bodies; images suggesting that secretory granules became part of these bodies were clear (Fig. 3).

The secretory granules of type 3 cells were strongly labelled with Ab-21 (Fig. 6). The paranuclear clusters of secretory granules were also reactive with Ab-21 (Fig. 4F). In some cells the crynophagic-like bodies were labelled with Ab-21, whereas in others they were not (Fig. 6). Ab-72 did not react with the secretory granules of this cell type (Table 1).

Type 4 cells These were round or elongated, with a major diameter ranging between 15 and 20 μm . These cells immunoreacted with Ab-21 but not with Ab-72. The Ab-21 immunoreactivity was seen throughout the cytoplasm (Fig. 4A, B and E). Type 4 cells were abundant in the caudal region of the PT, and were scarce or missing in the rostral third of the gland (Fig. 4A and B).

The outstanding ultrastructural feature of type 4 cells was the large number of granules, 350 nm in diameter, which occupied most of the cytoplasm. Ultrathin sections through the same cell revealed that all granules reacted with Ab-21 and anti-LH, and that none was labelled with Ab-72 (Fig. 5B).

Lectin binding in the bovine PT

When sections of bovine PT adjacent to those immunostained with Ab-21 were processed for WGA binding, cells of types 1, 3 and 4 displayed the same pattern of labelling with both probes (compare Fig. 4E and G). The paranuclear mass of Ab-21 immunoreactive material of type 3 cells was strongly and selectively labelled by WGA (Fig. 4G).

Immunoreactivity of the bovine PD with Ab-72 and Ab-21

At dilutions of 1:250, 1:500 and 1:1000 of Ab-21, numerous immunoreactive cells resembling the type 4 cells present in the PT (see above) were seen throughout the bovine PD. At dilutions of 1:2000 and 1:4000 of Ab-21, the immunoreactivity of the PD cells progressively decreased, so that at the 1:8000 dilution the PD immunoreactive cells were hard to recognise (Fig. 4D). However, this latter dilution did show types 1, 3 and 4 cells in the PT (Fig. 4C).

Immunoabsorption of Ab-21 with an extract of bovine PD drastically reduced the immunostaining of cells in PD, but the strong immunolabelling of the three cell populations of the PT was retained (data not shown).

Ab-72 did not react with cells of the bovine PD.

Ab-21, Ab-72 and anti- α chain immunoreactivities and lectin binding of the rat PT

The cells of the rat and hamster PT were strongly labelled with Ab-21. Most of the cells of the rat PT reacted with this antibody. Two cell populations were distinguished. The predominant cell population displayed a moderate immunoreaction throughout the cytoplasm and a strong immunoreaction of a well-defined paranuclear mass (Fig. 7A and B). A second, small population of cells displayed a strong reaction throughout the cytoplasm (Fig. 7B). The two populations of cells immunoreactive with Ab-21 also reacted with anti-TSH (Fig. 7B insert). The paranuclear mass of material that was labelled with Ab-21 was also reactive with anti-TSH (Fig. 7B insert).

The co-localisation study demonstrated that the same paranuclear mass reacted with Ab-21 and the antibody against the α chain of glycoprotein PD hormones (Fig. 7D–F). This paranuclear mass was also labelled with WGA (Fig. 7C). Adjacent sections processed for LFA and PNA binding showed that the cells of the rat PT lacked affinity for these lectins, whereas gonadotrophs and thyrotrophs in the PD were labelled by both lectins.

Ab-72 did not react with the rat PT. Neither Ab-21 nor Ab-72 reacted with cells of the rat PD.

Ab-21 and Ab-72 immunoreactivities in the hamster PT

The hamster PT displayed the same pattern of Ab-21 immunoreactivity as that described for the rat PT. It lacked Ab-72 immunoreactivity.

Figure 4 (A) Sagittal section of the bovine PT immunostained with Ab-21, at a dilution of 1:1000. Immunoreactive cells types 3 (thin arrows) and 4 (thick arrows) are shown. The rostral region of PT is towards the left (R), and the caudal region is towards the right (C). ME, median eminence. (B) High magnification of (A). The follicular arrangement of PT cells is obvious. Immunoreactive types 1 (arrowheads), 3 (thin arrows) and 4 (thick arrow) cells are indicated. The spot-like appearance of type 3 cells is clearly distinguished. V, blood vessel. (C) Bovine PT immunostained with Ab-21 at a dilution of 1:8000. Types 1 (arrowhead), 3 (thin arrow) and 4 (thick arrow) continue to be immunoreactive at this high dilution of the antibody. (D) Bovine PT immunostained with Ab-21 at a dilution of 1:8000. Some cells display a very weak immunoreactivity (arrows). (E) Detailed magnification of (B); immunoperoxidase staining with Ab-21 (1:1000 dilution). The structure of the follicles is distinguished, namely, non-reactive follicular cells, follicular lumen (f) and the location of the immunoreactive types 1 (arrowhead), 3 (thin arrows) and 4 (thick arrow) cells. (F) Ultrastructural immunocytochemistry using Ab-21 at a dilution of 1:1000. A type 3 cell is shown. Immunoreactive granules appear clustered in a paranuclear region (arrowhead). (G) Section adjacent to that of (E) processed for WGA binding. Binding sites are in types 1 (thick arrow) and 3 (thin arrows) cells, and in the content of the follicular lumen (f). Scale bars: (A) 30 μ m; (B) 15 μ m; (C and D) 7.5 μ m; (E and G) 3 μ m; (F) 600 nm.

Discussion

Ultrastructural and immunocytochemical studies have shown that the PT is formed by three distinct cell populations; namely, PT-specific cells, follicular cells and cells similar or identical to secretory cells of the PD (PD cells) (Wittkowski *et al.* 1999). The cytological characteristics and certain immunocytochemical properties of the PT-specific cells suggest an endocrine function for these cells (Stoeckel *et al.* 1973, 1993, 1994, Dellman *et al.* 1974, Fitzgerald 1979, Böckers *et al.* 1994, Bockmann *et al.* 1997b, Wittkowski *et al.* 1999). Subunits of glycoprotein PD hormones have been detected in PT-specific cells. With the exception of the rat and mouse PT-specific cells that express both the α and β subunits of TSH (Gross 1984, Wittkowski *et al.* 1988, 1999, Bockmann *et al.* 1997a), in the PT-specific cells of the other species the α subunit common to LH, FSH and TSH has been immunocytochemically detected. However, the specific β subunit of these hormones was missing from these cells (Böckers *et al.* 1994, Wittkowski *et al.* 1999). This has led to the suggestion that in the PT-specific cells the α subunit is associated with β subunit(s) which has not yet been identified (Stoeckel *et al.* 1994, cf. Wittkowski *et al.* 1999). Morgan *et al.* (1994) have reported synthesis and secretion by isolated PT cells of several labelled proteins, with an enhancement of this secretion by forskolin and an inhibition by melatonin. These authors have also obtained evidence that two or more factors secreted by the PT mediate PT–PD communication by affecting the secretion of PRL and of other PD hormones, not yet identified (Morgan *et al.* 1996).

The evidence indicates that the PT-specific cells may synthesise one or more novel glycoprotein hormone(s), that have tentatively been called 'tuberalin(s)' (Stoeckel *et al.* 1994, Morgan *et al.* 1996, Wittkowski *et al.* 1999). Isolation and identification of tuberalin(s) have not yet been reported. The present investigation has provided evidence allowing a tentative identification and cellular and subcellular distribution of at least two putative tuberalins, designated as tuberalin I and II.

Conditioned medium of explants of bovine PT as a source of tuberalins

It has been proposed that in the PT-specific, melatonin-responsive cells, secretion occurs in a constitutive manner, with regulation taking place at the level of synthesis (Morgan *et al.* 1994). Consequently, the amount of tuberalins stored in the PT is expected to be low, thus making the purification and identification of tuberalins using PT extracts a difficult task. The lack of specific probes for tuberalins makes their purification even more difficult. On the other hand, primary cultures of ovine PT have proven to be a useful tool to detect, in the conditioned medium, biosynthetically labelled proteins

secreted by the PT cells (Morgan *et al.* 1994). These authors, who used short-term (about 20 h) primary cultures of ovine PT cells, found that although there was a basal release of proteins with a broad range of molecular weights, only some of them were secreted at a higher rate in the presence of forskolin (Morgan *et al.* 1994). Conditioned media of short-term primary cultures of PT cells have also been used to investigate the PRL-releasing activity of tuberalins, and to partially characterise them (Morgan *et al.* 1996, Lafarque *et al.* 1998). Thus, conditioned medium of PT cultures appeared to be a potential good source of tuberalins. In a preliminary attempt, we compared the yield of proteins in conditioned media of primary cultures and of organ culture of bovine PT, and found the latter to be more efficient. Furthermore, the PT cells survived and retained their ultrastructural and immunocytochemical properties for several weeks. This allowed us to harvest and pool relatively high amounts of conditioned medium. This led us to use, in the present investigation, long-term organ culture without any kind of pharmacological treatment, as a source of proteins secreted by the PT. Furthermore, the use of bovine PT made it possible to run large-scale cultures of PT explants. Since only the rostral half of the PT was used for organ culture, a conditioned medium enriched with tuberalins was to be expected. This experimental protocol allowed us, finally, to run the conditioned medium through preparative gel electrophoresis, and to use selected bands as immunogen to raise antibodies.

Secretory granules of PT-specific cells are selectively labelled with antibodies raised against two proteins present in PT-conditioned medium (Ab-72 and Ab-21)

According to the present investigation, in cattle there are three types of PT-specific cells. None of them is labelled by antibodies against PD hormones, and they all react with Ab-72, or Ab-21, or with both. There is a fourth cell type (type 4) that could qualify as PT specific (Table 1). Taking into consideration all the findings (conventional electron microscopy, light and electron microscopy immunocytochemistry), the essential features of the four cell types are summarised in Table 1.

Evidence that the Ab-72 immunoreactive material corresponds to a protein, here designated as tuberalin I, secreted by a small population of PT-specific cells

The following findings may be taken as evidence that Ab-72 recognises a protein secreted by a population of bovine PT-specific cells: (1) The antibody was generated against a protein present in the conditioned medium collected after 5 and 8 days of culture of the rostral PT. (2) The antibody obtained (Ab-72) recognises, in immunoblot, only one band of 72 kDa in both the conditioned medium and the extracts of PT explants. (3) Ab-72

Figure 5 Ultrathin section of bovine PT immunostained with Ab-72. (A) Two immunoreactive type 1 cells (arrows) are shown. fc, follicular cell; asterisk, interfollicular connective tissue septum; bc, blood capillary. Scale bar: 400 nm. (B) An immunoreactive type 2 cell (2) and a non-reactive type 4 cell (4) are shown. In the former, immunoreactivity is confined to the secretory granules (thick arrows), in the latter no immunoreaction is seen in the secretory granules (thin arrows). Scale bar: 214 nm. (C) Detailed magnification of immunoreactive granules shown in (B). Scale bar: 88 nm.

Figure 6 Ultrathin section of bovine PT immunostained with Ab-21. Immunoreactivity is confined to granules of types 1 (arrowheads) and 3 (thin arrows) cells. Crynophagic-like structures of type 3 cells are not immunoreactive (thick arrows). fc, follicular cells. Scale bar: 285 nm. Insert, detailed magnification of immunoreactive granules of a type 3 cell. Scale bar: 105 nm.

immunostains a distinct population of PT-specific cells. (4) Within the latter, the immunoreaction is circumscribed to the content of the secretory granules. (5) Despite the fact that Ab-72 is a polyclonal antibody against a relatively large protein, it does not immunoreact with any cell of the bovine PD or pars intermedia. (6) The 140 nm secretory granules of type 2 cells exclusively react with Ab-72, indicating that these cells would be the storage site of the

72 kDa compound. (7) By biosynthetic labelling of proteins of primary culture of ovine PT cells, Morgan *et al.* (1994) have investigated the synthesis and secretion of a number of proteins. The most intensively labelled was a protein of 72 kDa, whose synthesis is increased by forskolin and inhibited by melatonin, indicating that the cells secreting the 72 kDa proteins are melatonin-responsive cells. Whether PT-specific cells types 1 and 2, containing Ab-72 immunoreactive granules, display melatonin receptors remains to be elucidated. Morgan *et al.* (1994) have provided strong evidence that the 72 kDa protein is secreted through the constitutive pathway, which would imply the absence or presence of only few secretory vesicles in the cells secreting this protein. This could be the case of type 1 cells, but not of type 2 cells; the latter, however, may represent the only source in the bovine PT of a 72 kDa secretory protein (see Table 1). This apparent contradiction needs to be investigated.

Chromogranin A, with a molecular weight of 48 000, but with an apparent molecular mass in SDS-PAGE of 75 kDa, and chromogranin B with a molecular weight of 71 500, have been found in the secretory cells of the

Table 1 Essential features of specific cells of the bovine PT

	Ab-21	Ab-72	Ab-LH	Diameter of secretory granules (nm)
PT-specific cell types				
Type 1	+	+	—	90
Type 2	—	+	—	140
Type 3	+	—	—	280
Type 4	+	—	+	350

Ab-LH, LH antiserum; + positive immunoreaction; — negative immunoreaction.

Figure 7 Paraffin sections of the rat medial basal hypothalamus. (A) Immunostaining using Ab-21 (AS-21) at a dilution of 1:1000. All cells of the PT are immunoreactive. ME, median eminence. (B) Detailed magnification of the area framed in (A). The strong immunoreaction of a paranuclear region gives most cells a spot-like appearance (thin arrows). A few cells appear strongly stained throughout the cytoplasm (thick arrow). Insert: section adjacent to that of (B), immunostained using anti-βTSH. The paranuclear spot shown by Ab-21 also reacts with this antiserum (thin arrow). A few fully reactive cells are also obvious (thick arrow). (C) Section adjacent to that of (B), processed for WGA binding. The paranuclear region immunostained with Ab-21 also binds the lectin (thin arrows). (D–F) The same section was sequentially immunostained using as primary antibody an antiserum against the α chain of glycoproteins PD hormones (AS-sub α) (D), anti-rabbit IgG (E) and Ab-21 (AS-21) (F). The α chain and the Ab-21 reactive material co-localise in the paranuclear spot (thin arrows) and in the whole cytoplasm of a few cells (thick arrows). Scale bars: (A) 15 μ m; (B–F) 7.5 μ m.

bovine PD, mainly in gonadotrophs and thyrotrophs (Winkler & Fischer-Colbrie 1992). Therefore, the possibility must be considered that the 72 kDa compound recognised by Ab-72 corresponds to a chromogranin. Furthermore, in cells of the chicken PT, chromogranin A and LH immunoreactivities co-localise in the same secretory granules (Kameda *et al.* 1998). However, the selective immunostaining of a small population of PT-specific cells by Ab-72, and the complete lack of Ab-72 immunoreaction in the bovine PD and in the whole adenohypophysis of the rat (including the PT) argue against the possibility that the Ab-72 immunoreactive material corresponds to one of the known chromogranins.

Ab-21 reveals proteins secreted by the predominant population of PT-specific cells

In immunoblots of the conditioned medium and in the explant proper, Ab-21 recognises four bands of 21, 22, 47 and 52. This makes the interpretation of the nature of the Ab-21 immunoreactive material difficult. Bearing in mind that the antisera were raised against bands that had been cut off the preparative gel, the immunoreaction of the four bands could be explained on the following grounds: the 21 kDa band generated its own antibody or the 22 kDa band also generated its own antibody because, due to its proximity to the 21 kDa band, it was included in the gel slices used as immunogen. An alternative is that only the 21 kDa band generated an antibody, and that this antibody cross-reacts with the 22 kDa protein. The immunoreaction of the 47 and 52 kDa bands suggests that these proteins, and the 21 kDa (and/or the 22 kDa) protein, share one or more epitope(s). The fact that Ab-21 immunoreacts with the secretory granules of two PT-specific cell types supports the possibility that this antiserum contains antibodies recognising two secretory proteins different from tuberalin I.

Evidence that type 3 cells, the predominant population of PT-specific cells, secrete an Ab-21 immunoreactive protein, here designated as tuberalin II

Type 3 cells represent the most distinct and numerous population of PT-specific cells. The following evidence strongly indicates that these cells secrete a protein (tuberalin II) recognised by Ab-21. (1) Ab-21 was generated against proteins present in the conditioned medium of organ cultures of the rostral PT. (2) Ab-21 selectively immunostains the content of the scarce secretory granules of type 3 cells. Since in most of these cells, the granules cluster in a region close to the nucleus, this gives these cells, under the light microscope, a spot-like appearance. Such an appearance has been shown to be a distinct characteristic of PT-specific cells of other species (Wittkowski *et al.* 1999). (3) The 280 nm secretory granules of type 3 cells exclusively react with Ab-21

(Table 1), indicating that these cells would be the storage site of tuberalin II. (4) The immunoreaction of type 3 cells with Ab-21 was not abolished by the immunoabsorption of Ab-21 with an extract of bovine PD. (5) The vast majority of the rat PT cells displayed a spot-like appearance after immunostaining with Ab-21. Cells with similar characteristics were missing from the rat and bovine PD.

Probable nature of tuberalin II

At present we cannot establish which one of the proteins immunoreactive with Ab-21 is present in the secretory granules of type 3 cells. In these cells the same granules, clustered as a paranuclear spot, immunoreact with Ab-21 and with an antibody against the common α chain of glycoprotein hormones of the PD. This finding supports the possibility that these secretory granules store a glycoprotein hormone with an α chain common to PD hormones, and a β chain being reactive with Ab-21; the latter would correspond to a specific secretory product of the PT, tuberalin II. The α chain has been detected in the cells of the Rathke's pouch a few days before the appearance of the α and β chains of glycoprotein hormones in the PD, which led to the suggestion that a 'free α chain' would play a role in the differentiation of the PD (Simmons *et al.* 1990, Stoeckel *et al.* 1993, Tanaka *et al.* 1997). Current immunocytochemical studies in our laboratory show that Ab-21 strongly immunostains the cells of the rat Rathke's pouch and the primordium of the PT at the same early developmental stage when the α chain is detected, further supporting the possibility that Ab-21 is recognising a β chain of a novel glycoprotein hormone.

The glycosylated nature of the material stored in the granules of type 3 cells is indicated by the labelling of these granules with WGA. Lectin histochemistry reveals, however, a striking difference between glycoproteins stored in the granules of PD gonadotrophs and thyrotrophs, and those present in the granules of type 3 cells of the PT. The former binds LFA, indicating that sialic acid is the terminal sugar residue of the carbohydrate moiety of the N-linked complex type glycoproteins; this agrees with the fact that α and β chains of glycoprotein hormones of the PD are core-glycosylated, with sialic acid as the terminal sugar residue (Ryan *et al.* 1988). On the other hand, the granules of type 3 cells in the PT bind WGA, but not LFA or PNA, indicating that sialic acid and galactose are missing, and that glucosamine would be the terminal sugar residue. If this sugar chain does indeed correspond to tuberalin II, it would imply that the carbohydrate moiety of this compound has a rather exceptional structure (cf. Rodríguez *et al.* 1988).

Immunocytochemistry and *in situ* hybridisation indicate that the rat PT-specific cells secrete the common α chain and the β chain of TSH (cf. Wittkowski *et al.* 1999). The lectin binding properties of these cells suggest, however, that the carbohydrate moiety of the TSH secreted by the

PT would be different from that of the TSH secreted by the PD. According to the present investigation, in the rat PT-specific cells, the β chain of TSH and the Ab-21 immunoreactive protein co-localise in the same paranuclear cluster of granules, giving support to the suggestion by Wittkowski *et al.* (1999) that these cells might co-express and release several active agents.

Type 1 cells might secrete a compound different from tuberalins I and II

The immunoreactivity of the 90 nm secretory granules of type 1 cells with both Ab-72 and Ab-21 could be due either to co-localisation within the same granules of two compounds, one reacting with Ab-72 (tuberalin I), and the other with Ab-21 (tuberalin II), or to the presence in these granules of a third compound, different from both tuberalins, displaying epitopes reacting with both antibodies. Although the immunoblot study using both antibodies supports the former possibility, this problem needs to be further investigated.

Are type 4 cells PT specific?

Type 4 cells are the only cell type containing granules immunoreacting with Ab-21 and with one of the antisera against PD hormones, the anti- β LH. Apparently, this cell type is also present in the PD. However, the dilution and immunoabsorption tests of Ab-21 point to the possibility that the type 4 cells of the PT contain an Ab-21 reactive compound which is homologous but not identical to LH and that, in the PD, Ab-21 immunoreacts with LH proper. The compound revealed by Ab-21 in type 4 cells could correspond to one of the proteins revealed by this antibody in immunoblots.

The use of both antibodies for the purification of Ab-21 and Ab-72 immunoreactive proteins, and for physiological experiments, are in progress.

Acknowledgements

We are grateful to Dr A F Parlow and the NIDDK's National Hormone and Pituitary Program for the donation of antisera against PD hormones. We also thank Vet. Dr Ricardo Alzola for his valuable co-operation in some aspects of this investigation, Mr L Delannoy for the excellent processing of samples for electron microscopy and Vet. Dr H Mimica, head of the Valdivia slaughterhouse, for providing all the facilities for the collection of bovine pituitary glands. During the whole period of this investigation, M G held a fellowship from the International Co-operation Agency of Spain. This work was supported by grant 1970627 from Fondecyt, Chile to E M R.

References

- Baker BL 1977 Cellular composition of the human pituitary pars tuberalis as revealed by immunocytochemistry. *Cell and Tissue Research* **182** 151–163.
- Baker BL & Yu YY 1975 Immunocytochemical analysis of cells in the pars tuberalis of the rat hypophysis with antisera to hormones of the pars distalis. *Cell and Tissue Research* **156** 443–449.
- Böckers TM, Sourgens H, Wittkowski W, Jekat A & Pera F 1990 Changes in TSH-immunoreactivity in the pars tuberalis and pars distalis of the fetal rat hypophysis following maternal administration of propylthiouracil and thyroxine. *Cell and Tissue Research* **260** 403–410.
- Böckers TM, Bockmann J, Fauteck JD, Kreutz MR, Bock R & Wittkowski W 1994 Pars tuberalis specific cells in the ovine pituitary do express the common α -chain of glycoprotein hormones: an *in situ* hybridization and immunocytochemical study. *European Journal of Endocrinology* **131** 540–546.
- Bockmann J, Böckers TM, Winter C, Wittkowski W, Winterhoff H, Deufel TH & Kreutz R 1997a Thyrotropin expression in hypophyseal pars tuberalis-specific cells is 3,5,3'-triiodothyronine, thyrotropin-releasing hormone, and Pit-1 independent. *Endocrinology* **138** 1019–1028.
- Bockmann J, Kreutz MR, Wittkowski W & Böckers TM 1997b TSH expression in murine hypophyseal pars tuberalis-specific cells. *Acta Anatomica* **160** 189–194.
- Bradford MM 1976 A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 278–281.
- Cameron E & Foster CL 1972 Some light and electron microscopical observations on the pars tuberalis of the pituitary gland of the rabbit. *Journal of Endocrinology* **54** 505–511.
- Cozzari C & Hartmann B 1980 Preparation of antibodies specific to choline acetyltransferase from bovine caudate nucleus and immunohistochemical localization of the enzyme. *PNAS* **77** 7453–7457.
- Dellmann HD, Stoeckel ME, Hindelang-Gertner C, Porte A & Stutinski F 1974 A comparative ultrastructural study of the pars tuberalis of various mammals, the chicken and the newt. *Cell and Tissue Research* **148** 313–329.
- Fitzgerald KT 1979 The structure and function of the pars tuberalis of the vertebrate adenohypophysis. *General and Comparative Endocrinology* **37** 383–399.
- González B & Rodríguez EM 1980 Ultrastructure and immunocytochemistry of neurons in the supraoptic and paraventricular nuclei of the lizard *Liolaemus cyanogaster*. *Cell and Tissue Research* **207** 463–477.
- Gross DS 1978 Effect of castration and steroid replacement on immunoreactive luteinizing hormone cells in the pars tuberalis of the rat. *Endocrinology* **103** 583–588.
- Gross DS 1983 Hormone production in the hypophyseal pars tuberalis of intact and hypophysectomized rats. *Endocrinology* **112** 733–744.
- Gross DS 1984 The mammalian hypophyseal pars tuberalis: a comparative immunocytochemical study. *General and Comparative Endocrinology* **56** 283–298.
- Herrera H & Rodríguez EM 1990 Secretory glycoproteins of the rat subcommissural organ are N-linked complex-type glycoproteins. Demonstration by combined use of lectins and specific glycosidases, and by the administration of Tunicamycin. *Histochemistry* **93** 607–615.
- Kameda Y, Miura M & Ohno S 1998 Localization and development of chromogranin A and luteinizing hormone immunoreactivities in the secretory specific cells of the hypophyseal pars tuberalis of the chicken. *Histochemistry and Cell Biology* **109** 211–222.
- Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* **227** 680–685.

- Lafarque M, Oliveros L & Aguado L 1998 Efecto de secreciones de pars tuberalis de la adenohipófisis sobre la liberación de prolactina desde pars distalis. *Medicina* **58** 36–40.
- Lis H & Sharon N 1986 Lectins as molecules and as tools. *Annual Review of Biochemistry* **55** 35–67.
- Morgan PJ, Williams LM, Davidson G, Lawson W & Howell E 1989 Melatonin receptors on ovine pars tuberalis. *Journal of Neuroendocrinology* **1** 1–4.
- Morgan PJ, Barrett P, Davidson G & Lawson W 1992 Melatonin regulates the synthesis and secretion of several proteins by pars tuberalis cells of the ovine pituitary. *Journal of Neuroendocrinology* **4** 557–563.
- Morgan PJ, Barret P, Davidson G, Lawson W & Hazlerig D 1994 p72, a marker protein for melatonin action in ovine pars tuberalis cells: its regulation by protein kinase A and protein kinase C and differential secretion relative to prolactin. *Neuroendocrinology* **59** 325–335.
- Morgan PJ, Webster CA, Mercer JG, Ross AW, Hazlerigg DG, McLean A & Barret P 1996 The ovine pars tuberalis secretes a factor(s) that regulates gene expression in both lactotropic and non lactotropic pituitary cells. *Endocrinology* **137** 4018–4026.
- Nualart F, Hein S, Rodríguez EM & Oksche A 1991 Identification and partial characterization of the secretory glycoproteins of the bovine subcommisural organ – Reissner's fiber complex. Evidence for the existence of two precursor forms. *Molecular Brain Research* **11** 227–238.
- Osamura Y & Watanabe K 1978 An immunohistochemical study of epithelial cells in the posterior lobe and pars tuberalis of the human adult pituitary gland. *Cell and Tissue Research* **194** 513–524.
- Rodríguez EM, Yulis R, Peruzzo B, Alvial G & Andrade R 1984 Standardization of various applications of methacrylate embedding and silver methenamine for light and electron microscopy immunocytochemistry. *Histochemistry* **81** 253–263.
- Rodríguez EM, Peruzzo B, Alfaro L & Herrera H 1988 Combined use of lectin histochemistry and immunocytochemistry for the study of neurosecretion. In *Neurosecretion: cellular aspects of the production and release of neuropeptides*, pp 71–88. BT Pickering, JB Wakerley & AJS Summerlee. New York, London: Plenum Press.
- Ryan RJ, Charlesworth C, McCormick DJ, Milius RP & Keutmann HT 1988 The glycoprotein hormones: recent studies of structure–function relationships. *FASEB Journal* **2** 2661–2669.
- Simmons DM, Voss JW, Ingraham HA, Holloway JM, Broide RS, Rosenfeld MG & Swanson LW 1990 Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. *Genes and Development* **4** 695–711.
- Sternberger LA, Hardy PH, Cuculis JJ & Meyer HG 1970 The unlabelled antibody–enzyme method of immunohistochemistry: preparation and properties of a soluble antigen–antibody complex (horseradish–peroxidase–antiperoxidase) and its use in identification of spirochetes. *Journal of Histochemistry and Cytochemistry* **18** 315–345.
- Stoeckel ME, Porte A, Hindelang-Gertner C & Dellmann HD 1973 A light and electron microscopic study of the pre and postnatal development and secretory differentiation of the pars tuberalis of the rat hypophysis. *Zeitschrift für Zellforschung* **142** 348–365.
- Stoeckel ME, Hindelang-Gertner C & Porte A 1979 Embryonic development and secretory differentiation in the pars tuberalis of the mouse hypophysis. *Cell and Tissue Research* **198** 465–476.
- Stoeckel ME, Hindelang C, Klein MJ, Poissonnier M & Felix JM 1993 Early expression of the glycoprotein hormone alpha-subunit in the pars tuberalis of the rat pituitary gland during ontogenesis. *Neuroendocrinology* **58** 616–624.
- Stoeckel ME, Hindelang C, Klein MJ, Poissonnier M & Felix JM 1994 Expression of the alpha-subunit of glycoprotein hormones in the pars tuberalis-specific glandular cells in rat, mouse and guinea pig. *Cell and Tissue Research* **278** 617–624.
- Tanaka S, Kurabuchi S, Mochida H, Hayashi H & Wakabayashi K 1997 Production and characterization of specific anti-peptide antiserum against free alpha-subunit of rat pituitary glycoprotein hormones. *Journal of Histochemistry and Cytochemistry* **45** 985–990.
- Towbin H, Staehelin T & Gordon J 1979 Electrophoretic transfer of proteins from polyacrilamide gel nitrocellulose sheets: procedure and some applications. *PNAS* **76** 4350–4354.
- Van Herck H, De Boer SF, Hesp AP, Van Lith HA, Baumans V & Beynen AC 1997 Orbital bleeding in rats while under diethylether anaesthesia does not influence telemetrically determined heart rate, body temperature, locomotor and eating activity when compared with anaesthesia alone. *Laboratory Animals* **31** 271–278.
- Williams LM & Morgan PJ 1988 Demonstration of melatonin binding sites on the pars tuberalis of the rat. *Journal of Endocrinology* **119** R1–R3.
- Wingstrand KG 1966 Comparative anatomy and evolution of the hypophysis. In *The Pituitary Gland*, vol. 1, pp 58–126. GW Harris & BT Donovan. London: Butterworths.
- Winkler H & Fischer-Colbrie R 1992 The chromogranins A and B: the first 25 years and future perspectives. *Neuroscience* **49** 497–528.
- Wittkowski W, Bergmann M, Hoffmann K & Pera F 1988 Photoperiod-dependent changes in TSH-like immunoreactivity of cells in the hypophyseal pars tuberalis of the Djungarian hamster, *Phodopus sungorus*. *Cell and Tissue Research* **251** 183–187.
- Wittkowski WH, Schultze-Bonhage AH & Böckers TM 1992 The pars tuberalis of the hypophysis: a modulator of the pars distalis? *Acta Endocrinologica* **126** 285–290.
- Wittkowski W, Bockmann J, Kreutz MR & Böckers T 1999 Cell and molecular biology of the pars tuberalis of the pituitary. *International Review of Cytology* **185** 157–194.
- Young BA, Foster CL & Cameron E 1966 Ultrastructural changes in the adenohypophysis of castrated rabbits. *Journal of Endocrinology* **35** 101–106.

Received 11 September 2000

Accepted 9 November 2000