ORIGINAL ARTICLE

Bovine pars tuberalis secretions release growth hormone from rat pars distalis of pituitary gland

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Abstract

The pituitary pars tuberalis (PT) is characterized by PT-specific secretory cells which have raised the possibility of an endocrine function for this portion of adenohypophysis.

OBJECTIVE: To investigate the effect of the secretion of bovine PT cells into culture medium on growth hormone (GH) response of dispersed pars distalis (PD) cells of rats.

METHODS AND RESULTS: 48-hour culture medium of all PT cells at 1 μg of protein concentration induced the greatest GH release from PD cells. After PT cells separation on a discontinuos Percoll gradient and culturing, only the culture medium of cells from 50 and 60% Percoll strength released GH from PD cells. Therefore, cells from 50 and 60% strenght Percoll were cultured together. Only 0.2 μg protein of this culture medium was required to induce the maximal GH release from PD cells, suggesting that these cells could be responsible for producing the factor(s) which affect PD somatotrophe cells. After protein separation by 12% SDS-PAGE of this PT culture medium bands were eluted. The biological activity, measured as ng/ml of GH from PD cells, corresponded to a protein(s) of molecular weight between 45 and 66 kDal.

CONCLUSIONS: The results indicate that there is an active proteic factor(s) secreted by the PT that acts upon PD cells to stimulate GH release and that PD could be an effector organ for some secretory product(s) of the PT.

Abbreviations and units

PT	pars tuberalis
PD	pars distalis
GH	growth hormone
BSA	bovine serum albumin
DNase	deoxyribonuclease
PBS	phosphate-buffered saline
h	hour
µg	microgram
kDa	kilodalton
°C	centigrade grades
g	gram
mg	milligram
min	minute
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SEM	standard error medium
ml	milliliter

Introduction

The pars tuberalis (PT) and pars distalis (PD) are constant subdivisions of the pituitary gland of vertebrates. The PT is a structurally distinct region of the adenohypophysis and it has been studied in rat, human, ovine and other species by electron microscopy and immunohistochemistry. The gonadotrophs and thyrotropes are located mainly in the ventrocaudal portion of the PT, in continuity with the pars distalis [1, 2]. However, the major portion of the PT consists of secretory cells specific to this portion of the pituitary [3]. Because of the close spatial relationship between the PT cells and the hypotalamic-hipophyseal portal capillaries, it has been suggested that the portal circulation might be the avenue for transport of the putative secretory product(s) of the PT to the PD [3, 4]. On the basis of this anatomical arrangement, Dellman hypothetised that the PD could be a target organ for the PT secretion(s).

Studies in a variety of photoperiodic species show that the PT contains a high density of melatonin receptors and suggest that it plays a major role in mediating the effects of melatonin on the neuroendocrine function [5, 6]. Prolactin, but not luteinizing hormone, secretion has been shown to be regulated by photoperiod-caused changes in the melatonin profile [5, 7]. Besides, the existence of an intrinsic, reversible photoperiod-circannual timer in pars tuberallis cells has been suggested [8].

Some studies have shown the possibility that factors released by the PT regulate the activity of lactotrophos in the PD. It has been reported that co-culture of ovine PD cells with PT cells causes prolactin secretion to increase to 50–100% above control levels over 24 h [9]. Also, it has been shown that a factor(s) released from ovine PT cells in a time-dependent and cycloheximide-sensitive manner increases c-fos expression in some, but not all, lactotrophs in PD cell cultures [10]. In addition, culture medium from bovine PT cells cultured for 48 h proved to have a potent stimulatory effect on PD cell prolactin secretion [11]. On the other hand, it is known that living pituitary cells could concurrently express the genes of both the growth hormone (GH) and PRL [12]. Furthermore**,** pituitary tumor GH3 cells synthesize and secrete both GH and PRL [13] and secretory granules of pituitary adenomas present antigenicity for GH and PRL, among other hormones [14]. However, there is no information about the influence of PT on the GH response from PD cells. Also, the physiological role and function of PT-specific cells is still elusive. The aim of this work is to establish whether PT cell secretions can affect the GH release from PD cells. To do this, bovine PT cells were isolated by Percoll gradient and culture and their culture medium was subsequently used to stimulate the GH release from rat PD cells.

Material and methods

Animals: Adult male Holtzman (250 g) rats bred in our animal facilities and maintained in a humidity and temperature (21–23 °C) controlled environment with a 12-h light:dark cycle were used. Tap water and pelleted rat chow (Cargill SACI, Argentina) were available ad libitum. The bovine pars tuberalis were collected from young bulls killed at a local abattoir, between 8:00 and 9:00 a.m., and at all times of the year. In all the procedure we followed the Revised Guide for the Care and Use of Laboratory Animals [15].

Chemicals: The following drugs were purchased from Sigma (St.Louis, MO, USA): 199 culture medium, crude trypsin, soybean trypsin inhibitor, collagenase type IV, DNase, penicillin G, streptomycin, nistatine, bovine serum albumin fraction V (BSA), molecular weight markers MW-SDS-200 and protease inhibitor coktail. All other chemicals were of reagent grade and were provided by Merck Laboratory, Buenos Aires, Argentina.

PT cell culture: Primary cultures of bovine PT were generated from 10–12 glands. The PT tissues were dissociated in 199 medium containing 0.25% crude trypsin by stirring at 37 °C and assisted by repeatedly drawing tissue fragments into a siliconized pipette. After 30 min 0.2 mg/ml trypsin inhibitor was added. The suspension was centrifuged at 100 x g for 10 min at 4 °C. The cell pellet was washed twice in 10 ml of 199 medium containing 2% penicillin G, 3% streptomycin and 0.04% nistatine and centrifuged. Then, the cell pellet was resuspended in the above indicated medium. Cell viability was determined to be 90% by trypan blue exclusion test. Cells (2×10^6 cells/ml) were cultured in 199 medium supplemented with antibiotic at 37 °C in 95% air-5% CO_2 for 24 h. The medium was removed, replaced by fresh medium added with antibiotics and cells were cultured for additional 24 h. After that, the culture medium was replaced again, as above described, and cells were cultured for 48 h in the same conditions. Finally, the culture medium was aspirated, added with a mix of protease inhibitors, centrifuged at 12,000 g for 20 min to pellet any cells, and used to stimulate PD cells.

Percoll gradient: Due to the existence of different cell populations in the bovine PT, a density gradient centrifugation of PT cells was performed on a discontinuous Percoll gradient to identify the cells whose

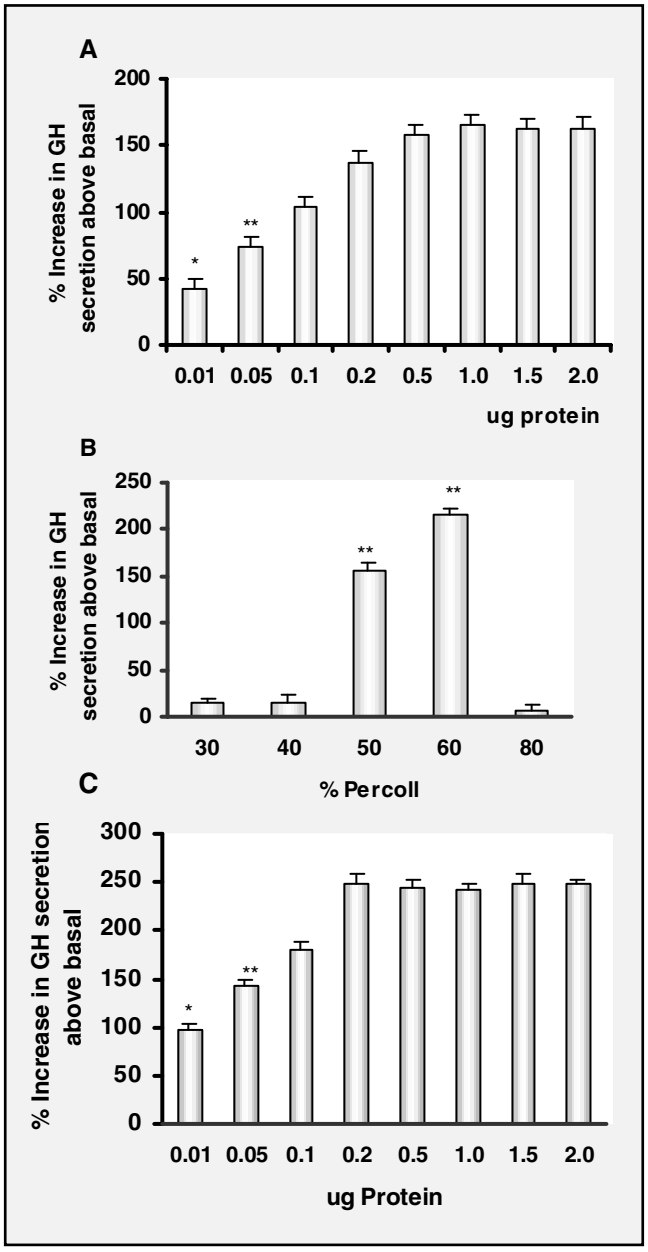

Fig. 1. GH release by dispersed rat PD cells incubated in vitro with culture medium of: **A**, all PT cell populations (dose-dependent effect); **B**, each of the fractions of PT cells separated on discontinuous Percoll gradients and, **C**, PT cells from 50+60 % Percoll fractions (dose-dependent effect). The output of PD GH is expressed as a percentage of the amount released from PD cells incubated with 199 medium alone (basal value). Bars represent mean ± SEM of duplicated measurements from five independent experiments with 4 tubes per point per experiment. Values were significantly different from sample basal value at $^{*}p< 0.005$ and $^{**}p< 0.001$.

secretions could be responsible for GH release from PD cells. Stock Percoll was diluted 1:10 with Hanks' balanced salt solution and then diluted again to different percentage (30, 40, 50, 60 and 80%) strengths of Percoll with Hanks' solution. Starting with 80% Percoll, 1.5 ml aliquots of decreasing strength were layered into a 12 ml conical centrifuge tube up to 30% Percoll. After dissociation and culture for 24 h twice as indicated above, the PT cells were layered above the 30% Percoll in 1 ml of Hanks'solution. The tube was then centrifuged at 400 x g for 15 min at 4 °C. Cells were harvested from each strength of Percoll, washed twice with 199 medium, tested by trypan blue test and cultured (2 x 10^6 viable cells/ml) for 48 h as indicated above. Cell viability was of 90–95% in all Percoll fractions.The collected culture media were used for subsequent experiments.

Dispersed pars distalis (PD) cells: Adult male rats were killed by decapitation. The brains and pituitaries were rapidly dissected out. The anterior lobes were dissected free from the neurointermediate lobe. PD cells were dispersed as described previously [16]. Briefly, each PD was cut into four pieces and placed into 1 ml 199 medium containing 0.2% collagenase. During shaking at 37 °C under 95% O_2 – 5% CO_2 atmosphere, remaining fragments of tissue were repeatedly (30–40 times) passed through a siliconized Pasteur pipette. Three minutes before the procedure was finished, 0.1 mg/ml DNase was added. Then, the suspension was centrifuged at 100 x g for 10 min at 4 °C. The cell pellet was washed twice in 5 ml 199 medium containing 0.25% BSA, 20 mg/l penicillin G and 30 mg/l streptomycin, centrifuged as described above and finally resuspended in the same medium. After viability determination, 1 x 10^5 cells/tube were placed in 0.5 ml of 199 medium and stabilized for 6 h at 37 °C under 95% O_2 – 5% CO_2 atmosphere. Then, the medium was replaced by 0.45 ml of fresh medium and cells were stimulated by addition of 50 µl of culture medium from PT cells, at a protein concentration depending on the experimental design. Medium from PD cells incubated with fresh medium alone was used as basal value. Tubes were incubated in a shaking bath for 30 min at 37 °C gassed with 95 % O_2 – 5 % CO_2. After centrifugation, the supernatants were kept at –70 °C until determination of GH. Protein concentrations were determined by the method of Bradford (1976) [17].

SDS-PAGE: Slab gel electrophoresis was performed using the method of Laemmli (1970) [18]. Molecular weight markers were visualized by Coomassie

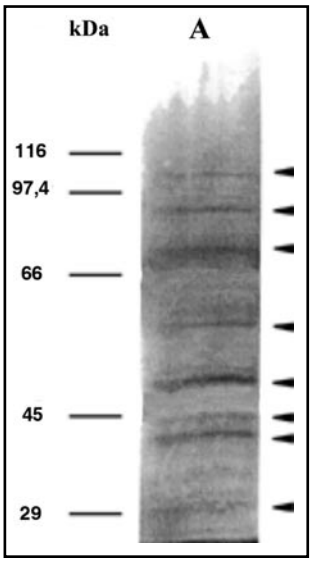

Fig. 2. SDS-PAGE (12%) gel of proteins secreted from bovine pars tuberalis cells of 50+60 % Percoll gradient. **kDa**, molecular weight markers. **A,** arrows indicate position of bands. Bands represent one of five experiments**.**

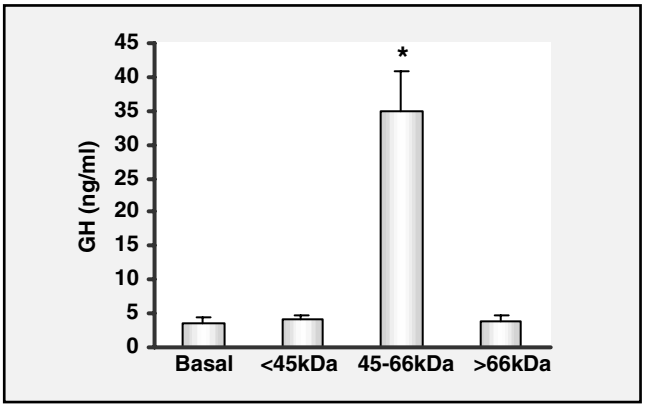

Fig. 3. GH release from PD cells stimulated with the protein (0.01 µg) eluted from SDS-PAGE (12%) gel after protein separation of culture medium from PT cells of 50+60% Percoll gradient. The eluates from bands of less than 45 kDa, between 45 and 66 kDa and more than 66 kDa were concentrated before testing their biological activities. Values are mean ± SEM from five experiments. *p<0.001.

blue staining, followed by destaining in glacial acetic acid/methanol/water (10:40:50, v/v/v) at room temperature.

After protein separation by 12% SDS-PAGE of PT cells culture medium from 50+60% Percoll gradient, the gel was cut into three pieces containing bands of less than 45 kDa, between 45 and 66 kDa and more than 66 kDa, respectively. The proteins contained in each piece of gel were eluted by incubation in phosphate-buffered saline (PBS) pH 7.5 for 4 h at 4 °C, with shaking. After concentration of the eluted protein by Microcom (OMEGA) filters**,** their biological activity was tested on PD cell incubations by determining the GH release.

Hormone assay: GH was measured by double antibody radioimmunoassay (RIA) using a kit provided by NIADDK of NIH (Dr. Raiti, NIADDK Rat Pituitary Hormone Distribution Program), as recommended. The results were expressed in terms of the rat GH RP-3 standard preparations. The assay sensitivity was less than 5 ng/ml and the inter- and intra-assay coefficients of variation were less than 10.0%.

Statistical analyses: Results are expressed as mean ± SEM. Significant differences among means were considered at a level of $p< 0.05$ and identified by one-way ANOVA. When only two means were compared the Student's t-test was used.

Results

Responsiveness of rat PD cells to culture medium of all PT cell populations, measured as GH release, is shown in Figure 1A. The concentration of GH released was proportional to the protein concentration in PT culture medium up to 1 µg protein ($p< 0.001$), when the maximal amount of GH was detected. All mean values were significantly different from the basal value.

The stimulation of PD cells with culture medium from each of the five fractions of PT cellular material distinguished by centrifugation over discotinuous Percoll gradients only induced a significantly higher GH release from PD cells in relation to the basal value when the culture medium of cells from 50 and 60% strenght Percoll ($p< 0.001$) were used, while the other Percoll fractions did not stimulate the GH release (Figure 1B). Because of this, cells from 50 and 60% strenght Percoll were cultured together to use their culture liquid for all the subsequent PD cell stimulation experiments. Figure 1C shows the GH release from PD cells incubated with different protein concentrations of culture medium of PT cells from 50+60% strength Percoll fractions. A significant GH response was observed with the lowest protein concentration tested (0.01 µg) and it was proportional to the concentration of protein in the PT culture medium up to 0.2 µg protein ($p< 0.001$). At this point, the maximal amount of GH was detected, which was more than two times the basal output. This response was maintained with higher protein concentrations. Results from Figures 1B and 1C suggest that PT cells from 50+60% Percoll fraction could be responsible for producing the factor(s) which affect(s) somatotrophe cells of PD.

Figure 2 shows the results from SDS-PAGE studies. After protein separation by 12% SDS-PAGE of the culture medium of PT cells from 50+60% Percoll gradient fractions, eight bands were visualized. The GH response obtained with the protein recovered from the three pieces of SDS gel is shown in Figure 3. The GH release was only detected ($p< 0.001$) when the PD cells were stimulated with the eluate from the piece of gel containing the two bands of molecular size between 45 and 66 kDa. In this case a high GH response was obtained with 0.01μg of protein. Protein eluted from the other two pieces of gel did not stimulate GH release from PD cells.

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Discussion

The pituitary pars tuberalis is characterized by PT-specific secretory cells the occurrence of which has raised the possibility of an endocrine function for this portion of adenohypophysis. In this study we report evidence for a factor(s) secreted by the PT of bovine pituitary that acts upon PD cells of rat to stimulate GH release.

The PT cellular material distinguished by centrifugation over discontinuous Percoll gradients allows to identify only two cell fractions, of 50 and 60 % of Percoll gradient, respectively, capable to induce the GH release from PD cells, the same effect produced by the culture medium from PT total cell populations. However, the release of GH by using the secretions of cell from 50+60% Percoll fractions occurred at a lower

(five times) protein concentration, suggesting that these PT cells could be responsible for producing the factor(s) which affect to somatolactotrophe cells of PD. This also evidences the sensibility of PD cells to the unknown PT factor(s).

The stimulation of PD cells cannot be attributed to the presence of hypothalamic factors in the culture medium of these cells, since they were cultured long enough to eliminate the ending nerves which could be present in the PT dissection. Additionally, the partial purification of PT cells on Percoll gradients and the successive cell washes to eliminate Percoll solution also contributes to the PT cultures being exempt of hypothalamic factors. It is not possible that the concentration of GH measured come from the PT cells since immunocytochemical studies in different animal species has indicated that GHs are not present within PT [19].

Our results suggest a possible direct modulating effect of PT, at least on the somatotrope cells of PD, and increase the possibility that PD can be a probable target organ for the PT secretion(s), as we and other authors have previously suggested [9–11].

Although the exact chemical identity of the factor(s) involved is not yet known, in this work we have found the biological activity on the basis of a protein of molecular size between 45- and 66-kDa. It is not yet clear, however, whether this is due to one or two molecular components since two bands were eluted together from the piece of gel.

The ability to detect the involved PT factor(s) in our experimental scheme may have been due to artificial conditions created in vitro, which cannot fully represent in vivo conditions. However, the density of PT cells used for culturing was not so high so as to assume that they might have produced an artificially high concentration of factor(s) above the threshold required to release GH in PD cells.

Due to the peculiar location of PT in close contact to the primary plexus of the portal system and to hypothalamic nerve endings of the median eminence, it has been indicated that the secretory products of PT could reach the perivascular space and/or the subarachnoid space through the intercellular channels of the PT [20]. This could also suggest that in *in vivo* conditions the probable target organ for the secretion of the PT could be certain region(s) of the brain in order to affect the neuroendocrine axis via a short-loop feed-back system. On the other hand, the possibility that, in vivo, PT factor(s) is acting on some other cells of PD cannot be excluded. In this regard, PT specific factor(s) could have a paracrine action on the surrounding somatotropes of PD.

In spite of the fact that the exact nature of this secreted PT factor(s) is still unknown, the results of this study provide evidence of its protein nature, and also of its molecular size. Although the endocrine function of the PT remains to be well elucidated, this study reveals the potential influence of the PT on a second endocrine axis and also extends our knowledge of pituitary PT.

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REFERENCES

1 Gross DS. The mammalian hypophyseal pars tuberalis: a comparative immuno- cytochemical study. Gen Comp Endocrinol 1984; **56**:283–298.
2 Skinner DC, Herbison AE, Robinson JE. Immunocytochemical identification of oestrogen receptors in the ovine pars tuberalis: Localization within gonadotrophs. J Neuroendocrinol 1992; **4**: 659–662.
3 Dellman H-D, Stoeckel ME, Hindelang-Gertner C, Porte A, Stutinsky F. A comparative ultrastructural study of the pars tuberalis of various mammals, the chicken and the newt. Cell Tissue Res 1974; **148**:313–329.
4 Fitzgerald KT. The structure and function of the pars tuberalis of the vertebrate adenohypophysis. General Comparative Endocrinol 1979; **37**:383–399.
5 Morgan PJ, Barrett P, Howell HE, Helliwell R. Melatonin receptors: localisation, molecular pharmacology and physiological significance. Neurochem Internat 1994; **24**:101–146.
6 Barrett P, Messager S, Schuster C, Moar KM, Mercer JG, Morgan PJ. Pituitary adenylate cyclase-activating polypeptide acts as a paracrine regulator of melatonin-responsive cells of the ovine pars tuberalis. Neuroendocrinology 2002; 143:2366–2375.
7 Malpaux B, Skinner DC, Maurice F. The ovine pars tuberalis does not appear to be targeted by melatonin to modulate luteinizing hormone secretion, but may be important for prolactin release. J Neuroendocrinol 1995; **7**:199–206.
8 Lincoln GA, Andersson H, Hazlerigg D. Clock genes and the long-term regulation of prolactin secretion: evidence for a photoperiod/circannual timer in the pars tuberalis. J Neuroendocrinol 2003; **15**:390–397.
9 Hazlerigg DG, Hastings MH, Morgan PJ. Production of a prolactin releasing factor by the ovine pars tuberalis. Mol Endocrinol 1996; **8**:489–492.
10 Morgan PJ, Webster CA, Mercer JG, Ross AW, Hazlerigg AM, MacLean A, et al. The ovine pars tuberalis secretes a factor(s) that regulates gene expression in both lactotropic and nonlactotropic pituitary cells. Endocrinology 1996; **137**:4018–4026.
11 Lafarque MM, Oliveros LB, Aguado LI. Effect of secretions of pars tuberalis of adenohypophysis on the release of prolactin from pars distalis. Medicina 1998; **58**:36–40.
12 Willard ST, Amstutz MD, Abraham EJ, Castano JP, Leaumont DC, Faught WJ, Frawley LS. Simultaneous indirect activity measurements of GH and PRL genes in the same, living mammosomatotrope. Am J Physiol 1999; **277**:1150–1153.
13 Kakeya T, Takeuchi S, Takahashi S. Epidermal growth factor, insulin, and estrogen stimulate development of prolactin-secreting cells in cultures of GH3 cells. Cell Tissue Res 2000; **299**: 237–243.
14 Kamitani H, Masuzawa H, Kanazawa I, Kubo T. Secretory granules of pituitary adenomas: quantitative study of hormonal antigenicity. Acta Neuropathol 2000; **99**:263–270.
15 Bayne K. Revised Guide for the Care and Use of Laboratoy Animals available. American Physiological Society. Physiologist 1996; **199**:208–211.
16 Spinedi E, Johnston C, Negro-Vilar A. Increased responsiveness of the hypothalamic-pituitary axis after neurotoxin-induced hypothalamic denervation. Endocrinology 1984; **115**:267–272.
17 Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; **72**:248–254.
18 Laemmli UK. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 1970; **227**:680–685.
19 Antunes JL, Carmel PW, Zimmerman EA, Ferin M. The pars tuberalis of the rhesus monkey secretes luteinizing hormone. Brain Res 1979; **166**:49–55.
20 Aguado LI, Schoebitz K, Rodríguez EM. Intercellular channels in the pars tuberalis of the rat hypophysis and their relationship to the subarachnoid space. Cell Tissue Res 1981; **281**:345–354.