BIOCELL
2008, 32(3): 211-218

ISSN 0327 - 9545
PRINTED IN ARGENTINA

A 66-kDa protein of bovine hypophyseal *Pars tuberalis* induces luteinizing hormone release from rat *Pars distalis*

MARTHA LAFARQUE AND LILIANA OLIVEROS

Cátedra de Fisiología. Laboratorio de Biología de la Reproducción (LABIR), Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, San Luis, Argentina.

Key words: adenohypophysis, gonadotropes, lactotrophs, follicle stimulating hormone, prolactin

ABSTRACT: In this study, evidence for a factor secreted by bovine hypophyseal *pars tuberalis* that stimulates luteinizing hormone (LH) release from rat *pars distalis* cells is shown. The secretion products of bovine *pars tuberalis* cells into the culture medium were assayed on dispersed rat *pars distalis* cells in 30 min incubations and superfusion experiments. The culture medium from *pars tuberalis* total cell populations, added at a dose of 6 μg per tube, induced the greater LH release from *pars distalis* cells, without effect on follicle stimulating hormone (FSH) release. After *pars tuberalis* cells separation on a discontinuos Percoll gradient, only the culture medium of cells from 50 and 60% strength Percoll were able to release LH from rat *pars distalis* cells. Therefore, cell fractions from 50 and 60% strenght Percoll were cultured together. To elicit maximal LH release (6 times the basal output), with the addition of 2 μg of *pars tuberalis* protein was required, suggesting that these cells produce the factor or factors which affect *pars distalis* gonadotrope cells. After applying the *pars tuberalis* culture medium on 12% SDS-PAGE, the band with biological activity was that of 66-kDal. Fifty ng protein of its eluate released almost 9 times the basal output of LH from *pars distalis* cells. Results suggest a modulating effect of a protein from the bovine *pars tuberalis* on rat cultured gonadotrope cells from the *pars distalis.*

Introduction

Pars tuberalis is a structurally distinct region of the adenohypophysis and it has been studied in rat, human, ovine and other species (Gross *et al*., 1984; Skinner *et al*., 1992). *Pars tuberalis* of most mammalian species contains specific cells which are structurally and functionally different from *pars distalis* cells (Bock *et al*., 2001). *Pars tuberalis* cell types include the follicular cells, *pars distalis* cells and the so-called *pars tuberalis*-specific cells. The latter are distinct endocrine cells displaying melatonin receptors (Wittkowski *et al*., 1999).

It is generally accepted that LH and FSH are stored and secreted from different secretory granules within *pars distalis* bihormonal cells (Thomas and Clarke, 1997; Farnworth, 1995). However, numerous gonadotropes have been detected in the anteroventral region of the *pars tuberalis* (Skinner and Robinson, 1996). It has been hypothesized that LH is released from *pars tuberalis* gonadotropes and diffuses into the medial eminence where it suppresses LHRH release from the terminals of the LHRH neurons, either by a direct action or by an action of other neuronal terminals (Nakazawa *et al*., 1991). Few LHRH fibers have been reported to be present in the adjacent *pars tuberalis* of the rat pituitary by fluorescence immunohistochemistry for LHRH and electron-microscopy. It has been postulated that the LHRH message might be transmitted not only by the

Address correspondence to: Liliana Oliveros. Cátedra de Fisiología. Laboratorio de Biología de la Reproducción (LABIR), Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, San Luis, ARGENTINA.
E-mail: *lolive@unsl.edu.ar*
Received on September 17, 2007. Accepted on June 21, 2008.

established hypophyseal portal vein system but also via the folliculo-stellate cells in the *pars tuberalis* to aid in LH release modulation (Mabuchi *et al*., 2004). Due to the close spatial relationship between *pars tuberalis* cells and the hypotalamic-hypophyseal portal capillaries, it has also been suggested that the portal circulation might be the avenue for the transport of the putative secretory product(s) from *pars tuberalis*, thus reaching the *pars distalis* (Dellmann *et al*., 1974; Fitzgerald, 1979). On the basis of this anatomical arrangement, Dellmann *et al*. (1974) hypothesized that *pars distalis* is a target organ for *pars tuberalis* secretion(s).

A few studies have shown the possibility that factors released by *pars tuberalis* regulate the activity of lactotrophs in *pars tuberalis.* It has been reported that co-culture of ovine *pars distalis* cells with *pars tuberalis* cells causes prolactin secretion, with an increase of 50-100% above control levels over 24 h (Hazlerigg *et al*.,1996). We have demonstrated that conditioned medium of cultured bovine cells of the *pars tuberalis* for 48 h has a potent stimulatory effect on cell prolactin and GH secretions by *pars distalis* cells (Lafarque *et al*., 1998; 2004).

Although the nature of the secretory product(s) of the *pars tuberalis*-specific cells has not yet been clarified, for practical reasons, previous authors have designated the unidentified *pars tuberalis* hormone(s) as tuberalin(s) (Morgan *et al*., 1996). *Pars tuberalis*-specific cells synthesize the common a subunit of the *pars distalis* glycoprotein hormones. It has been suggested that tuberalin II, a 21 kDa protein secreted by a predominant population of *pars tuberalis*-specific cells (type 3 cells) would correspond to the beta chain of a specific glycoprotein secreted by those *pars tuberalis*-specific cells (Guerra and Rodriguez, 2001). However, the endocrine function of *pars tuberalis* is currently unknown.

The aim of this study was to establish whether *pars tuberalis* cell secretions can affect LH and FSH release from *pars distalis* cells. With this objective, bovine *pars tuberalis* cells were isolated by Percoll gradient and cultured, and the culture medium was subsequently used to stimulate LH and FSH release from rat *pars distalis* cells.

Materials and Methods

Animals

Adult male Holtzman rats (250 g) bred in our animal facilities and maintained under conditions of controlled humidity and temperature (21-23ºC) and a 12-h light/dark cycle were used. Tap water and pelleted rat chow (Cargill SACI, Argentina) were available ad libitum. The bovine *partes tuberalis* were collected from young bulls killed at a local abattoir, between 8:00 and 9:00 a.m., throughout the year.

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 and bovine serum albumin fraction V (BSA). All other chemicals were of reagent grade and were provided by Merck Laboratory, Buenos Aires, Argentina. LH and FSH kits were kindly provided by NIADDK of NIH (Dr. Raiti, NIADDK Rat Pituitary Hormone Distribution Program).

Pars tuberalis *cell culture*

Primary cultures of bovine *pars tuberalis* were generated from 10-12 glands. The *pars tuberalis* tissues were dissociated in 199 medium containing 0.25% crude trypsin by stirring at 37ºC and assisted by repeatedly drawing tissue fragments into a siliconized pipette. After 30 min 0.2 mg/mLtrypsin inhibitor was added. The suspension was centrifuged at 100 x g for 10 min at 4ºC. 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 x 10^6 cells/mL) were cultured in 199 medium supplemented with antibiotics at 37ºC in 95% air-5% CO_2 for 24 h. The medium was removed, replaced by fresh medium with antibiotics and cells were cultured for additional 24 h. After that, the culture medium was replaced again, as described, and cells were cultured for 48 h in the same conditions. Finally, the culture medium was aspirated, centrifuged to remove cellular debris, supplemented with a mix of protease inhibitors and used to stimulate pars distalis cells.

Percoll gradient

Due to the existence of different cell populations in bovine *pars tuberalis*, a density gradient centrifugation of *pars tuberalis* cells was performed on a discontinuous Percoll gradient to identify the cells whose secretions could be responsible for LH and FSH release

from *pars distalis* 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, *pars tuberalis* 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 4ºC. 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 *cells*

Adult male rats were killed by decapitation. The brains and pituitaries were rapidly dissected out. The anterior lobes were separated from the neurointermediate lobes. *Pars distalis* cells were dispersed as previously described (Spinedi *et al*., 1984). Briefly, each *pars distalis* was cut into four pieces and placed into 1 mL 199 medium containing 0.2% collagenase. During shaking at 37ºC 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 4ºC. The cell pellet was washed twice in 5 mL 199 medium containing

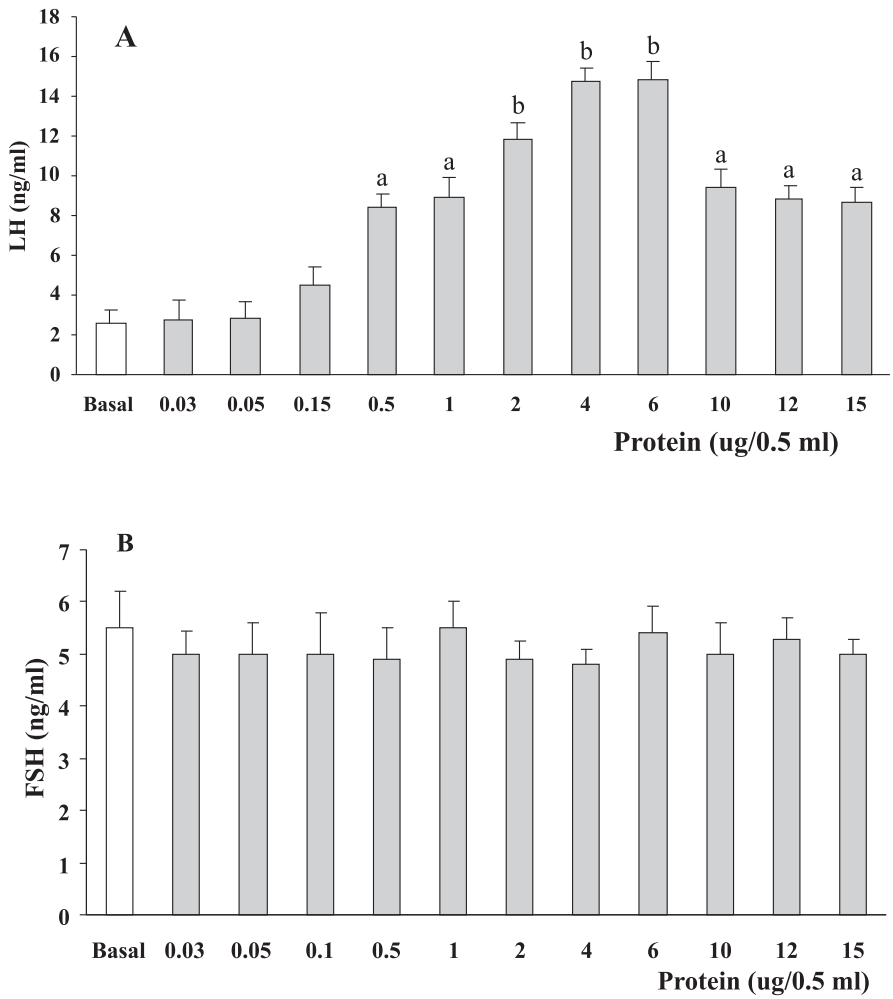

FIGURE 1. LH (**A**) and FSH (**B**) release by dispersed cells from rat *pars distalis* that were incubated *in vitro* with culture medium of all *pars tuberalis* cell populations (dose-dependent effect). The μg of protein represents the amount of *pars tuberalis* protein in 500 μl of final volume of incubation. Bars represent mean ± SEM of duplicate measurements from four independent experiments with 3 tubes per point per experiment. Letters indicate values with significant differences vs basal value, $^aP < 0.01$ and $^bP < 0.001$.

0.25% BSA, 20 mg/L penicillin G and 30 mg/L streptomycin, centrifuged as described 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 37ºC 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 different doses (0.03-15 µg protein), dissolved in 50 µL of medium from cultured *pars tuberalis* cells. Basal secretions of LH and FSH were obtained by *pars distalis* cells incubation containing only fresh 199 medium with 0.25 BSA and antibiotics. Tubes were incubated in a shaking bath for 30 min at 37ºC gassed with 95% O_2 - 5% CO_2. After centrifugation, the supernatants were kept at -70ºC until LH and FSH determinations. Protein concentrations were determined by the method of Bradford *et al*. (1976).

Superfusion of pars distalis *dispersed cells*

Cells (2 x 10^6 viable cells) were resuspended in Biogel P2 (200 - 400 mesh), previously hydrated with 199 culture medium at 4ºC for 12 hs. This cell preparation was packed into a plastic column and superfused with fresh 199 medium supplemented with 0.25% BSA, 20 mg/L ascorbic acid and antibiotics, with a flow of 0.75 mL/min for 10 min. Then, cells were stimulated with 1 mL 28 mM KCl in the medium and the superfusion was continued with medium alone. Fractions were collected every 2 min, for 20 min. After that, the column was washed with fresh 199 medium and cells were stimulated with 1 mL of culture medium from *pars* tuberalis cells of 50-60% strenght Percoll containing 3 and 0.3 µg protein per mL. Fractions were collected for 20 min, every 2 min. Collected fractions were stored at -70ºC until LH measurement.

SDS-PAGE

Slab gel electrophoresis was performed using the method of Laemmli (1970). Molecular weight markers were visualized by Coomassie 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 medium from cultured *pars tuberalis* cells from 50+60% Percoll gradient, individual bands were cut and the proteins contained in each piece of gel were eluted by incubation in phosphate-buffered saline (PBS) pH 7.5 at 4ºC for 4 h, in a shaking bath. After concentrating the eluted protein by Microcom (OMEGA) filters, their biological activity was tested by incubations on *pars distalis* cells by determining LH release.

LH and FSH radioimmunoassays

LH and FSH were measured in duplicate by double antibody radioimmunoassay (RIA) using a kit provided by the NIADDK of NIH, as recommended. Results were expressed in terms of rat LH or FSH RP-2 standard preparations. The assay sensitivity was less than 5 ng/mL and the inter- and intra-assay coefficients of variation was less than 10.0.

Statistical analysis

Data are expressed as mean ± SEM. The statistical significance of the differences between means was assessed by one-way ANOVA followed by Tukey post-test. A P value of <0.05 was considered significant.

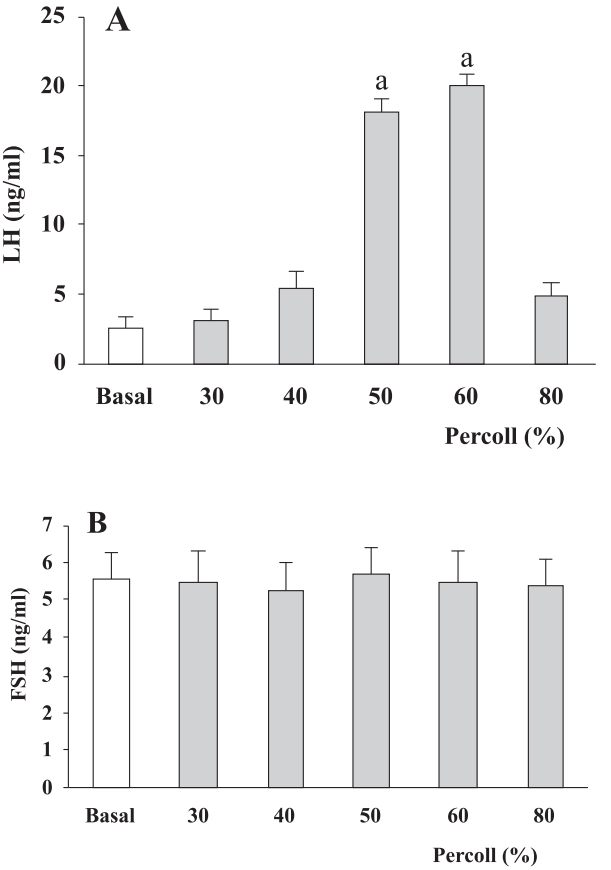

FIGURE 2. LH (**A**) and FSH (**B**) release by dispersed cells from rat *pars distalis* incubated *in vitro* with 6 µg of protein from the culture medium of each of the fractions of *pars tuberalis* cells separated on discontinuous Percoll gradients in 500 µl of final volume of incubation. Bars represent mean ± SEM of duplicate measurements as indicated in Fig. 1. Letters indicate values with significant differences vs basal value, $^aP < 0.001$.

Results

The response of rat *pars distalis* cells to culture medium from total *pars tuberalis* cell populations (without Percoll separation) measured as the release of LH and FSH is shown in figure 1. The concentration of released LH was proportional to the amount of protein in the *pars tuberalis* culture medium (50 μL) until 6 μg of protein were present in the final volume of *pars distalis* cells incubation (500 μL), where the maximal amount of LH was detected. With higher protein concentrations the LH release was less than with 6 μg of protein (Fig. 1A). No effect on FSH release was observed with any of the used doses (Fig. 1B).

When the stimulations of *pars distalis* cells were done with 6 μg of protein obtained from cultures of each of the five Percoll bands of *pars tuberalis* cells, LH release was observed with culture media obtained from only two cell bands (50 and 60% Percoll; Fig. 2A). Again no effect on FSH release was observed (Fig. 2B).

Therefore, cells from 50 and 60% strength Percoll were cultured together. Figure 3A shows LH release from *pars distalis* cells that were incubated with different protein concentrations of culture medium from 50-

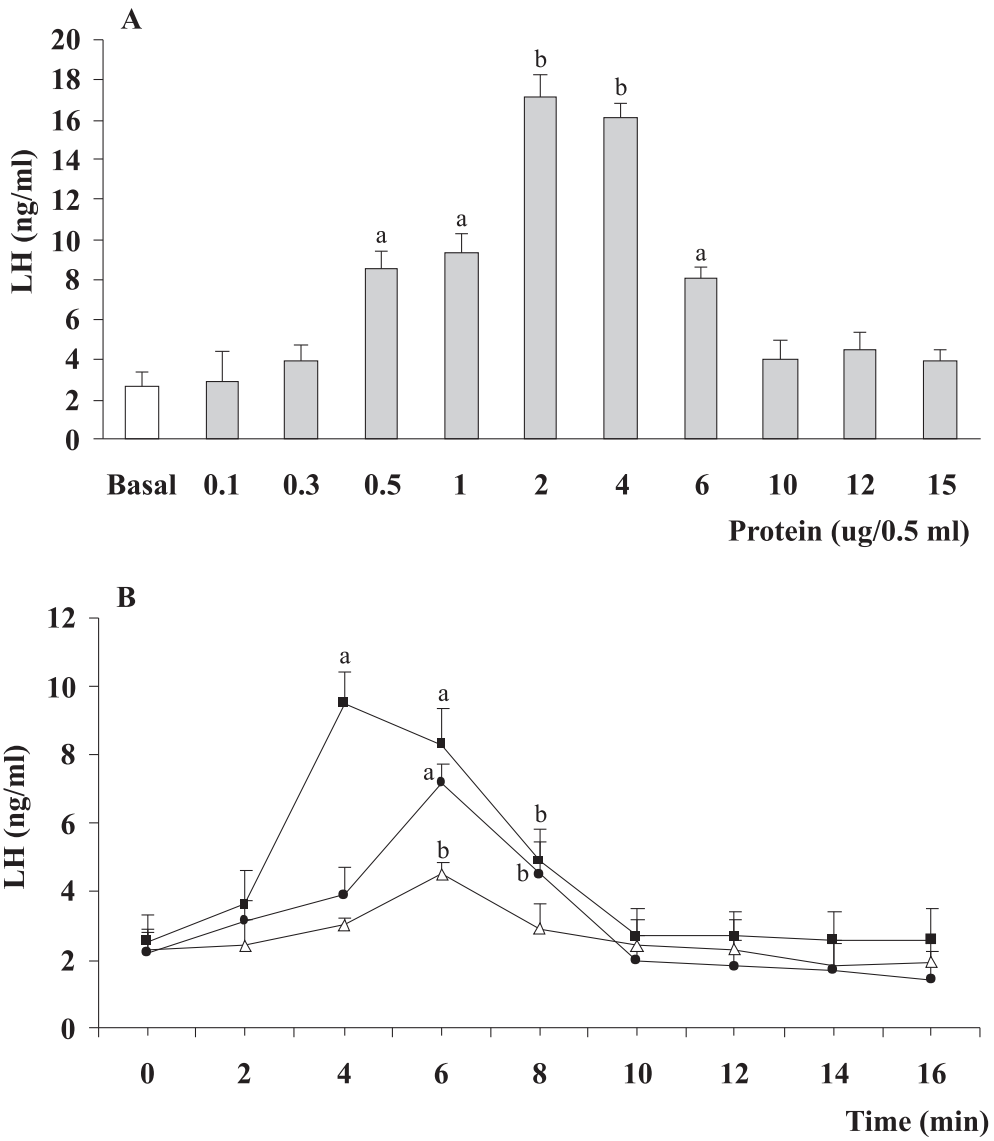

FIGURE 3. LH release by dispersed cells from rat *pars distalis* stimulated with culture medium of *pars tuberalis* cells from 50+60% Percoll fractions. **A**, incubation experiments (dose-dependent effect). Bars represent mean ± SEM of duplicate measurements as indicated in Fig. 1. [a]p< 0.01 and [b]p< 0.001 vs basal value. **B**, superfusion experiments. Stimulation with (- ■ -) 1mL of KCl 28 mM, and with 1 mL of culture medium from *pars tuberalis* cells of 50-60% strenght Percoll containing 3 (- ● -) and 0.3 (- Δ -) μg protein per mL. Statistical differences (mean ± SEM) of 4 different experiments [a]*P* < 0.001 and [b]*P* <0.01 vs basal value.

60% strength Percoll cells fractions. The maximal response of *pars distalis* cells was observed when they were stimulated with 2 and 4 μg of *pars tuberalis* protein in the final volume of incubation (500 μl). This response was not mantained with higher protein concentrations.

Results from the superfusion experiments are shown in figure 3B. After unspecific stimulation with 28 mM KCL (black squares) a significant increase in the release of LH from *pars distalis* cells was induced over baseline values after 4 min. Also a significant response of *pars distalis* cells was observed when they were stimulated with culture medium of *pars tuberalis* cells from 50-60% Percoll fractions, containing either 3 or 0.3 μg of protein per mL (black dots and white triangles, respectively).

Figure 4A shows the results from SDS-PAGE studies. After protein separation by 12% SDS-PAGE of the culture medium obtained from 50-60% Percoll cell fractions, eight bands were visualized. The LH release response obtained with the protein recovered from each band is shown in Fig. 4B. LH release was only detected ($p< 0.001$) when *pars distalis* cells were stimulated with the eluate from the band of molecular size of 66 kDa. In this case a high LH response was obtained with 50 ng of *pars tuberalis* protein in the final volume of incubation (500 μl).

Discussion

Several types of secretory cells have been described in the *pars tuberalis* of the hypophysis. Previously we have shown that culture medium from total bovine cell populations of *pars tuberalis* stimulate the release of prolactin and GH from rat *pars distalis* cells (Lafarque *et al*., 1998; 2004). In this study we report evidence for a factor of 66 kDa secreted by the *pars tuberalis* of bovine pituitary that shows an LH releasing action on rat *pars distalis* cells.

The *pars tuberalis* cellular populations were fractioned over a discotinuous Percoll gradients allowed to identify only two of the cell fractions (the 50 and 60% Percoll fractions) that were capable to induce LH release from *pars distalis* cells. As expected, the maximum LH release induced by secretions from the 50+60% Percoll fractions occurred at a three times lower protein concentration, suggesting that *pars tuberalis* cells in those fractions could be responsible for producing the factor(s) which affect *pars distalis* gonadotrope cells.

Studies in the rat indicate that the mode of hormone synthesis and/or release from LH cells of the *pars tuberalis* is different from those of the *pars distalis* (Gross *et al*., 1984; Watanabe, 1986). The secreting LH capacity of *pars tuberalis* cells appears to be smaller and inconsistent, with low LHRH sensitivity (Watanabe,

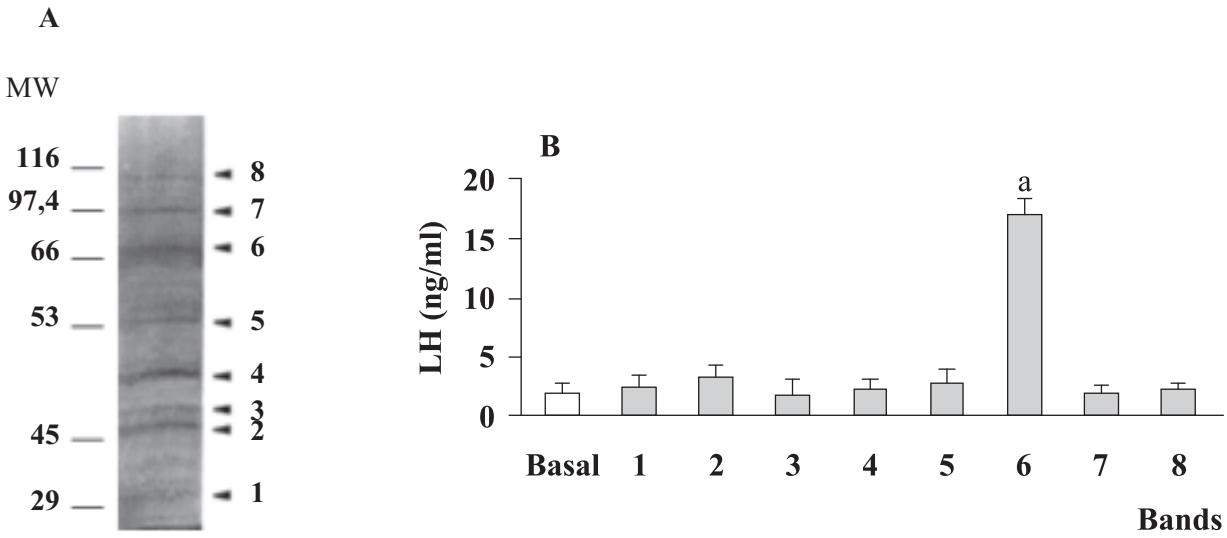

FIGURE 4. A, SDS-PAGE (12%) gel of culture medium from *pars tuberalis* cells of 50-60% Percoll fractions, bands 1 to 8. MW: molecular weight markers. **B**, LH release from dispersed *pars distalis* cells stimulated with the protein (50 ng) eluted from each band from the SDS-PAGE (12%) gel. Bars represent mean ± SEM of duplicate measurements of 4 independent experiments. $^{a}P < 0.001$ band 6 corresponding to 66 kDa vs basal value.

1986). In our case, the measured LH comes from *pars distalis* cells, and not from *pars tuberalis* cells, since LH was not detected in the culture medium of *pars tuberalis* cells using the specific LH antibody for rats.

Also, it is not likely that the measured LH comes from new synthesis in *pars distalis* cells, since in superfusion experiments the LH response was observed at shorter times after stimulation with *pars tuberalis* cell secretions.

In the rat, numerous LHRH-positive fibers have been observed as tiny lines with several varicosities both in the primary vascular plexus and in the hypothalamus corresponding to the posterior half of the portal vein area. LHRH-positive fibers have also been noted around S-100-positive cells in the *pars tuberalis* (Mabuchi *et al*., 2004). In our experimental conditions, the stimulation of *pars distalis* 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 nerve endings which could be present in the *pars tuberalis* dissection. Additionally, the partial purification of *pars tuberalis* cells on Percoll gradients and the successive cell washes to eliminate Percoll solution also contributes to the *pars tuberalis* cultures being exempt of hypothalamic factors.

After *pars distalis* cell stimulation with culture medium of *pars tuberalis* cells from 50+60% Percoll fractions containing 6 μg protein or more in the final volume of *pars distalis* cells incubation, LH release decreased. This fact could be explained by a possible direct inhibitory effect of prolactin on LH secretion. In the same experimental model, we have previously reported that secretions of *pars tuberalis* cells from 50+60% Percoll fractions containing 4 μg protein or more in 500 μl of final incubation volume increase prolactin release from *pars distalis* cells (Lafarque *et al*., 1998). Studies in the rat have shown that *in vitro* treatment with prolactin is able to suppress both basal and LHRH-stimulated LH release from cultured pituitary fragments (Cheung, 1983; Foreman and Porter, 1981) and reduce the percentage of pituitary cells secreting LH *in vitro* (Sortino and Wise, 1989). Similarly, the *in vivo* and *in vitro* LH response to exogenous LHRH administration was shown to be reduced in hyperprolactinemic rats, compared with controls (Smith, 1982).

Culture medium from *pars tuberalis* cells did not induce FSH release from *pars distalis* cells. The intracellular mechanism for the differential LH and FSH release after stimulation with *pars tuberalis* secretions might be explained by the fact that gonadotropins are stored and secreted from different secretory granules within bihormonal cells (Thomas and Clarke, 1997; Farnworth, 1995).

Our results suggest a possible modulating effect of *pars tuberalis* on *pars distalis* gonadotrope cells. Although the exact nature of the factor involved in LH release is not known yet, in this study we have found the biological activity on the basis of a *pars tuberalis* protein of molecular size of 66-kDa.

Future studies will be carried out to identify the structure of these *pars tuberalis* proteins and also to determine the signalling pathway involved in LH release from *pars distalis* cells. It has been shown that a factor(s) released from ovine *pars tuberalis* cells increases c-fos expression in some, but not all, lactotrophs in *pars distalis* cell cultures (Morgan *et al*., 1996). This study reveals the potential influence of the *pars tuberalis* on a second endocrine axis and also extends our knowledge of pituitary regulation.

Acknowledgments

This work was supported by grants Proyect 9302 and PROIPRO 20306 from San Luis University, Argentina.

References

Bradford M (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72: 248-254.

Bock N, Böckers TM, Bockmann J, Nowak P, Buse E, Wittkowski W (2001). The pars tuberalis of the monkey (*Macaca fascicularis*) hypophysis: Cell types and hormone expression. Cells Tissues Organs. 169: 55-63.

Cheung CY (1983). Prolactin suppresses luteinizing hormone secretion and pituitary responsiveness to luteinizing hormone-releasing hormone by a direct action at the anterior pituitary. Endocrinology. 113: 632-638.

Dellmann HD, Stoeckel ME, Hindelang-Gertner C, Porte A, Stutinsky F (1974). A comparative ultrastructural study of the pars tuberalis of various mammals, the chicken and the newt. Cell Tissue Res. 148: 313-329.

Farnworth PG (1995). Gonadotrophin secretion revisited. How many ways can FSH leave a gonadotroph? J Endocrinol. 145: 387-395.

Fitzgerald KT (1979). The structure and function of the pars tuberalis of the vertebrate adenohypophysis. Gen Comp Endocrinol. 37: 383-399.

Foreman MM, Porter JC (1981). Prolactin augmentation of dopamine and norepinephrine release from superfused medial basal hypothalamic fragments. Endocrinology. 108: 800-804.

Gross DS, Turgeon JL, Waring DW (1984). The ovine pars tuberalis: a naturally occurring source of partially purified gonadotropes which secrete luteinizing hormone *in vitro*. Endocrinology 114: 2084-2091.

Guerra M, Rodriguez EM (2001). Identification, cellular and subcellular distribution of 21 and 72 kDa proteins (tuberalins?) secreted by specific cells of the pars tuberalis. J Endocrinol. 168: 363-379.

Hazlerigg DG, Hastings MH, Morgan PJ (1996). Production of a prolactin releasing factor by the ovine pars tuberalis. Neuroendocrinol. 8: 489-492.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature. 227: 680-685.

Lafarque MM, Ezquer M, Aguado LI, Oliveros LB (2004). Bovine pars tuberalis secretions release growth hormone from rat pars distalis of pituitary gland. Neuroendocrinol Lett. 25: 273-277.

Lafarque MM, Oliveros LB, Aguado LI (1998). Efecto de secreciones de *pars tuberalis* de la adenohipófisis sobre la liberación de prolactina desde pars distalis. Medicina (Buenos Aires) 58: 36-40.

Mabuchi Y, Shirasawa N, Sakuma E, Hashimoto Y, Kuno M, Coombs RJ, Herbert DC, Soji T (2004). Intercellular communication within the rat anterior pituitary: relationship between LH-RH neurons and folliculo-stellate cells in the pars tuberalis. Cell Tissue Res. 317: 79-90.

Morgan PJ, Webster CA, Mercer JG, Ross AW, Hazlerigg DG, MacLean A, Barrett P (1996). The ovine pars tuberalis secretes a factor(s) that regulates gene expression in both lactotropic and nonlactotropic pituitary cells. Endocrinology 137: 4018-4026.

Nakazawa K, Marubayashi U, McCann SM (1991). Mediation of the short-loop negative feedback of luteinizing hormone (LH) on LH-releasing hormone by melatonin-induced inhibition of LH release from the *pars tuberalis*. Proc Natl Acad Sci USA. 88: 7576-7579.

Skinner DC, Herbison AE, Robinson JE (1992). Immunocytochemical identification of oestrogen receptors in the ovine *pars tuberalis*: Localization within gonadotrophs. J Neuroendocrinol. 4: 659-662.

Skinner DC, Robinson JE (1996). The *pars tuberalis* of the ewe: no effect of season or ovariectomy on the distribution, density or presence of immunoreactive cells. Cell Tissue Res. 284: 117-123.

Smith MS (1982). Effect of pulsatile gonadotropin-releasing hormone on the release of luteinizing hormone and follicle-stimulating hormone *in vitro* by anterior pituitaries from lactating and cycling rats. Endocrinology. 110: 882-891.

Spinedi E, Johnston C, Negro-Vilar A (1984). Increased responsiveness of the hypothalamic-pituitary axis after neurotoxin-induced hypothalamic denervation. Endocrinology 115: 267-272.

Sortino MA, Wise PM (1989). Effect of hyperprolactinemia on luteinizing hormone and prolactin secretion assessed using the reverse hemolytic plaque assay. Biol Reprod. 41: 618-625.

Thomas SG, Clarke IJ (1997). The positive feedback effects of estrogen mobilizes LH-containing but not FSH-containing secretory granules in ovine gonadotropes. Endocrinology 138: 1347-1350.

Watanabe YG (1986). A comparative *in vitro* study on LHRH responsiveness of LH cells of the *pars tuberalis* and pars distalis. Cell Tissue Res. 245: 369-375.

Wittkowski W, Bockmann J, Kreutz MR, Böckers TM (1999). Cell and molecular biology of the *pars tuberalis* of the pituitary. Int Rev Cytol. 185: 157-194.