Effects of activin on hormone secretion by single female rat pituitary cells: analysis by cell immunoblot assay

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Abstract

We investigated the effect of activin A on secretion of LH, FSH, and prolactin (PRL) by female cultured rat pituitary cells at the single-cell level by means of the cell immunoblot assay. Anterior pituitary cells from 8-week-old female rats were preincubated with or without activin A for 24 h, after which they were monodispersed and immediately used for cell immunoblot assay.

The percentages of LH-, FSH- and PRL-immunoreactive cell blots detected were 5.5, 5.3 and 43.1%, respectively, of all pituitary cells applied to the transfer membrane. The percentage of LH-secreting cells and mean LH secretion per cell did not change after treatment with activin. In contrast, activin significantly

increased the percentage of FSH-secreting cells and mean FSH secretion per cell to 136.0 and 114.5% respectively. In addition, activin significantly decreased the percentage of PRL-secreting cells and mean PRL secretion per cell to 52.2 and 72.0% respectively.

These results suggest that (1) activin A has effects on female rat pituitary cells that increase not only the amount of FSH secretion per cell but also the number of FSH-secreting cells, and (2) activin A decreases both the amount of PRL secretion per cell and the number of PRL-secreting cells.

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Introduction

Originally isolated from the gonads, activin has been detected in the rat anterior pituitary (Meunier *et al.* 1988, Roberts *et al.* 1989, Bilezikjian *et al.* 1993, Halvorson *et al.* 1994, Liu *et al.* 1996), where it may play an autocrine/paracrine role in the regulation of anterior pituitary hormones (Corrigan *et al.* 1991, Besecke *et al.* 1996). Recent studies have demonstrated that it has specific stimulatory effects on follicle-stimulating hormone (FSH) gene expression in the pituitary and FSH secretion in culture but has no effect with regard to luteinizing hormone (LH) (Vale *et al.* 1986, Ling *et al.* 1986, Kitaoka *et al.* 1987, Ying 1988). Attardi & Miklos (1990) used static plated cultures and reported increased FSH secretion in activin-treated male rat pituitary. Katayama *et al.* (1990), on the other hand, in an immunocytochemical study showed that treatment with activin A increases the number of immunoreactive FSH-containing cells in male rat pituitary cell cultures without affecting the proportion of immunoreactive LH-containing cells. Activin has also been shown to inhibit secretion of prolactin (PRL), growth hormone (Kitaoka *et al.* 1988, Murata & Ying 1991) and adrenocorticotrophic hormone (Bilezikjian *et al.* 1991) in culture.

Recent improvements in techniques, such as the cell immunoblot assay (CIBA) (Kendall & Hymer 1987, Arita *et al.* 1991, Arita 1994) and the reverse hemolytic plaque assay (Neill & Frawley 1983), have made it possible to quantify the amount of hormones secreted by individual endocrine cells and to estimate the relative abundance of hormone-secreting cells. The results of such analyses at the single-cell level have suggested that endocrine cells that secrete a specific hormone are functionally heterogeneous with respect to both basal and secretagogue-induced secretion.

The objectives of the present study were to investigate how activin A changes the secretions of FSH and PRL in cultured pituitary cells. For this purpose, we analyzed the effects of activin A on LH, FSH, and PRL secretion by cultured female rat pituitary cells at the single-cell level by using the chamber CIBA technique.

Materials and Methods

Reagents

Recombinant human activin A was a gift from the Central Research Laboratory of Ajinomoto Co. (Kawasaki, Japan)

(Eto *et al.* 1987). Dulbecco's modified Eagle's medium (DMEM) with Hepes buffer (DMEMH) and PBS with Hepes buffer (PBSH) were prepared from powdered DMEM (Nissui, Tokyo, Japan), powdered Dulbecco's PBS (Nissui), NaHCO_3 (1.8 g/liter), penicillin G (100 U/ml), streptomycin sulfate (100 µg/ml) and Hepes (20 mM). Fetal calf serum and horse serum were purchased from Filtron Pty Ltd (Brooklyn, Victoria, Australia) and heat-inactivated before use. The rat LH, FSH and PRL (rat LH RP-3, rat FSH RP-2 and rat PRL RP-3 respectively) used as the standards in the CIBA were generously provided by the NIADDK. Rabbit antiserum to rat LH (A536/R4H) and rabbit antiserum to rat PRL (i539/002) were purchased from UCB-bioproducts S A (Braine-Allaud, Belgium). Rabbit antiserum to rat FSH (anti-rat FSH-IC-1) was obtained through the National Hormone and Pituitary Program of the NIDDK (Bethesda, MD, USA). Biotinylated donkey anti-rabbit immunoglobulin antibody and streptavidin-alkaline phosphatase conjugate were purchased from Amersham (Arlington Heights, IL, USA). Trypsin 1:250 and pancreatin were purchased from Sigma (St Louis, MO, USA). BSA (fraction V) and the chemicals used were obtained from Wako Pure Chemical Industries Co. Ltd (Osaka, Japan) unless otherwise specified.

Rat female pituitary cell cultures

All animal experimentation was conducted in accordance with the ethical standards of the institutional animal care and use committee of the University of Tokushima.

Eight-week-old female Wistar rats (Charles River Japan, Inc., Yokohama, Japan) at random stages of the estrous cycle were used. Dispersed anterior pituitary cells were prepared by trypsin digestion as described previously (Yokoyama *et al.* 1995), with some modifications. Anterior pituitaries were removed after decapitation and minced into small fragments. The fragments were incubated with shaking at 37°C for 40 min in freshly prepared solution containing 0.25% (w/v) trypsin 1:250 in 20 ml 0.3% BSA/PBSH, then mechanically dispersed with a Pasteur pipette and passed through a 100 µm metal mesh until monodispersed cells were obtained. After incubation in solutions containing 0.1% pancreatin for 1 min in 10 ml 0.3% BSA/PBSH, the monodispersed cells were washed three times in DMEM with 2.5% fetal calf serum and 10% horse serum (DMEMS).

Cell yield per pituitary was $(2.0\text{--}2.5) \times 10^6$ cells, and cell viability, as judged by trypan blue exclusion, was more than 95%. Pituitary cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 /95% air with fresh DMEMS with or without 10 nM activin A at a density of 1.5×10^6 cells/35 mm well. After 24 h, the culture medium was removed and the cells were completely monodispersed with 0.01% trypsin 1:250 and washed twice with

DMEMH. Cell yield per well was $(1.2\text{--}1.4) \times 10^6$ cells, and viability was more than 95%.

CIBA

CIBA was performed by the method described previously (Arita 1994). In brief, pieces (50×25 mm) of polyvinylidene difluoride transfer membrane (Immobilon; Millipore, Bedford, MA, USA) were prewetted in methanol for 20 s, rinsed in distilled water for 5 min, and equilibrated with DMEMH for 60 min before use in cell blotting. The prewetted transfer membrane was placed on a glass microscope slide with the water-repellent side up to form the floor of the chamber. Two small glass coverslips (18×24 mm, thickness 0.14 mm) were placed 20 mm apart on the transfer membrane, and a large glass coverslip (60×24 mm) was then lowered on to the small glass coverslips to form the roof of the chamber. The incubation chamber had an area of 360 mm², a height of 0.15 mm, and an average volume of 54 µl. Then, 100 µl of monodispersed pituitary cell suspension was infused into the chamber. Cell density was 1.5×10^6 cells/ml in the CIBA for LH and FSH, and 5×10^3 cells/ml in the CIBA for PRL. The chambers were incubated at 37°C in a humidified atmosphere of 5% CO_2 /95% air for 0.5 to 6 h. Various amounts (78.125–2500 pg) of rat LH, FSH or PRL in 250 nl 20 mM Tris-buffered saline, pH 7.6 (TBS), supplemented with 0.8% Tween 20, were applied as the standard on dry transfer membranes. After incubation for 30 min, the transfer membranes for the standards were immersed and rocked in 100% methanol for 10 s, washed in distilled water and DMEMH for 2 and 10 min respectively, and then processed in exactly the same way as those for cell blots.

Transfer membranes incubated with pituitary cells or to which standards had been applied were immersed in TBS containing 10% BSA for 2 h to block any unoccupied binding sites. After the blocking procedure, the anterior pituitary hormones bound to the transfer membranes were immunostained specifically with the following solutions at room temperature with shaking: (1) 10% normal donkey serum (Chemicon, Temecula, CA, USA) in 50 mM Tris-HCl, pH 7.6, containing 500 mM NaCl, 0.5% Tween 20, 1% BSA and 0.05% NaN_3 (hTBS-T) for 30 min; (2) primary antiserum against the anterior pituitary hormone in hTBS-T containing 10% normal donkey serum for 12 h (rabbit antisera to rat LH and PRL at a dilution of 1:10 000, and rabbit antiserum to rat FSH at a dilution of 1:2000); (3) biotinylated donkey anti-rabbit immunoglobulin in hTBS-T containing 10% normal donkey serum at a dilution of 1:500 for 40 min; (4) streptavidin-alkaline phosphatase conjugate at a dilution of 1:3000 in hTBS-T for 40 min; and (5) the enzyme substrate nitroblue tetrazolium (0.4 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.2 mg/ml; BRL,

Gaithersburg, MD, USA) in 0.1 M Tris-HCl (pH 9.5), containing 0.1 M NaCl and 50 mM MgCl₂ for 3–4 min. During each interval between treatments, the transfer membranes were rinsed several times in TBS containing 0.1% Tween 20 for 30–60 min. After the color reaction, the transfer membranes were rinsed with distilled water for 15 min and allowed to dry in air.

These cell blots were demonstrated to be specific because (1) omission of the primary or secondary antiserum and replacement of the primary antiserum with normal rabbit serum of the same dilution produced no cell blots, and (2) preabsorption of the primary antiserum with 10 ng/ml rat LH, FSH or PRL for 48 h before use in the CIBA blocked formation of the cell blots.

Image analysis

The immunostained transfer membranes were photographed with a stereo microscope (SZ6045TR; Olympus, Tokyo, Japan) equipped with 35 mm photomicrography and a fiber-optic ring light system, and the amounts of anterior pituitary hormones secreted by single cells were quantified with a Macintosh computer (Apple Japan, Tokyo, Japan) using the public domain NIH Image program (NIH Image 1.62) by the following processes.

(1) Stained transfer membranes were illuminated with constant light, magnified with a stereo microscope, and then photographed with 35 mm films (Fujichrome 100; Fujifilm, Tokyo, Japan).

(2) The photomicrographs obtained were digitized with a 35 mm film scanner (QuickScan 35; Minolta, Tokyo, Japan) and saved as an array (1920 × 1266 pixels) of gray levels in TIFF format. The mean area of the LH and FSH blots was smaller than that of the PRL blots at the same magnification, so they were scanned at double magnifying power. For this reason, a pixel in the saved images corresponded to 2.69 µm in actual distance in the LH and FSH analysis, and 5.38 µm in the PRL analysis.

(3) After shading error correction, the gray values of cell blots based on the optical density of the saved images were converted to values based on the amount of each anterior pituitary hormone per pixel by referring to the standards.

(4) After the gray level of the background field neighboring the cell blot to be measured had been computed, the threshold value for the binary image was calculated by adding the value that corresponded to 2 fg/pixel (usually 20–25) to the average gray value of the background field.

(5) Using the binary image, the area of cell blots was determined by computing the number of pixels above the threshold in each cell blot (usually 50–2000 pixels/cell blot).

(6) The amount of hormone secreted by single cells was calculated by multiplying the area of cell blots by the difference between the average gray value of the cell blots and the threshold value.

Statistical analysis

The number of hormone-secreting cells in a 5.2 × 3.4 mm area of the incubation chamber in the LH and FSH analysis, and in a 10.3 × 6.8 mm area in the PRL analysis, and the mean amounts of hormone secretion per cell were determined from five transfer membranes in each experiment, and the mean and s.e. values were calculated from the values in five independent experiments. Differences were statistically analyzed by ANOVA. In addition, $P < 0.05$ was considered to be statistically significant.

Results

Photomicrograph and standard CIBA curves

Figure 1 shows a photomicrograph of rat anterior pituitary hormone-immunoreactive cell blots in a CIBA chamber with (Fig. 1, A-2, B-2 and C-2) and without (Fig. 1, A-1, B-1 and C-1) activin A. Activin A was effective in increasing FSH immunoreactivity (Fig. 1, B-1 and B-2) and in decreasing PRL immunoreactivity (Fig. 1, C-1 and C-2), but its presence did not alter LH immunoreactivity (Fig. 1, A-1 and A-2). The standard curves used in the CIBA for LH, FSH and PRL are shown in Fig. 2. Conversion of the gray level for the optical density of cell blots to the gray levels for the amount of LH, FSH or PRL by referring to the respective standard curves made it possible to quantify the amount of hormone secreted by single cells.

Time dependence of the number of hormone-secreting cells and amount of secretion by individual cells

The number of hormone-secreting cells, defined as the number of all immunoreactive cell blots detected in a 5.2 × 3.4 mm area of the incubation chamber in the analysis for LH and FSH and in a 10.3 × 6.8 mm area in the analysis for PRL, increased with incubation time and plateaued at between 1.5 and 6 h (Fig. 3). Accordingly, an incubation time of 3 h was chosen for use in the present study. The number of LH-, FSH- and PRL-immunoreactive cell blots detected after 3 h incubation accounted for 5.5 ± 0.3 , 5.3 ± 0.6 and $43.1 \pm 4.4\%$ respectively of the total number of pituitary cells applied to the transfer membrane.

Mean LH secretion and mean FSH secretion by individual cells increased with incubation time, with plateaus between 1.5 and 6 h, while mean PRL secretion increased in a time-dependent manner up to 6 h (Fig. 3).

Effect of activin on frequency distribution of hormone-secreting cells

The frequency distribution of LH, FSH or PRL secretion by single cells during a 3 h incubation was compared

Figure 1 Photomicrographs of representative anterior pituitary hormone-immunoreactive cell blots. (A) LH, (B) FSH, (C) PRL, (1) no treatment and (2) treatment with 10 nM activin A. Bar=500 μ m.

between control and activin-pretreated cells (Fig. 4). The amount of LH secretion by single cells varied from 0.03 to 31.37 pg/cell per 3 h in the control group. More than 90% of the total LH-secreting cells secreted less than 1 pg/3 h. No significant changes in LH secretion by individual cells occurred in response to the addition of activin, as shown in a representative sample in Fig. 1, A-2.

On the other hand, while the amount of FSH secretion by single cells varied from 0.01 to 25.45 pg/cell per 3 h in the control group, activin administration increased the number of FSH blots, as shown in a representative sample in Fig. 1, B-2.

By contrast, the amount of PRL secretion by single cells varied from 0.03 to 16.34 pg/cell per 3 h in the control

Figure 2 A standard curve used in the CIBA for LH, FSH and PRL. Various amounts of rat LH (NIADDK RP-3), FSH (NIADDK RP-2) or PRL (NIADDK RP-3) in 250 nl TBS-T were applied to transfer membranes and immunostained by standard immunocytochemical procedures. The gray level on the ordinate was obtained by subtraction of the actual gray level of the standards from the gray level of the background. As the area of LH-immunoreactive blots produced by applying the standard solution was approximately $8\,670\,000\ \mu\text{m}^2$ on average, an LH amount of 2500 pg/250 nl solution on the abscissa, for example, corresponded to $288.3\ \text{ag}/\mu\text{m}^2$ of blots.

group, and addition of activin A caused marked suppression of PRL secretion by individual cells, as shown in a representative sample in Fig. 1, C-2.

Figure 3 LH, FSH and PRL secretion from individual anterior pituitary cells as a function of incubation time. The number of all LH-, FSH- or PRL-immunoreactive blots that were detected in an area of $5.2 \times 3.4\ \text{mm}$ (LH and FSH) or $10.3 \times 6.8\ \text{mm}$ (PRL) of incubation chamber ($\square$) and the mean amount of LH, FSH or PRL secreted from single pituitary cells expressed as pg/cell ($\bullet$) were determined after 0.5, 1, 1.5, 3 and 6 h incubation. Values are the mean $\pm$ S.E. of five transfer membranes.

Effect of activin on number of hormone-secreting cells, mean hormone secretion and total hormone secretion

The number of hormone-secreting cells, mean hormone secretion by single cells and total hormone secretion, calculated by multiplying the number of hormone-secreting cells by the mean hormone secretion by single cells during a 3 h incubation, were compared between control and activin-pretreated cells (Fig. 5). In the control group, the number of LH-secreting cells was almost identical with the number of FSH-secreting cells (216.0 ± 11.7 vs 208.6 ± 2.5 , $P=0.63$) (Fig. 5, top). When activin was added, however, the number of LH-secreting

Figure 4 Frequency distribution of LH, FSH and PRL secretion from single pituitary cells. Cells were maintained with either medium alone (□) or medium containing 10 nM activin (■) for 24 h, and then used for CIBA. The range of each class is 0.5 pg/cell.

cells (216.0 ± 11.7 vs 220.0 ± 16.7 , $P=0.76$), mean LH secretion per cell (0.62 ± 0.07 vs 0.68 ± 0.08 pg, $P=0.56$) and total LH secretion (134.6 ± 13.0 vs 148.5 ± 13.4 pg, $P=0.45$) did not change significantly (Fig. 5).

In contrast, administration of activin A resulted in a significant increase in the number of FSH-secreting cells, to 136.0% (208.6 ± 2.5 vs 283.6 ± 8.7 , $P<0.01$) (Fig. 5, top), and mean FSH secretion and total FSH secretion significantly increased, to 114.5% (2.14 ± 0.09 vs 2.45 ± 0.09 pg, $P<0.02$) and 155.6% (446.1 ± 9.5 vs 694.0 ± 17.9 pg, $P<0.01$) respectively.

On the other hand, administration of activin A resulted in a significant decrease in the number of PRL-secreting cells, to 52.2% (273.3 ± 27.3 vs 142.7 ± 12.5 , $P<0.01$) (Fig. 5, top), and mean PRL secretion and total PRL secretion decreased significantly, to 72.0% (2.88 ± 0.07 vs 2.07 ± 0.06 pg, $P<0.01$) and 37.6% (785.8 ± 107.1 vs 295.4 ± 43.1 pg, $P<0.01$) respectively.

Figure 5 Effects of activin treatment on hormone secretion by individual anterior pituitary cells. Cells were maintained with either medium alone (□) or medium containing 10 nM activin (■) for 24 h, and then used for CIBA. The top panel shows the number of all LH-, FSH- or PRL-immunoreactive blots that were detected in an area of 5.2×3.4 mm (LH and FSH) or 10.3×6.8 mm (PRL) of incubation chamber; the middle panel shows the mean amount of LH, FSH or PRL secreted from single pituitary cells expressed as pg/cell; the bottom panel shows the total amount of LH, FSH or PRL secretion obtained by multiplying the number of hormone-secreting cells by the mean hormone secretion. Values are mean $\pm$ S.E. for five transfer membranes.

Discussion

CIBA using an incubation chamber makes it possible not only to quantify hormone secreted by single endocrine cells accurately but to determine simultaneously the percentage of hormone-secreting cells by counting immunoreactive cell blots detected in a given area of transfer membrane after incubation with cell suspension of a known cell density. Because of these advantages of chamber CIBA, we were able to investigate whether the increased FSH secretion and decreased PRL secretion in response to activin treatment *in vitro* were due to changes in the number of hormone-secreting cells and/or changes in the amount of hormone secreted per cell.

Approximately 5–6% of all pituitary cells were found to be LH- and FSH-secreting cells in the present study. Smith *et al.* (1984) have demonstrated that approximately 5–6% of all pituitary cells immunostained as LH-containing cells, but that only some of these cells actually

secrete LH, while Noguchi *et al.* (1996) reported finding that approximately 4–5% of all pituitary cells are FSH-secreting cells when CIBA was used. As LH and FSH are known to be secreted by the same cells under basal conditions, the present results are consistent with the findings in these earlier studies.

The present study has shown that LH and FSH are secreted quite heterogeneously by individual cells. The amount of LH secreted by single cells under basal conditions differs by approximately 1000-fold, and the corresponding amount of FSH differs by no less than 2000-fold. The finding that gonadotrophs are heterogeneous with respect to their FSH-secreting properties is consistent with the findings of Noguchi *et al.* (1996).

Using the CIBA for LH and FSH, the present study demonstrated that activin has the dual effect of increasing not only the percentage of gonadotrophs that actually secrete FSH but also the mean FSH secretion per cell, but that activin has no effect on the percentage of gonadotrophs that secrete LH or on mean LH secretion per cell, confirming a stimulatory action of activin on FSH secretion, with no effect on LH secretion *in vitro* (Vale *et al.* 1986, Ling *et al.* 1986, Kitaoka *et al.* 1987, Ying 1988).

Administration of activin A significantly increased the number of FSH-secreting cells, to 136.0%, but had no effect on the number of LH-secreting cells. This finding may mean that activin increases the proportion of FSH-secreting monohormonal cells without affecting the proportion of LH-FSH-secreting bihormonal cells. An immunocytochemical study by Katayama *et al.* (1990) showed that treatment with activin A (10 ng/ml) for 72 h increased the number of immunoreactive FSH-containing cells to 125–141% in male rat pituitary cell culture, but did not affect the proportion of immunoreactive LH-containing cells. As the fact that a cell contains a particular hormone does not necessarily mean that it actually secretes it (Smith *et al.* 1984, Arita *et al.* 1993), our findings suggest that activin increases the number of FSH-secreting cells as well as the number of FSH-containing cells.

On the other hand, the use of CIBA for PRL in this study showed that activin decreases both the percentage of lactotrophs that actually secrete PRL and mean PRL secretion by individual cells, confirming the inhibitory action of activin on PRL secretion *in vitro* (Kitaoka *et al.* 1988, Murata & Ying 1991).

Approximately 43% of all pituitary cells were demonstrated to be PRL-secreting cells in the present study. Boockfor & Frawley (1987) demonstrated, using reverse hemolytic plaque assay, that approximately 40–50% of all pituitary cells are PRL-secreting cells, and Arita *et al.* (1991) demonstrated, using CIBA, that 55–66% of all pituitary cells are PRL-secreting cells. One possible explanation for this difference in percentage of PRL-secreting cells may be the difference in time after trypsin dispersion. Arita *et al.* preincubated the pituitary cells for

8–9 days before using CIBA, whereas we preincubated them for 24 h.

The present study has shown that PRL is secreted quite heterogeneously by individual cells. The amount of PRL secreted by individual cells differs by approximately 600-fold. The finding that lactotrophs are heterogeneous with respect to their PRL secretory properties is consistent with the findings of Arita *et al.* (1991).

Administration of activin A significantly decreased both the number of PRL-secreting cells and mean PRL secretion per cell, to 52.2 and 72.0% respectively in the present study. It has been reported that activin A reduces thyrotropin-releasing hormone-mediated (Kitaoka *et al.* 1988) and basal (Murata & Ying 1991) PRL secretion in rat pituitary cell cultures. These reports, however, stated that activin A did not alter intracellular PRL content (Kitaoka *et al.* 1988) or that it increased it (Murata & Ying 1991), and the immunocytochemical study by Katayama *et al.* (1990) suggested that activin A did not change the percentage of immunoreactive PRL-containing cells. This discrepancy between the effects of activin A on PRL-containing cells and PRL-secreting cells may suggest that activin regulates not only the production of PRL by pituitary cells but also its release.

In conclusion, our results suggest that (1) activin A has effects on female rat pituitary cells that increase not only the amount of FSH secretion per cell, but also the number of FSH-secreting cells, and (2) activin A also decreases both the amount of PRL secretion per cell and the number of PRL-secreting cells.

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