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Regulation of Immunoreactive Activin A Secretion from Cultured Rat Anterior Pituitary Cells

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Abstract. Immunoreactive activin A (ir-activin A) release from cultured rat anterior pituitary cells was examined by measuring ir-activin A in culture medium by a specific radioimmunoassay. Ir-activin A release into the medium increased over 1–18 days, and reached a maximal level at 12–15 days. The basal levels of ir-activin A in the culture media were 0.70 ± 0.10 (mean ± SD), 1.30 ± 0.36 and 1.83 ± 0.44 ng/10^6 cells, when cultured for 6 days with 0, 2 and 10% fetal calf serum, respectively. LHRH induced an approximate 1.4-fold increase in ir-activin A release in contrast to a 40–60% inhibition with FSH, but LH did not affect the activin A release. In the presence of 12-o-tetradecanoylphorbol acetate (TPA), ir-activin A release was enhanced, but no significant effect was induced by forskolin. Activin A was distinctly immunostained in cultured rat anterior pituitary cells. These results suggested that activin A release from the pituitary is modified by FSH and LHRH, and that the activation of protein kinase C may be involved in the action of LHRH.

Key words: Activin, Radioimmunoassay, Follicle-stimulating hormone, Luteinizing hormone-releasing hormone, Anterior pituitary cells, Protein kinase C

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ACTIVIN, a homodimer or heterodimer of the βA and βB subunits of inhibin, has various functions in gonadal and extragonadal tissues, and it is a member of the transforming growth factor-β gene superfamily [1]. Activins and inhibins exhibit opposite effects on follicle-stimulating hormone (FSH) release from the pituitary gland despite their related structure [1–4]. Besides stimulating FSH secretion, activin inhibits the secretion of growth hormone (GH), prolactin and adrenocorticotropic hormone (ACTH) [5–8], and stimulates the secretion of follistatin, an activin-binding protein, from cultured rat anterior pituitary cells [9]. Furthermore, activin modifies the secretion of inhibin and sex steroids from cultured rat granulosa cells [10, 11], and enhances the meiotic maturation of rat oocytes and spermatogonial proliferation [12, 13]. These observations suggest that activin plays important roles in the regulation of the pituitary-gonadal axis.

Anterior pituitary cells are controlled by various autocrine/paracrine factors produced locally, including activin, inhibin and follistatin [9, 14–16]. Activin βA-subunit mRNA and βA-subunit immunoreactivity have also been detected in rat anterior pituitary cells [15, 17], but it still remains unknown whether and how anterior pituitary cells secrete mature activin A. We investigated the regulatory mechanism of immunoreactive (ir-) activin A secretion from anterior pituitary cells by a specific radioimmunoassay (RIA) for activin A.

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Materials and Methods

Activin A and other proteins

Recombinant human (rh-) activin A was provided by Dr. Y. Eto (Ajinomoto Central Research Laboratories, Kawasaki, Japan). Porcine activin AB and activin B were gifts from Professor H. Sugino (Institute for Enzyme Research, The University of Tokushima, Japan). Bovine inhibin was provided by Professor Y. Hasegawa (Kitasato University School of Veterinary Medicine and Animal Sciences, Towada, Japan). Rat follicle-stimulating hormone (rFSH) and luteinizing hormone (rLH) were provided by NIDDK (USA). Synthetic luteinizing hormone-releasing hormone (LHRH) was purchased from Tanabe Pharmaceutical Co. (Tokyo, Japan).

Pituitary cell culture

The secretion of ir-activin A was assessed with dispersed rat anterior pituitary cells. For each experiment, 40 female Wistar rats at random cycling were decapitated and the anterior pituitary glands were removed quickly. The pituitary cells were then dispersed with collagenase/DNase and cultured according to the documented procedures [19, 20]. The cells were allowed to recover for 72 h at a concentration of 1×10^6 cells/m*l*/well in HEPES-buffered Dulbecco's Modified Eagle's Medium (DMEM, GIBCO Laboratories, NY, USA) supplemented with 10% fetal calf serum (FCS, GIBCO Laboratories, NY, USA). The cells were cultured in fresh DMEM containing 2% FCS for a further 72 h, rinsed twice with FCS-free DMEM and incubated in 2% FCS-DMEM in the presence or absence of rLH, rFSH, LHRH, 12-o-tetradecanoylphorbol acetate (TPA) or forskolin, respectively. The culture media were collected according to the protocol of each experiment and stored frozen at −20 °C until ir-activin A was assayed. The pecentage of viable cells estimated by trypan blue dye exclusion was about 90% at the beginning of the experiment.

Radioimmunoassay of activin A

The ir-activin A concentration in the culture medium was measured by means of an RIA after extraction as reported [21, 22]. In brief, collected culture media were precipitated by adding 3.35 volumes of 99.3% acetonitrile/0.7% trifluoroacetic acid followed by vigorous mixing for 30 min. The supernatant was harvested after centrifugation at 2,000 × g for 30 min at 4 °C and chilled to −20 °C for 40 min. The lower portions of the separated layers were collected, evaporated and lyophilized. The lyophilized fraction was reconstituted with RIA buffer before assay. The recovery rate of rh-activin A was 82–107%.

The ir-activin A level was determined by a specific RIA that we developed [23]. The lowest detectable concentration in this assay was 0.24 ng/m*l*, and within- and between-assay coefficients of variation were 3.7 and 6.7%, respectively. The antibody used in the assay crossreacted slightly with bovine inhibin (3.3%) and porcine activin AB (9.5%), but not with porcine activin B, LHRH, rFSH, rLH or rGH (<0.1%).

Immunohistochemistry

Rat anterior pituitary cells were dispersed and allowed to grow on coverslips for 7 days. The adherent cells were washed with 0.01 M phosphate-buffered saline (PBS), pH 7.4, and fixed for 30 min with methanol. After incubation for 30 min in 0.3% H_2O_2 to quench endogenous peroxidase activity, the coverslips were blocked in 1% Block Ace (Dainippon Pharmaceutical Co., Tokyo, Japan) for 30 min at room temperature. The cells were then incubated with anti-activin A rabbit antiserum (1:200) in 0.01 M PBS, pH 7.4, containing 0.2% normal goat serum for 60 min at room temperature, and stained by the conventional avidin-biotin-peroxidase (ABC) method with the complete diaminobenzidine terahydro-chloride (DAB) substrate kit (Sigma, MO, USA) as a chromogen. The immunostained cells were observed under the light microscope. Non-immune rabbit serum and immune serum preabsorbed with 5 μg/m*l* rh-activin A were used for control studies.

Statistical analysis

All results are expressed as the mean ± SD of triplicates. Statistical analyses were performed by means of Student's *t* test.

Results

Basal secretion of ir-activin A

The ir-activin A concentration in the culture medium was measured 1, 3, 6, 9, 12, 15 and 18 days after rat anterior pituitary cells (1×10^6 cells/well) had been incubated for 6 days. The time course of ir-activin A accumulation in the culture medium is shown in Fig. 1. The ir-activin A level increased gradually with the period in culture and reached a maximal level of 3.2–4.2 ng/10^6 cells at 12–15 days of culture in two independent experiments.

In cultures ranging from $0.25–2 \times 10^6$ cells/well, basal ir-activin A secretion increased in a concentration-dependent manner (Fig. 2A). We therefore used a concentration of 1×10^6 cells/well in the following experiments. As shown in Fig. 2B, basal secretion of ir-activin A was increased by about 1.8-fold (1.30 ± 0.36 ng/ml, $P<0.05$) with 2% and 2.6-fold (1.83 ± 0.44 ng/ml, $P<0.01$) with 10% FCS-DMEM as compared with the control culture (0.71 ± 0.10 ng/ml).

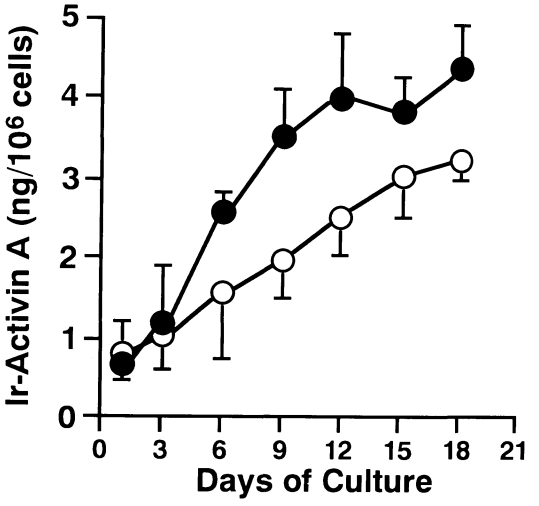

Fig. 1. Time course of the ir-activin A concentration in the culture medium. Rat anterior pituitary cells were cultured in DMEM supplemented with 2% FCS at a concentration of 1×10^6 viable cells/well for 1–18 days. The results of two independent experiments are shown. (●), experiment 1; (○), experiment 2.

The effects of gonadotropin and LHRH on ir-activin A release

When the cells were incubated for 72 h with LH or FSH, ir-activin A release was inhibited by about 40–60% by FSH at a concentration of 2–20 nM ($P< 0.05$), but there was no significant change with LH (0.2–20 nM) (Fig. 3). In contrast, 10 nM LHRH increased ir-activin A release 1.4-fold ($P< 0.05$), but 100 nM LHRH had no significant effect.

The effects of TPA and forskolin on ir-activin A release

As shown in Fig. 4, 20–100 nM of TPA dose- and time-dependently stimulated ir-activin A release from cultured pituitary cells ($P<0.05$), but no significant effect was induced by forskolin. The time course of ir-activin A release in the presence of forskolin tended to increase, but it was not statistically significant (Fig. 4B).

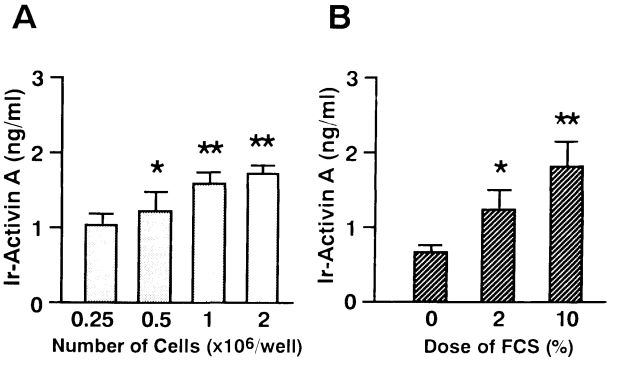

Fig. 2. Basal release of ir-activin A from cultured rat anterior pituitary cells. A: The effects of cell density upon ir-activin A release from rat anterior pituitary cells cultured in DMEM supplemented with 2% FCS for 6 days. Ir-activin A release into the culture medium reached a maximum level at a concentration of 1×10^6 cells/well. $*P<0.05$, $**P<0.01$ compared with cell culture at a concentration of 0.25×10^6 cells/well. B: Dose-related effects of FCS on ir-activin A release from rat anterior pituitary cells cultured for 6 days in DMEM supplemented with 0, 2 and 10% FCS, respectively. The pituitary cells were cultured at a concentration of 1×10^6 cells/well. $*P<0.05$, $**P<0.01$ compared with cell culture in DMEM without FCS.

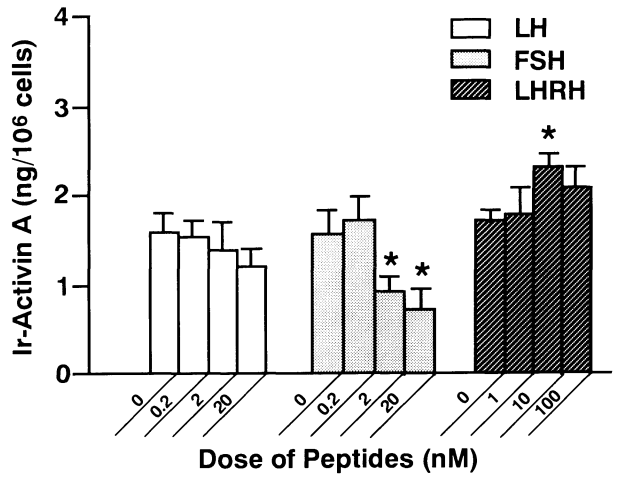

Fig. 3. Ir-activin A release in response to LH, FSH or LHRH. Anterior pituitary cells were cultured for 3 days in 2% FCS-DMEM at a concentration of 1×10^6 cells/well in the absence or presence of LH, FSH or LHRH. *$P<0.05$ compared with cell control.

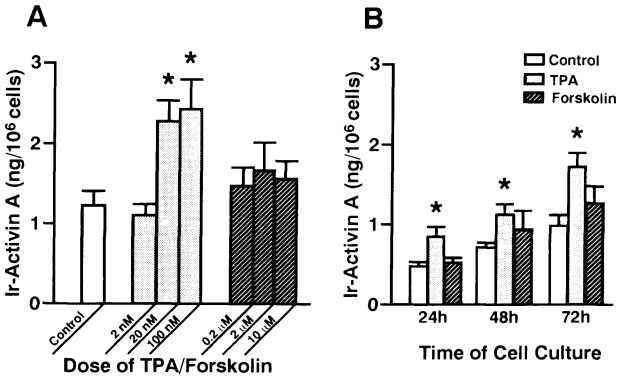

Fig. 4. Effects of TPA and forskolin on ir-activin A release from cultured pituitary cells. A: The effect of TPA on ir-activin A release was dose-dependent in rat anterior pituitary cells at a concentration of 1×10^6 cells/well cultured for 3 days in 2% FCS-DMEM, but not forskolin. B: The increase in ir-activin A release was time-dependent in rat anterior pituitary cells cultured with 20 nM TPA. 2 μM of forskolin tended to increase ir-activin A release but it was not statistically significant. The pituitary cells were cultured at a concentration of 1×10^6 cells/well. *$P<0.05$ compared with cell control.

Immunohistochemical staining of activin A

As shown in Fig. 5, the cytoplasm of cultured anterior pituitary cells was immunostained with anti-activin A rabbit antiserum. The immunostained cells accounted for about 10% of the cultured cells. The controls were not immunostained for activin A (data not shown).

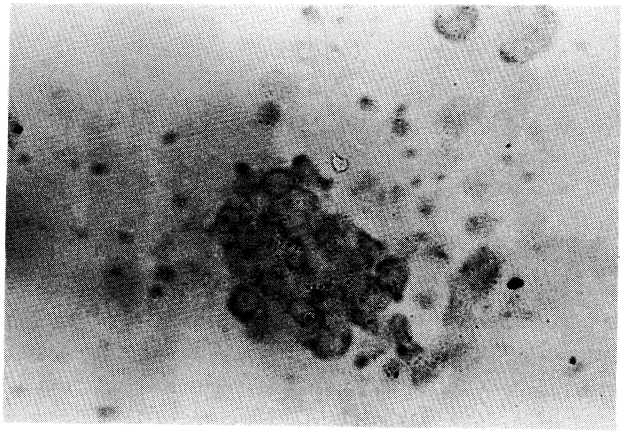

Fig. 5. Immunohistochemical staining for activin A in cultured rat anterior pituitary cells. Activin A was distinctly immunostained in the cytoplasm of cultured rat anterior pituitary cells.

Discussion

We developed a specific RIA and used it to show that activin A is secreted from rat anterior pituitary cells. The basal secretion of ir-activin A increased with time in culture and was significantly accelerated in the presence of fetal calf serum. Furthermore, we obtained direct evidence that activin A exists in the pituitary cells by immunostaining the cytoplasm of cultured anterior pituitary cells, but we did not identify which type of cells were immunostained in the pituitary.

Activin B has been regarded as a local regulator for FSH secretion in the rat pituitary, but not activin A [15, 18]. The report that the selective immunoneutralization of activin B inhibited FSH secretion from cultured pituitary cells supported the view that activin B is a potent positive regulator of FSH in the pituitary [24], but our data indicated that activin A is produced by anterior pituitary cells, suggesting that it also acts as a local regulator in the pituitary. There have been several recent reports regarding the presence of activin A in the pitutiary. Halvorson *et al.* have demonstrated by means of reverse transcription polymerase chain reaction that the βA-subunit mRNA is expressed in rat anterior pituitary cells

[17], and Demura *et al.* have revealed that the βA subunit mRNA is invariably expressed in human pituitary adenomas [25]. These reports are in accordance with our results and support the view that activin A as well as activin B is actually produced in anterior pituitary cells. Considering that the expression of βA- and βB-subunits is differentially regulated in the gonad [26], activin A and B may be differentially regulated in the pituitary as well and play different roles, but it remains to be studied further whether and how these activins interact with each other in regulating the secretion of pituitary hormones.

Activin modifies gonadotropin secretion from the anterior pituitary. We therefore investigated whether ir-activin A release is regulated by gonadotropin and LHRH. FSH inhibited ir-activin A release in a dose-dependent manner, suggesting that activin A secretion is regulated by the local feedback of FSH. In contrast, LHRH enhanced ir-activin A release significantly at a concentration of 10 nM. Although the physiological significance of these results is not yet clear, pituitary activin A may be involved at least in part in the increased FSH secretion in castrated and aged rats, in which LHRH secretion from the hypothalamus is increased. Furthermore, pituitary activin A may interfere with other substances produced in the pituitary. Follistatin, an activin-binding protein, is also present in the pituitary and its secretion is enhanced by activin [9]. DePaolo *et al.* recently demonstrated that pituitary follistatin mRNA expression is stimulated after ovariectomy by the mediation of pituitary activin [27]. Accordingly, it may be postulated that pituitary activin A, increased after castration, contributes to the increased secretion of FSH and follistatin from the pituitary. As a result, the increased FSH secretion may inhibit pituitary activin A secretion by the local feed back, and locally secreted follistatin may attenuate the activin action to prevent a further increase in FSH secretion, but further investigation is necessary to elucidate the interaction of these substances.

Incubating rat anterior pituitary cells with TPA or forskolin *in vitro* enhance the release of activin B and follistatin [9, 15]. These facts indicate that the activation of the PKA and PKC-dependent signal transduction pathways is involved in regulating activin B and follistatin production in the pituitary. But our results indicated that only TPA and LHRH, activators of the PKC pathway, enhanced ir-activin A release from cultured rat anterior pituitary cells. In contrast, forskolin, an activator of the PKA pathway, did not excert a significant effect. These findings suggested that activin A secretion from the pituitary is regulated by a different mechanism from that of activin B and follistatin as found in rat granulosa cells [26].

In conclusion, this study demonstrated that ir-activin A is secreted from rat anterior pituitary cells and its release is regulated by FSH and LHRH, and that the activation of PKC may be involved in the action of LHRH. The roles and regulatory mechanisms of activin A in the pituitary require further investigation.

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