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Activin-A, but not inhibin, regulates 17β-hydroxysteroid dehydrogenase type 1 activity and expression in cultured rat granulosa cells

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Abstract

17β-Hydroxysteroid dehydrogenase type 1 (17HSD type 1) catalyzes the reduction of estrone (E_1) to biologically more active estradiol (E_2). In the present study, the effect of activin, inhibin, and follistatin on 17HSD activity and 17HSD type 1 expression in cultured, unluteinized rat granulosa cells was examined. Furthermore, the effects of these hormones on 17HSD type 1 expression were compared with the expression of P450 aromatase (P450arom). Rat granulosa cells were pre-incubated in serum-free media for 3 days, followed by a 2-day treatment with activin, inhibin, follistatin and 8-Br-cAMP. Activin in increasing concentrations appeared to effect a dose-dependent increase in 17HSD activity. In addition, increasing concentrations of activin also increased 17HSD type 1 mRNA expression. Addition of 8-Br-cAMP at concentrations of 0.25 and 1.5 mmol/l together with activin significantly augmented the stimulatory effects of activin alone in the cultured cells. Neither inhibin, nor follistatin, either alone or in combination with 8-Br-cAMP, had any notable effects on 17HSD activity and 17HSD type 1 expression. Preincubation of activin with increasing concentrations of follistatin significantly diminished the stimulatory effect of activin. In the presence of follistatin, activin did not significantly increase the 8-Br-cAMP-induced 17HSD activity and 17HSD type 1 expression. The culturing of granulosa cells in the presence or the absence of inhibin or follistatin with or without 8-Br-cAMP did not alter the effect of these peptides on P450arom expression in rat granulosa cells as judged by Northern blot analysis of total RNA. However, cAMP-induced P450arom expression was enhanced by activin treatment, except when follistatin was present. This is in line with the suggested role of follistatin as an activin-binding protein, which limits the bioavailability of activin to its membrane receptors. Thus, the results support the notion of a paracrine/autocrine role of activin in follicular steroidogenesis of growing follicles.

Keywords: Activin-A; Inhibin; 17β-Hydroxysteroid dehydrogenase type 1; Granulosa cells

1. Introduction

17β-Hydroxysteroid dehydrogenases (17HSDs) catalyzes the conversion of 17-ketosteroids into the highly active 17β-hydroxysteroids. Thus, these enzymes are essential in the biosynthesis and metabolism of estrogens and androgens. 17β-hydroxysteroid dehydrogenase type 1 (17HSD type 1) catalyzes the reduction of estrone (E_1) to more biologically active estradiol (E_2) [1–3]. Studies on the 17HSDs so far cloned in humans and rodents suggest that the type 1 enzyme is closely associated with gonadal E_2 production in mammals.

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17HSD type 1 expression in ovarian granulosa cells has been shown to be under multi-hormonal regulation. In immature rat ovaries, the expression of 17HSD type 1 is increased by estrogens and follicle stimulating hormone (FSH) and the effect of FSH is modulated by estrogens, androgens and growth factors [4–7].

Activin, inhibin and follistatin are synthesized in the granulosa and theca cells of growing follicles [8–10]. These hormones are suggested to be involved in the regulation of FSH secretion by the pituitary and in the paracrine and autocrine regulation of growth and functional differentiation of ovarian follicles [11–13]. Apart from the gonads, inhibin, activin and follistatin are also produced in several other tissues and organs, including the pituitary, adrenals, bone marrow, kidney, pancreas and placenta [11,14–16], where they are suggested to play a key role in development and differentiation. Inhibin is a 32-kilodalton (kDa) heterodimer glycoprotein consisting of two subunits, an alpha-subunit and either of two related beta-subunits, βA and βB, thus forming inhibin A (α–βA) and inhibin B (α–βB). Activin is a 24 kDa homodimer consisting of two inhibin β-subunits; thus forming activin A (βA–βA), activin B (βB–βB) and activin AB (βA–βB). However, four isoforms of the β-subunit exists, namely βA, βB, βC which are present in the liver, and βD found only in the Xenopus [17,18]. Thus, the different combinations of β-subunits result in a greater number of activin and inhibin isoforms. Follistatin is a single-chain, monomeric, cystein-rich glycosylated polypeptide with no structural relationship to inhibin and activin. Due to alternative splicing of the follistatin gene, at least three (32, 35 and 39 kDa) mRNA transcripts of follistatin have been presently identified [13].

Several studies have shown that activin enhances gonadotropin-responsive aromatase activity in granulosa cells [19,20]. Follistatin has been reported to potentially block most in vitro activin activities [21–23], and it has also been demonstrated to inhibit gonadotropin-responsive aromatase activity of cultured granulosa cells [24]. Conversely, inhibin has been reported to act locally to increase LH-stimulated cytochrome-P450c17 enzyme activity and androgen synthesis in theca interna cells [25,26]. However, its role in granulosa cell steroidogenesis remains equivocal. Activin, inhibin and follistatin appear to be important local regulators of ovarian folliculogenesis and steroidogenesis. One therefore examined here, using enzyme activity measurements and Northern blot analysis, the modulatory impacts of these key autocrine/paracrine factors on the expression and activity of the 17HSD type 1 enzyme in cultured rat granulosa cells, for which no data are currently available. The influence of these hormones on P450 aromatase (P450arom) expression was also compared to that of 17HSD type 1.

2. Materials and methods

2.1. Chemicals and hormones

[α-^{32}P]dCTP (3000 Ci/mmol) and [2,4,6,7-^{3}H]E_1 (114 Ci/mmol) were purchased from Amersham International (Bucks, UK). Estrone was from Steraloids (Wilton, NH), diethylstilbestrol (DES) from Innovative Research of America (Sarasota, FL) and 8-Br-cAMP was from Boehringer Mannheim (Mannheim, Germany). Porcine inhibin was obtained from Sigma (St. Louis, MO). Human recombinant activin A and follistatin were kindly provided by National Hormone and Pituitary Program (NHPP), National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (NIDDK), USA. Other reagents not mentioned in the text were either from Sigma or Merck AG (Germany) and were of the highest purity grade available.

2.2. Granulosa cell culture

Immature (23–24-days-old) Sprague–Dawley female rats were implanted subcutaneously with DES-pellets, releasing 1.2 mg of DES/day. On the fifth day of DES treatment the animals were sacrificed and the ovaries were removed aseptically and transferred to culture medium. Granulosa cells were harvested by puncturing the follicles with fine (26 gauge) needles, allowing expulsion of the cells. Cells were recovered by centrifugation, washed with fresh medium and counted in a hemocytometer. Cell viability was routinely between 42 and 52% as measured by the trypan blue exclusion test. The culture medium used was DMEM/Ham's F-12 (Seromed, Biochrom KG, Germany) supplemented with Hepes-buffer (25 mmol/l), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mmol/l) and 0.1% (wt./vol.) BSA. For 17HSD activity measurements, the cells were cultured in 24-well plates (Costar, Cambridge, MA) at a density of 2×10^5 viable cells/well in 0.9 ml of culture medium. For RNA extraction, the cells were seeded into 6-well plates (Nunc, Roskilde, Denmark) at a density of 2×10^6 viable cells/well in 2 ml of medium. The cells were initially cultured for 72 h with no other treatment and thereafter, incubated in fresh culture medium with or without test reagents for an additional 48 h. All cultures were performed under a water-saturated atmosphere of 95% air/5% CO_2 at 37°C. None of the applied treatments affected the viability of the cells as assessed by MTT assays (data not shown).

2.3. 17HSD activity measurement

At the end of the culture period, 100 μl of culture medium/well, containing 10 μmol/l of [^{3}H]E_1 (2×10^5 cpm/well) were added. After 3 h incubation, the media

were removed and frozen in an ethanol-dry ice bath, after which the samples were stored at −20°C until the steroids were extracted into diethyl ether–ethyl acetate (9:1). The organic phases were then evaporated to dryness and resuspended in acetonitrile–water (50:50, vol/vol). Thereafter, E_1 and E_2 were separated in a Symmetry C18 reverse-phase chromatography column (3.9 × 150 mm, Waters, Milford, MA) connected to a HPLC system (Waters, Milford, MA). Radioactivity was measured by an on-line β-counter (150 TR, FLO-ONE Radiomatic, Packard, Meridan, CT) connected to the HPLC-system, using Ecoscint A scintillation solution (National Diagnostic, Atlanta, GA). 17HSD activities were expressed as pmol of E_2 formed per 10^5 cells/h.

2.4. RNA isolation and Northern blot analyses

Total RNA from cultured granulosa cells was isolated using TRIzol Reagent (Life Technologies, Grand Island, NY), according to the manufacturer's instructions. Ten or 20 μg of total RNA were subjected to 1% (wt./vol.) agarose-formaldehyde gel electrophoresis and then transferred onto Hybond nylon membrane (Amersham International, UK) by capillary blotting and fixed by UV radiation. The membranes were subsequently prehybridized for 4 h at 42°C in 5 × SSPE (1 × SSPE = 0.15 mol/l NaCl, 10 mmol/l sodium phosphate buffer, and 0.1 mmol/l EDTA, pH 7.4) with formamide (50%), BSA (0.1%), Ficoll (0.1%), polyvinylpyrrolidone (0.1%), sodium dodecyl sulfate (SDS; 0.5%) and 20 mg salmon sperm DNA per liter. Hybridizations with ^{32}P-labeled full length cDNAs of rat 17HSD type 1 [4] and rat P450arom (kindly provided by Professor JoAnne Richards, Department of Cell Biology, Baylor College of Medicine, Houston, TX) were then carried out overnight at 42°C. The nylon membranes were then washed twice in 2 × SSPE–0.1% SDS at 42°C for 15 min each and once in 1 × SSPE–0.1% SDS at 42°C for 10–15 min and exposed to Kodak X-AR films (Eastman Kodak, Rochester, NY) at −70°C. Densitometric scanning of the signals on the X-ray films was performed with a laser densitometer (Molecular Dynamics, Santa Ana Court, CA) and results were referred to the signal obtained by hybridizing the membranes with a rat glyceraldehyde-3-phosphate dehydrogenase cDNA probe.

2.5. Data analysis

All experiments were performed three or four times. The biochemical determinations in each experiment were carried out in triplicate. The significance of the difference between mean results was analyzed by Student's *t*-test or one-way analysis of variance, followed by the modified *t*-test using Bonferroni method. $P < 0.05$ was considered statistically significant. Representative results of Northern blot analysis are presented.

3. Results

Granulosa cells were obtained from the ovaries of immature Sprague–Dawley rats primed with DES pellets. As has been previously shown [7], freshly isolated granulosa cells from DES-primed immature female rats showed a relatively high basal 17HSD activity and 17HSD type 1 expression, which declined to about 5% of its initial value following a 3-day culture in serum-free media. Therefore, to exclude the effects of in vivo DES-priming on 17HSD activity and 17HSD type 1 expression, freshly isolated cells were cultured in serum-free media for 3 days before a further 2-day incubation of the cells with the tested peptides.

A dose-dependent up-regulation of 17HSD activity was detected following a 2-day treatment of the cultured cells with activin (1–100 ng/ml, Fig. 1). The ED50 of the activin effect was 30 ng/ml, and an activin concentration of 20 ng/ml was sufficient to increase significantly the 17HSD activity of the cells. The 17HSD activity induced by 8-Br-cAMP (0.25 and 1.5 mmol/l) was significantly augmented in the presence of increasing concentrations of activin (Fig. 1). Furthermore, Northern analysis of total RNA isolated from the cultured cells after treatment with activin at concentrations of 20 and 100 ng/ml showed 3- and 5-fold increases, respectively, in 17HSD type 1 expression (Fig. 2). However, P450arom expression was not affected by activin treatment. The higher concentration of activin (100 ng/ml) further increased the expression of 17HSD type 1 mRNA in the presence of 0.25 and 1.5 mmol/l of 8-Br-cAMP 10- and 2-fold, respectively (Fig. 3). In addition, P450arom expression induced by 0.25 and 1.5 mmol/l of 8-Br-cAMP was also increased by co-treatment with 100 ng/ml of activin 5- and 3-fold, respectively (Fig. 3).

The co-treatment of the cultured cells with follistatin (200 ng/ml) and activin (100 ng/ml) resulted in a strong inhibition of the stimulatory effect of activin on 17HSD type 1 expression (Fig. 3). The activin enhanced cAMP-induced expression of both 17HSD type 1 and P450arom was also strongly inhibited by co-treatment with 200 ng/ml of follistatin (Fig. 3). Similarly, when granulosa cells were treated with increasing concentrations of activin together with follistatin (100 or 200 ng/ml), the latter significantly diminished the activin-induced 17HSD activity (Fig. 4). Follistatin with or without 8-Br-cAMP (0.25 and 1.5 mmol/l) had no significant effect on 17HSD activity of cultured cells. Furthermore, follistatin (100 and 200 ng/ml) significantly diminished the stimulatory effects of activin (1–100 ng/ml) in the presence of 0.25 and 1.5 mmol/l of 8-Br-cAMP on 17HSD activity (Fig. 5).

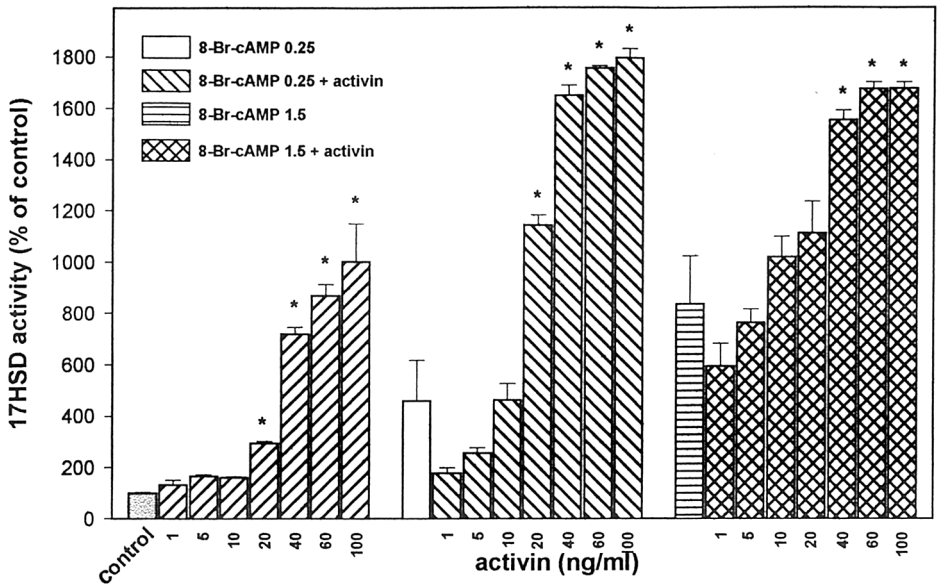

Fig. 1. Dose-dependent effect of activin on 17β-hydroxysteroid dehydrogenase (17HSD) activity in the absence or presence of 8-Br-cAMP. Granulosa cells obtained from diethylstilbestrol (DES)-treated immature rats were cultured for 72 h without treatment and then treated with increasing concentrations of activin (1–100 ng/ml) alone or in combination with 8-Br-cAMP (0.25 or 1.5 mmol/l) for 48 h. Each point represents the mean ± S.E. of triplicate determinations. * $P < 0.05$.

Addition of inhibin (1–100 ng/ml) alone or with 1.5 mmol/l 8-Br-cAMP, or in combination with follistatin (200 ng/ml) and 8-Br-cAMP (1.5 mmol/ml) did not significantly affect the 17HSD activity of the cultured cells (data not shown). Also, Northern analysis revealed no significant change in the expression of 17HSD type 1 or P450arom mRNAs in the cultured cells following inhibin (10 and 100 ng/ml) treatments alone and in combination with 8-Br-cAMP (1.5 mmol/l) and follistatin (200 ng/ml) (Fig. 6).

4. Discussion

Ovarian follicular growth and development in mammals is reflected in the notable variations of the expression and regulation of ovarian steroidogenic enzymes [12,27–29]. Gonadotropins play a key role in these processes. The actions of gonadotropins are, however, modulated by several hormones, such as androgens and estrogens, and a number of growth factors [12,29–31]. Increasing evidence has accumulated in recent years on the local autocrine/paracrine actions of activin, inhibin and follistatin on ovarian folliculogenesis and streroidogenesis [9,11,24,32]. The present results show that activin-A increases the basal and cAMP-stimulated 17HSD activity and 17HSD type 1 expression. They further demonstrate that human activin-A, but not inhibin or follistatin, directly modulates steroidogenesis in cultured unluteinized granulosa cells. Thus, the results support previous findings on the modulatory role of activin in follicular steroidogenesis [24,33].

Activin has been shown to augment FSH-stimulated P450arom activity and to induce FSH and LH receptors in unluteinized granulosa cells [20,23,24]. In granulosa cells from large preovulatory follicles in some

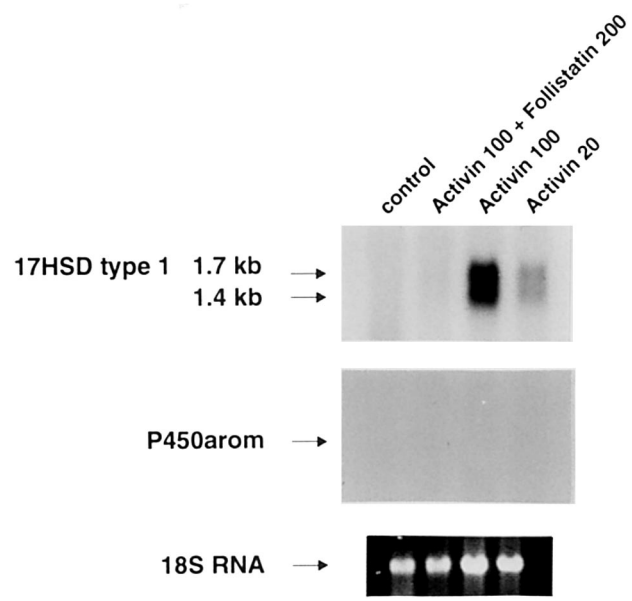

Fig. 2. Northern blot analysis (20 μg total RNA per lane) of RNA extracted from granulosa cells cultured without treatment (control, lane 1), with activin (100 ng/ml) in combination with follistatin (200 ng/ml), or with activin alone (100 ng/ml, lane 3; 20 ng/ml, lane 4). Full-length cDNAs for rat 17β-hydroxysteroid dehydrogenase type 1 (17HSD type 1) and rat P450 aromatase (P450arom) were used as probes, and sample loading was controlled by ethidium bromide staining of 18S ribosomal RNA.

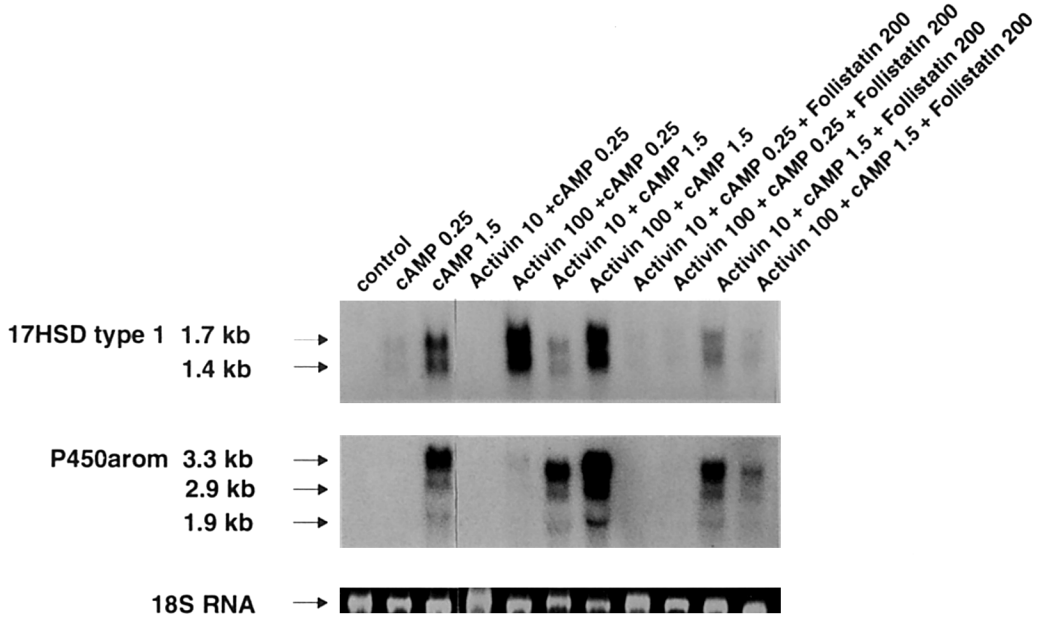

Fig. 3. Northern blot analysis (10 μg total RNA per lane) of RNA extracted from granulosa cells cultured without treatment (control, lane 1), with 8-Br-cAMP (0.25 mmol/l, lane 2; 1.5 mmol/l, lane 3), or with activin (10 ng/ml, 100 ng/ml) in combination with 0.25 nmol/l of 8-Br-cAMP (lanes 4 and 5) or 1.5 mmol/l of 8-Br-cAMP (lanes 6 and 7). Lanes 8–11 represent activin (10 or 100 ng/ml) in combination with 8-Br-cAMP (0.25 or 1.5 mmol/l) and follistatin (200 ng/ml). Full-length cDNAs for rat 17β-hydroxysteroid dehydrogenase type 1 (17HSD type 1) and rat P450 aromatase (P450arom) were used as probes, and sample loading was controlled by ethidium bromide staining of 18S ribosomal RNA.

species, activin has been shown to inhibit spontaneous luteinization by stimulating gonadotropin-responsive E_2 production and activity of P450arom, and by supressing simultaneously progestrone production [35]. In the present study, activin induced in a dose dependent manner both 17HSD activity and 17HSD type 1 expression in granulosa cells. Thus, the reported anti-luteinizing effects of activin could also be reflected in the up-regulation of the 17HSD activity and 17HSD type 1 expression.

FSH acts via cAMP-dependent pathway to induce follicular 17HSD type 1 and P450arom enzyme expressions [36–39]. Previous reports have shown that activin enhances the FSH-, forskolin- and cAMP-induced P450arom activity [21,34]. The present results demonstrate that activin alone or in the presence of cAMP strongly up-regulates 17HSD activity, 17HSD type 1, and cAMP-induced P450arom expression in cultured rat granulosa cells. However, the exact mechanisms of action of activin remains to be clarified. At present, there are contradictory reports on the role of cAMP as a possible mediator of effects of activin. Some investigators have suggested a cAMP-dependent mechanism of action for activin [21]. However, the present results showing that activin further increases cAMP-induced 17HSD type 1 expression and activity and cAMP-induced P450arom expression, together with the findings of others [34] showing an activin stimulation of cAMP-induced P450arom activity in cultured granulosa cells, suggest that the effects of cAMP and activin in steroidogenesis in granulosa cells are complementary. Furthermore, activin alone was not able to induce P450arom expression.

The role of inhibin as an autocrine/paracrine ovarian regulator has remained controversial. A previous study reported inhibitory effects of inhibin on gonadotropin-stimulated P450arom activities and estrogen production

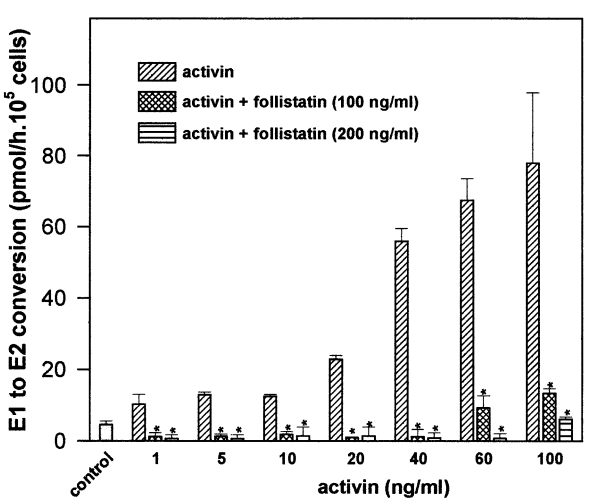

Fig. 4. Effect of follistatin on activin-induced 17β-hydroxysteroid dehydrogenase (17HSD) activity. Granulosa cells obtained from diethylstilbestrol (DES)-treated immature rats were cultured for 72 h without treatment, and then treated for 48 h with increasing concentrations of activin (1–100 ng/ml) alone or in combination with follistatin (100 or 200 ng/ml). Each point represents the mean ± S.E. of triplicate determinations. * $P < 0.05$.

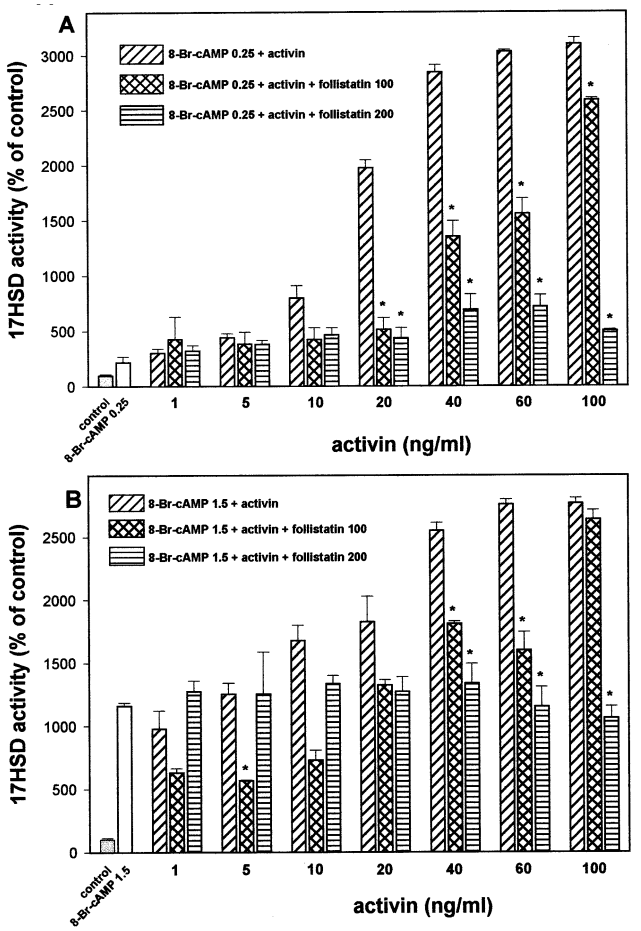

Fig. 5. Effect of follistatin (100 or 200 ng/ml) on (8-Br-cAMP + activin)-induced 17β-hydroxysteroid dehydrogenase (17HSD) activity. Granulosa cells obtained from diethylstilbestrol (DES)-treated immature rats were cultured for 72 h without treatment and then treated for 48 h with 8-Br-cAMP (0.25 mmol/l, A; 1.5 mmol/l, B) and increasing doses of activin (1–100 ng/ml). Each point represents the mean ± S.E. of triplicate determinations. * $P < 0.05$.

in cultured rat granulosa cells [40]. However, these results have not been confirmed so far [19,20,34,41]. The results did not demonstrate any effects of inhibin at concentrations as high as 100 ng/ml on basal or cAMP-stimulated 17HSD activity, 17HSD type 1 mRNA or P450arom mRNA expression in unluteinized granulosa cells obtained from DES-primed immature rats. Thus, the present result supports the notion [13,19,41] that inhibin probably does not exert any effect on steroidogenesis in unluteinized granulosa cell.

Previous studies have shown that treatment with follistatin suppresses FSH-induced P450arom activity and inhibin production, and increases FSH-induced progesterone production by rat granulosa cells [22,24]. Presumably by binding activin and to a lesser extent inhibin molecules through their common β-subunits [42], follistatin effectively neutralizes their bioactivity. In addition, through its association with cell membrane-anchored heparin sulfate proteoglycans [43], follistatin might effectively compete with membrane-associated activin receptors [44] for activin, thus further reducing activin bioavailability and bioactivity. The present results showed that follistatin has no significant effects on the basal level and cAMP-induced 17HSD activity, 17HSD type 1 and P450arom expresion in unluteinized granulosa cells. Furthermore, the present study showed that the stimulatory activity of activin on 17HSD activity and 17HSD type 1 expression in cultured rat unluteinized granulosa cells was strongly blocked by follistatin. This supports the notion that most intraovarian actions of follistatin may be attributable to its ability to neutralise activin bioactivity by binding the activin β-subunits.

In conclusion, the present results support possible paracrine/autocrine activities of activin in ovarian steroidogenesis, which also can be also modulated by activin/follistatin interactions.

Acknowledgements

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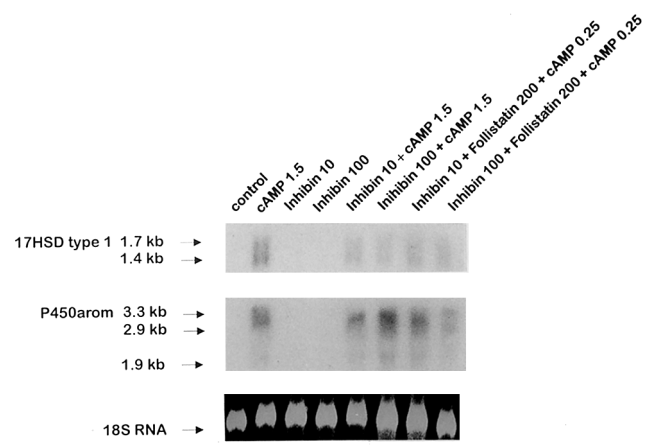

Fig. 6. Northern blot analysis (10 μg total RNA per lane) of RNA extracted from granulosa cells cultured without treatment (control, lane 1), with 8-Br-cAMP (1.5 mmol/l, lane 2), and with inhibin (10 ng/ml, lane 3; 100 ng/ml, lane 4). Lanes 5–8 represents inhibin (10 or 100 ng/ml) in combinations with 8-Br-cAMP (0.25, 1.5 mmol/l) and follistatin 200 ng/ml. Full-length cDNAs for rat 17β-hydroxysteroid dehydrogenase (17HSD) type 1 and rat P450 aromatase (P450arom) were used as probes, and sample loading was controlled by ethidium bromide staining of 18S ribosomal RNA.

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