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Differential Control of Activin, Inhibin and Follistatin Proteins in Cultured Rat Granulosa Cells

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Follistatin, activin and inhibin proteins are produced by granulosa cells, but the mechanisms controlling their production remain unclear. Here, we examined how the protein kinase A (PKA) and protein kinase C (PKC) pathways act and interact to regulate production of these proteins. Granulosa cells from immature rats were cultured with activators of the PKA pathway (100 ng/ml FSH, 10 μM forskolin) and/or activators of the PKC pathway (100 nM GnRH agonist, 100nM 2-0-tetradecanoyl-phorbol-13-acetate, TPA). Conditioned media were assayed for inhibin and activin by ligand blotting using recombinant human ^{125}I-follistatin and for follistatin by double ligand blotting using cold activin plus ^{125}I-follistatin. FSH and forskolin stimulated inhibin but not activin production. In contrast, GnRH and TPA stimulated activin, and to a lesser degree, inhibin production; significantly, this is the first demonstration of activin dimer production by granulosa cells. Activators of the PKA pathway antagonized the actions of PKC effectors and vice versa. All agents increased follistatin protein production, and the PKA and PKC activators interacted to generate further increases in follistatin production. These results show that the FSH-PKA signalling pathway favors formation of αβ inhibin dimers while the GnRH-PKC pathway favors formation of β-subunit activin dimers. Both pathways act to increase follistatin protein production.

There are a wide variety of polypeptide factors that can influence the differentiated state of ovarian cells (1). Many of these factors, such as inhibin, activin and follistatin are produced locally within the ovary and presumably function as autocrine/paracrine regulators (2,3). The physiological role of these proteins within the ovary remains unclear; however, a functional role of inhibin and activin in follicle growth and atresia has been reported (4,5). Because follistatin is an activin binding protein (6), it has been implicated in modulating the bioactivity of activin (2,3).

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Abbreviations used are:
cAMP, cyclic AMP; FSH, follicle-stimulating hormone; GnRH, gonadotropin releasing hormone; kDa, kiloDalton; PKA, protein kinase A; PKC, protein kinase C; rhFS, recombinant human follistatin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPA, 2-0-tetradecanoyl-phorbol-13-acetate.

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Previous studies have found that the mRNAs for inhibin/activin α, βA and βB subunits (7,8) and follistatin (9,10) are expressed in granulosa cells. FSH increases the levels of these mRNAs (2,3,9,11-13). As expected, the FSH effects are mimicked by agents that increase cAMP levels (14,15). Thus, one can conclude that the FSH cAMP/protein kinase A (PKA) pathway is coupled to increases in the levels of the mRNAs encoding these proteins. Activators of the PKC pathway, such as GnRH or phorbol esters, are able to block the stimulatory effects of FSH on the α and β inhibin subunit mRNAs (14) and the inhibin protein (16,17) in ovarian granulosa cells. Taken together, these results indicate that cross-talk between the PKA and PKC signalling pathway functions in regulating the levels of the inhibin/activin subunit mRNAs expressed in granulosa cells.

In spite of these findings, the results are still equivocal because of the current lack of dependable analytical procedures to measure the dimeric proteins as well as follistatin. The development of a specific assay system to quantitate these proteins is required for understanding the regulatory mechanisms controlling their production. Here we describe a system for semi-quantitation of these proteins.

Materials And Methods

Granulosa Cell Culture

Animals were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and procedures were approved by the Institutional Animal Care and Use Committee. Ovaries from untreated 26-day old female Sprague-Dawley rats (Harlan) were removed and granulosa cells collected. Cells (1.2-2.0 x 10^5 viable cells/ml) were cultured in Dulbecco's Modified Eagle's Medium and Ham's F12 (15). Cells were pre-cultured for 2 days in 10% fetal bovine serum, washed and re-cultured for an additional 2 days in serum-free medium as control (no additions), or with FSH (100 ng/ml), GnRH agonist (100 nM), forskolin (10 μM), and/or TPA (100 nM).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed under non-reducing conditions using a Xcell mini-cell system (Novex, Encinitas, CA). Conditioned media were concentrated 40 times by using Immersible-CX10 ultrafilters (10,000 molecular weight cut-off, Millipore, Bedford, MA). Concentrated media were loaded onto 12% polyacrylamide gels (Tris/glycine percentage gel, Novex, Encinitas, CA), electrophoresed at a constant voltage of 100 V for 2h and then transferred to 0.45 μm nitrocellulose membranes (Bio-Rad, Richmond, CA) for 2h at 150 mA using the Mini Blot module (Novex).

Ligand Blotting and Double Ligand Blotting

Ligand blotting was carried out essentially as described (18). After soaking in 3% NP-40 for 30 min, the membrane was blocked in 1% casein/Tris-buffered saline with 0.1% Tween 20 (TBST) for 1h at room temperature. The membrane was then incubated with 20 ml of casein/TBST buffer containing 2 X 10^5 cpm/ml of ^{125}I labeled recombinant human follistatin-288 (rhFS-288) (19) overnight at 4C. After washing with TBST, the membrane was exposed to X-ray film with an intensifying screen at -80C.

Double ligand blotting was performed as described (18). After washing in 3% NP-40, the membrane was blocked with casein/TBST, then incubated with 10 ml of casein/TBST containing 10 nM of activin-A for 2h at room temperature. After washing the membrane with TBST, it was incubated with 20 ml of casein/TBST containing the ^{125}I-rhFS-288 (2 X 10^5 cpm/ml) overnight at 4C. Filter was then washed and the membrane was exposed to X-ray film. Fig. 1 shows the

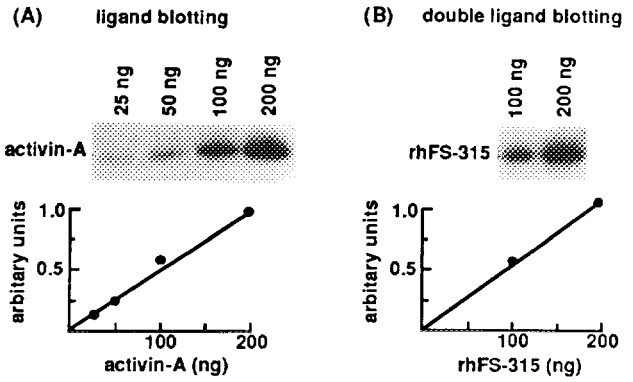

Fig. 1. Ligand and double ligand blotting analysis of purified activin A (A) and rhFS-315 (B) showing dose dependency. Relative intensity is graphed in arbitrary units.

results of the dose- response effects of the ligand and double ligand blot, demonstrating the semi-quantitative nature of the assay.

Data Analysis

Relative densities of the radioactive bands obtained by ligand blotting and double ligand blotting were determined by transmittance scanning densitometry. Experiments were repeated two or three times for FSH/GnRH or forskolin/TPA treated cells, respectively. Values are expressed as percentages relative to the highest density in that group.

Results

Regulation of Inhibin, Activin and Follistatin by FSH and GnRH

Figure 2 shows the data obtained when granulosa cells were treated with FSH and/or GnRH. The signal obtained with a 50 ng standard of activin-A was stronger than that of 200 ng of inhibin-A (Fig. 2A). This can be explained by the differences in the number of β subunits between inhibin and activin molecule (1 vs 2, respectively), their affinity for follistatin, and the topology of the activin/inhibin molecules on the filter (18). Therefore, the relative strength of the activin and inhibin signals obtained from the culture media must be compared with their respective standards, not with one another.

The levels of the 24 kDa activin and 32 kDa inhibin proteins were low in control media (Fig. 2A). Secretion of inhibin dimer was markedly increased in the presence of FSH. GnRH also enhanced inhibin secretion, but to a lesser degree than FSH. However, the pronounced stimulation of inhibin by FSH was antagonized by co-incubation with GnRH.

In contrast, activin secretion from granulosa cells was blocked in the presence of FSH, while GnRH substantially increased activin levels in conditioned media. The effect of GnRH on activin secretion was slightly decreased in the presence of FSH. GnRH (with and without FSH) produced increases in high molecular mass bands (65 kDa) which bind ^{125}I-follistatin. These bands may be pro-activin molecules because their intensities correlate with those of the activin bands.

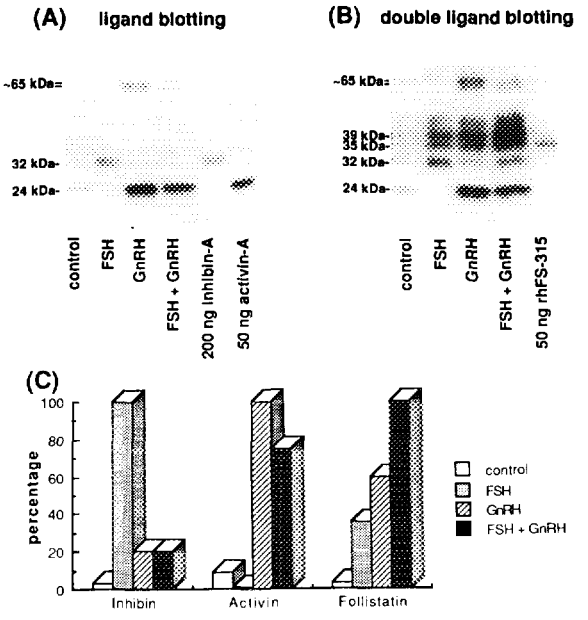

Fig. 2. Ligand and double ligand blotting analysis of inhibin, activin and follistatin (FS) proteins in media from FSH and GnRH-treated granulosa cells. Cells were incubated for 2 days as controls or with 100ng/ml FSH and/or 100 nM GnRH agonist. (A) ligand blotting was used to quantitate inhibin and activin levels and (B) double ligand blotting was used to determine follistatin. Numbers at left indicate molecular sizes of the various bands. (C) Representative results are expressed as percentages relative to the highest density value in each group.

The double ligand blotting method is known to identify follistatin, plus activin and inhibin (18). As seen in Figure 2B, a 50 ng standard of 35 kDa rhFS-315 is easily visualized. Control media contained weak bands migrating at 24, 32, 35 and 39 kDa (Fig. 2B). The 24 kDa band is activin, while the 35 and 39 kDa bands are isoforms of follistatin with different degrees of glycosylation and heterogeneity at the C-terminal region of the molecule (19). The 32 kDa band, however, likely represents a mixture of inhibin and the 32 kDa follistatin isoform (19). In view of this and the predominance of the 35 kDa form in follicular fluid, the 35 kDa band was scanned for follistatin.

Both FSH and GnRH increased the levels of 35 kDa follistatin (Fig. 2C). Co-treatment with FSH and GnRH caused greater elevation in follistatin (35 and 39 kDa species) compared to that of either hormone alone. The faint bands above 35 kDa are probably higher molecular weight isoforms of follistatin, possibly caused by partial reduction of the 18 disulfide bonds in the molecule.

Regulation of Inhibin, Activin and Follistatin by Forskolin and TPA

Cells were also treated with forskolin and TPA which activate the PKA and PKC pathways, respectively. As seen in Figure 3A, B, and C, results obtained using forskolin and/or TPA were almost identical to those obtained with FSH and/or GnRH (compare Figs. 2 and 3).

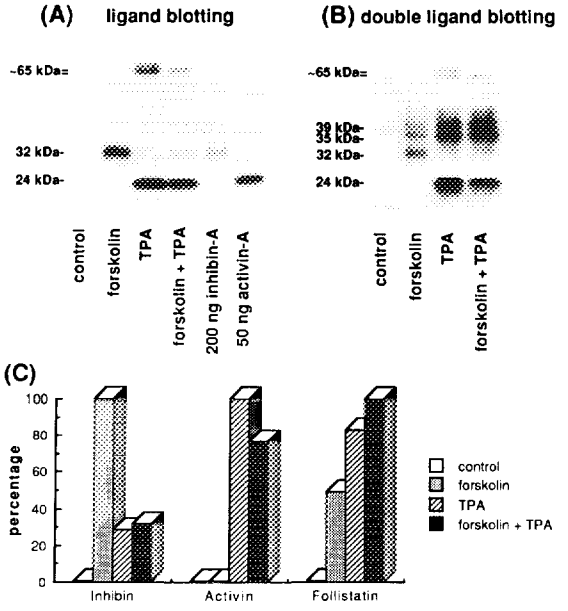

Fig. 3. Ligand and double ligand blotting analysis of inhibin, activin and follistatin proteins in media from forskolin and TPA-treated cultures. Cells were incubated for 2 days as controls or with 10 μM forskolin, and/or 100 nM TPA. Representative results are expressed as percentages relative to the highest density value in each group.

Discussion

Our results confirm the earlier observation that FSH and forskolin stimulate inhibin protein production by cultured rat granulosa cells and that GnRH andTPA can block the FSH effect (16, 17). Now we report the interesting and potentially important findings that 1) the cAMP-PKA and PKC pathways are coupled preferentially to inhibin and activin production, respectively. This result supports the novel hypothesis that different mechanisms exist for the establishment and maintenance of inhibin and activin protein synthesis; and 2) both signal transduction pathways function to stimulate follistatin protein production. Accordingly, ligands that increase intracellular cAMP and/or diacylglycerol levels would be expected to increase follistatin protein production. At present, it remains unclear how our results relate to *in vivo* physiology. However, our model should be very useful for future progress in understanding this question.

There is substantial evidence that elevated levels of inhibin are uniquely associated with dominant follicles (2-5, 11-12). The functional role of FSH in the mechanism of selection of dominant follicles is also clear (1). Our data indicate that activation of the FSH signal transduction pathway causes the α and β subunits in granulosa cells to be assembled into inhibin, but not activin, dimers. Although the mechanisms controlling the assembly of the inhibin/activin dimers are not known, it is tempting to speculate that the translated products from the α, βA and βB subunit mRNAs in granulosa cells of dominant follicles are processed selectively into inhibin proteins. These data would be consistent with a selectogenic role for inhibin in the process of folliculogenesis.

At the opposite extreme, activin dimers are produced following activation of the PKC signalling pathway. In recent experiments, we have found that exogenous GnRH agonist initiates a wave of atresia in preantral follicles of estrogen-primed, immature rats *in vivo* (Erickson, unpublished data). Thus, ligands that activate the PKC signalling pathway (and presumably β subunit activin dimer production) are able to cause atresia. Such results would be consistent with the results of Woodruff, *et al.* (4) in which exogenous activin caused follicle atresia.

Recent studies showed that forskolin and TPA stimulated follistatin mRNA expression in this model (15). Our experiments now extend these findings by demonstrating that both these signal transduction pathways dramatically increase follistatin protein synthesis. Thus, one can conclude that follistatin production is positively coupled to two different second messenger systems. Experiments are in progress to identify the significance of this finding.

Collectively, our data indicate that receptors linked through different G-proteins interact at some downstream points of the two signal transduction cascades to specify how the α and β subunits are associated into inhibin or activin. A similar process could be involved in controlling follistatin protein expression. With the availability of our *in vitro* systems it should be possible to characterize the mechanisms responsible for the inhibin, activin and follistatin biosynthesis.

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