Control of peptides regulating mitosis of granulosa cells in immature rat ovary by oestrogen and gonadotrophin

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Previous studies in this laboratory have demonstrated the presence of a peptide that inhibits granulosa cell proliferation in medium sized follicles. This peptide was produced after 72–96 h of exposure to diethylstilboestrol (DES). This study analyses oestrogen and gonadotrophin modulation of this and another stimulatory peptide found in large follicles. Intact, immature, female rats were assigned to the following study groups: (i) one to four injections of DES (2 mg per rat) given at intervals of 24 h, animals killed 24 h after the last injection; (ii) DES at 0 and 24 h, animals killed 24 or 48 h after the last injection; and (iii) DES plus pentobarbital (37 mg kg⁻¹ body weight) at 30 h, animals killed 48 h after the last injection. Small, medium and large follicles (diameters of < 200, 200–400 and > 400 µm, respectively) were isolated from ovaries, granulosa cells were harvested and follicular fluid supernatant (FFS) was collected. FFS proteins were tested for their effects on incorporation of [³H]thymidine into granulosa cell DNA. Results showed that unfractionated FFS protein (150 µg) from medium follicles in groups (i) (three and four injections only), (ii) and (iii) inhibited [³H]thymidine incorporation. Pentobarbital blockage of gonadotrophin secretion had no effect on the inhibitory peptide activity and two injections of diethylstilboestrol were enough to stimulate synthesis of the inhibitory peptide, provided sufficient time was allowed; FFS protein (150 µg) from large follicles was stimulatory, but only when it was collected 24 h after the second injection, and the effect was abolished with pentobarbital treatment. Fractionation of pooled FFS into proteins with three molecular mass ranges (< 10 kDa, 10–30 kDa and > 30 kDa) showed that the inhibitory activity was in the < 10 kDa fraction while the > 30 kDa fraction stimulated thymidine incorporation. The number of medium and large follicles increased 24 h after the second DES injection, but the number of granulosa cells in the large follicles was significantly reduced after pentobarbital blockage of gonadotrophins. Taken together, these findings show that (i) the inhibitory peptide is induced in effective amounts in the medium follicles 48 h after the second DES injection and induction is not modulated by gonadotrophins; and (ii) stimulatory activity seen in the large follicles is transient and under gonadotrophic control, since blockage of follicle-stimulating hormone (FSH) secretion led to loss of stimulatory activity and a resulting reduction in number of granulosa cells in large follicles. We, therefore, propose that, besides recruiting small follicles, FSH boosts large follicle growth in the penultimate stages of follicle development via the synthesis of a stimulatory peptide of molecular mass > 30 kDa. Medium follicle growth, however, is regulated more by the oestrogen-induced inhibitory peptide.

Introduction

Ovarian folliculogenesis is characterized by rapid growth of small primary follicles, with few granulosa cells, to mature pre-ovulatory follicles with several layers of cells. Thus, granulosa cell proliferation is an important feature in this developmental process. The well-known mitotic regulators are follicle stimulating hormone (FSH), oestradiol and possibly luteinizing hormone (LH; Richards, 1980; Bogovich *et al.*, 1981). The nature of the proliferative response to these hormones depends on the

specific stage of development, and hence the size of the follicles. Whereas Hirshfield (1985) has shown that, in rats, FSH stimulates only certain classes of follicle to grow more rapidly, studies from our laboratory have shown that oestrogens stimulate small follicles and inhibit medium follicle growth (Chakravorty *et al.*, 1991b). This suggests that the rate of granulosa cell proliferation is controlled at each stage by separate hormonal cues.

The exact mechanism by which gonadotrophins and oestradiol control granulosa cell mitosis is not completely understood. Both FSH and oestrogens are known to be mitogenic *in vivo*, but neither hormone exhibits a marked effect *in vitro*, suggesting that these hormones might act indirectly by stimulating

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mitogenic factors. There is substantial evidence that several peptide growth factors are involved (Gospodarowicz and Bialecki, 1979). Some of these factors are present in the ovary and are produced locally (Adashi *et al.*, 1985; Dorrington *et al.*, 1988; Lobb *et al.*, 1988), regulating granulosa cell proliferation in an autocrine or paracrine manner. Some of these factors may also be present in follicular fluid. The present study analyses the growth inhibitory and stimulatory properties of factors present in follicular fluid from small, medium and large follicles on [³H]thymidine incorporation in response to diethylstilboestrol (DES) and pituitary gonadotrophins. Previous studies in our laboratory have shown that there is a gonadotrophin surge at 36 h when DES was given to intact, immature rats at 0 and 24 h (Chakravorty *et al.*, 1991a) and that growth of medium and large follicles is stimulated 12 h after this surge. In contrast, 72–96 h of DES treatment caused an inhibition of growth, specifically of medium follicles. We have shown that the inhibition is due to the synthesis of a peptide factor, which we call 'granulosa cell mitostatic peptide' (GCMP) since it inhibits granulosa cell mitosis. The objective of the present study was to characterize GCMP and detect a stimulatory peptide found in follicular fluid. The studies were designed to investigate gonadotrophic modulation of the activity of these peptides by blocking the endogenous gonadotrophin surges by treatment with pentobarbital and testing for activity of the peptides in follicular fluid supernatant on [³H]thymidine incorporation into granulosa cell DNA.

Materials and Methods

Animals

Intact, immature female Sprague–Dawley rats were obtained from Holtzman Laboratories (Madison, WI). The animals were housed in temperature-controlled (22°C) quarters with 14 h light and 10 h darkness and provided with commercial rat chow and water *ad libitum*.

Experimental protocol

Four sets of studies were done with animals assigned to different groups for different regimens of treatment either with 2 mg of diethylstilboestrol (Steraloids Inc., Wilton, NH) per rat subcutaneously in 0.2 ml of propylene glycol or with pregnant mares' serum gonadotrophin (PMSG).

Experiment 1. Animals were assigned to four treatment groups according to the number of DES injections given: one, two, three or four injections at intervals of 24 h. All animals were killed by decapitation at 28 days of age and 24 h after the last injection. The treatment groups were designated by the number of DES injections given at times before killing (indicated in parentheses): one (24 h), two (48 h), three (72 h) and four (96 h).

Experiment 2. Two injections of DES at 0 and 24 h were given and animals were killed at 48 or 72 h, i.e. 24 or 48 h after the second injection.

Experiment 3. Two injections of DES at 0 and 24 h were given, with or without treatment with pentobarbital (37 mg kg⁻¹ body weight) given intraperitoneally 6 h after the second injection (at 30 h). The animals were killed at 72 h (48 h after the second injection of DES).

Experiment 4. A single injection of PMSG, 8 iu per rat in 0.2 ml of 0.9% (w/v) saline, was given at 0 h to two groups of animals 26 days old. One group was killed at 24 h and the other group was killed at 48 h after PMSG administration. Follicular fluid supernatant (FFS) was collected and tested for effects on [³H]thymidine incorporation into granulosa cell DNA.

All animals were kept on a lighting schedule of 14 h light and 10 h darkness, lights being on between 05:00 and 19:00 h Eastern Standard Time (USA). All injections at 0 h were given at 06:00 h.

Follicle isolation, granulosa cell extraction and collection of follicular supernatant fluid

The ovaries were removed and cleaned of fat; follicles were isolated and separated by size; and granulosa cells were then harvested from follicles of three sizes as described previously (Chakravorty *et al.*, 1991a). Rates of [³H]thymidine incorporation in these cells were measured as described previously (Chakravorty *et al.*, 1991a). Follicular fluid supernatant was collected (Chakravorty *et al.*, 1991a, b) after rupturing the follicles in a measured amount of buffer and separating the granulosa cells by centrifugation (50 g for 5 min). Protein content of FFS samples was measured (Lowry *et al.*, 1951).

Cell culture for effects of follicular fluid proteins

The granulosa cell culture system used for testing the effects of FFS on [³H]thymidine has been described (Chakravorty *et al.*, 1991a). Briefly, 1.25×10^5 viable granulosa cells from medium-sized follicles, collected after two injections of DES, were plated in 22.6 mm wells containing McCoy's 5A buffer with Hepes and L-glutamine and 1% fetal calf serum (Gibco, treated with charcoal to remove steroids). These cells, chosen for high mitotic activity, were incubated without (control) or with FFS protein in duplicate, or with 150 µg of bovine serum albumin (to eliminate non-specific protein effects). Two doses (150 and 300 µg) of unfractionated FFS protein from small, medium and large follicles, collected after the different treatment regimens, were added to the cell culture. The cells were incubated for 36 h, then 0.5 µCi [methyl-³H]thymidine (ICN Radiochemicals, Irvine, CA; specific activity = 37 Ci mmol⁻¹) was added to each well and the culture continued for an additional 12 h. The cells were then washed three times with ice-cold buffer (containing a 100-fold excess of unlabelled thymidine) to remove excess radiolabelled thymidine and the cultured cells were dispersed with KOH (0.1 mol l⁻¹) with 1% Triton-X 100. The DNA was precipitated with trichloroacetic acid (Fisher Scientific, 10% w/v) and the radioactivity of the pellet was measured.

Partial purification of follicular fluid supernatant

The FFS collected from small, medium and large follicles was pooled (to maximize the amount of protein recovered) and

centrifuged at 7000g for 15 min to remove any particulate components. The clear supernatant was then serially filtered (by centrifugation) through micro-concentrators (Centricon filters, Amicon, Danvers, MA) with membranes with molecular mass cutoff points of 10 kDa and 30 kDa; this yielded three fractions containing proteins in the molecular mass ranges <10, 10–30 and >30 kDa. Different doses of each of these fractions were tested for their effects on [³H]thymidine incorporation as described above.

Polyacrylamide gel electrophoresis of FFS fractions

The fractionated FFS was subjected to polyacrylamide-gel electrophoresis (PAGE). One-dimensional PAGE was performed under denaturing conditions with sodium dodecyl sulfate PAGE (SDS–PAGE) and also under non-denaturing conditions according to the methods described by Laemmli (1970) and Maizel (1974) using 1 mm × 16 mm gels. Resolving gels contained 18% acrylamide (acrylamide:bis = 37.5:1) and stacking gels were 4% acrylamide and measured 2 cm. Two sets of standards were used: (i) Sigma MW-ND-500 (Sigma Chemicals, St Louis, MO): albumin, bovine serum (66 kDa); albumin, chicken egg (45 kDa); carbonic anhydrase, bovine erythrocytes (29 kDa); α -lactalbumin, bovine milk (14.2 kDa); and (ii) Sigma GF-70 (Sigma Chemicals): albumin, bovine serum (66 kDa); carbonic anhydrase (29 kDa); cytochrome *c* (12.5 kDa); aprotinin (6.5 kDa).

Standards were prepared by dissolving a mixture of 1 mg each of the four proteins in 1 ml of electrophoresis buffer and 10–20 μ l of the mixed solution was loaded on the wells. Partially purified FFS protein samples were mixed with 10% glycerol (30 μ l in 300 μ l of FFS fraction) and 0.1% of bromophenol blue (10 μ l of a stock solution containing 3 g ml⁻¹) and loaded on to 1 cm wells.

FFS proteins were separated electrophoretically by applying a current of 30–50 mA per gel until the tracking dye reached the bottom edge of the gel (about 3–4 h). For the non-denaturing PAGE analysis, prior to application of samples, the gels were conditioned by pre-electrophoresis for 1 h to remove the harmful agents used in acrylamide polymerization.

Protein bands in the gels were stained for 3 h with Coomassie Blue and then destained. For the non-denaturing PAGE, the whole gel was sliced longitudinally into two halves, one containing the lanes with the standards and the other with lanes containing the FFS proteins; the half containing the standards was stained and the remaining half was quickly frozen on dry ice for subsequent bioassay of eluted proteins.

Assay for activity of proteins separated by non-denaturing PAGE

The activity of proteins localized on non-denaturing PAGE was tested by their effect on incorporation of thymidine into granulosa cell DNA in a short-term bioassay (8 h). The frozen gel was sectioned into 1 mm slices using a Mickles Gel Slicer (Brinkman Instruments, NY) and the slices were transferred to 10 mm × 75 mm glass tubes (five slices per tube) containing 700 μ l of cell culture buffer (McCoy's 5A with 1% fetal calf serum). The tubes were incubated at 37°C with gentle shaking for 6 h to elute the protein.

Aliquots of 500 μ l of the buffer containing the eluted proteins were added to 400 μ l of cell suspension containing

1.25×10^5 viable granulosa cells. The cells were incubated with the eluted FFS proteins for 36 h at 37°C and then [³H]thymidine was added (0.5 μ Ci per well in 0.1 ml) and incubation was continued for another 4 h. Incubation was terminated and the incorporation rate was measured as described previously.

Blood sampling and gonadotrophin assays

For determination of the exact timing of the gonadotrophin surges and their blockage, blood samples were collected from two groups of rats every 2 h, between 32 and 40 h after the first DES injection. One group received pentobarbital (37 mg kg⁻¹ body weight in 0.2 ml of saline) at 30 h; the intraperitoneally other group (controls) received saline only as vehicle. Concentrations of LH and FSH in serum samples were determined by the double antibody radioimmunoassay method as described by O'Connor *et al.* (1982) and Rao and Mahesh (1986). Purified LH and FSH standards and first antibodies for LH (NIAMDD-rLH-S7; dilution = 1:234 125) and FSH (NIAMDD-rFSH-S11; dilution = 1:125 000) were obtained from the Rat Pituitary Distribution Program of the National Institute of Arthritis, Metabolic and Digestive Diseases (NIAMDD). Second antibody (dilution = 1:250) was purchased from Amell Inc. (Brooklyn, NY). Standard curve ranges of the assays were 1–256 ng LH per tube and 4–512 ng FSH per tube and percentage bindings were 16–20 and 25–30%, respectively. Intra- and interassay variations were 5.8 and 10.8%, respectively, for FSH and 7.9 and 11.2%, respectively, for LH.

Statistical analysis

Results were analysed by one-way analysis of variance (ANOVA) and Tukey's multiple-range test using the STATGRAPHICS statistical program (Statistical Graphics Corporation, STSC, Inc).

Results

Effect of diethylstilboestrol treatment on [³H]thymidine incorporation and time of killing

Incorporation rates in granulosa cells from small follicles (Fig. 1) did not vary significantly with increasing number of DES injections or with variation in time of kill after two injections (Expt 1); but in the medium follicles, the significant stimulatory effect seen 24 h after the second injection was lost when 48 h was allowed to elapse between the second injection and killing. Furthermore, three and four injections were inhibitory to incorporation in these follicles, as reported earlier (Chakravorty *et al.*, 1991b). In the large follicles, significant stimulation was seen after two injections, but this effect was not sustained with increasing time or number of injections.

Effects of variation of time of killing after the second injection

Protein content of FFS (Fig. 2a) was measured and no significant differences were found in the small follicles with variation of time of killing after the second injection (Expt 2). Protein

Fig. 1. Incorporation of [^3H]thymidine in rat granulosa cells from small, medium and large follicles after (□) one, (■, ▨) two, (▩) three or (▧) four injections of diethylstilboestrol (2 mg per rat). Animals receiving two injections were killed either 24 (■) or 48 h (▨) after the second injection. Data are means $\pm$ SEM from four experiments, each with pooled ovaries from 10–12 rats. Values within each follicle size were compared between treatments and those for medium and large follicles were significantly different between treatment groups (* $P < 0.01$).

content of the large follicles increased 24 h after the second injection, but this increase was not maintained 48 h after the second injection. Protein content in the medium follicles increased at 48 h.

When tested for effects on [^3H]thymidine incorporation (Fig. 2b), FFS protein from the large follicles was stimulatory at 24 h after the second injection, but this effect was reduced after a further 24 h, indicating that it was transient. In contrast, FFS protein from the medium follicles had no effect at the earlier time, but significantly inhibited incorporation 48 h after the second DES treatment. FFS protein from the small follicles had no effect.

Protein content of FFS after treatment with pentobarbital to block gonadotrophin surges

It has been shown that two injections of diethylstilboestrol given to intact rats produce a gonadotrophin surge at 36 h (Chakravorty *et al.*, 1991a). The effect of these surges on FFS protein content and peptide activity was investigated by blocking both FSH and LH surges with pentobarbital, given at 30 h (Expt 3). When pentobarbital was given 6 h before the expected surge time and serum content of gonadotrophins was assayed every 2 h for the following 10 h, both FSH (Fig. 3a) and LH (Fig. 3b) were completely blocked. Removal of the gonadotrophin surges increased follicular protein content in the medium follicles (Fig. 4), but decreased it in the large follicles.

Effects of FFS protein after pentobarbital treatment

In animals that were killed 48 h after the second DES injection, FFS protein from medium follicles (Fig. 5a) significantly inhibited thymidine incorporation; 150 and 300 μg of unfractionated FFS protein inhibited incorporation in a dose-dependent manner. Furthermore, pentobarbital blockage of gonadotrophin surges

Fig. 2. (a) Protein content of small, medium and large rat follicles and (b) effects of the follicular fluid protein on [^3H]thymidine incorporation following one (□) or two injections of diethylstilboestrol (DES, 2 mg per rat), the latter groups being killed at 24 (■) or 48 h (▨) after the second injection. Data are means $\pm$ SEM from four experiments, each with pooled ovaries from 10–12 rats. Control values $\pm$ SEM in (b) are denoted by the shaded area. Values have been compared for each follicle size between treatment groups (* $P < 0.05$). Protein content and inhibitory activity of follicular fluid supernatant (FFS) from medium follicles increased at 48 h, whereas protein content increased in the large follicles and it was stimulatory at 24 h after the second DES injection.

did not affect the degree of inhibitory activity in the FFS; there was no significant difference between pentobarbital- or vehicle-treated groups. FFS protein from the medium and large follicles was stimulatory when FFS was collected 24 h after the second DES injection. The effect was greater in the large follicles and at a lower dose of the total protein (150 μg), but the stimulatory effects were lost when FFS was collected 48 h after the second injection. However, in the large follicles, pentobarbital treatment not only abolished the stimulatory effects seen with 150 μg (Fig. 5a), but was also significantly inhibitory at a dose of 300 μg of the total FFS protein (Fig. 5b). The decrease in protein synthesis in large follicles seen after gonadotrophin blockage (Fig. 4) could have stimulated granulosa cell proliferation particularly, since an increase in FFS protein in large follicles 12 h after the gonadotrophin surge correlated with a concomitant increase in thymidine incorporation with 150 μg of the protein at this time. Furthermore, a decline in concentration or a loss of activity of this peptide took place with time,

Fig. 3. Serum concentrations of (a) follicle-stimulating hormone (FSH) and (b) luteinizing hormone (LH) following treatment of rats with vehicle (□) or pentobarbital (■, 37 mg kg⁻¹ body weight), given 6 h after the second diethylstilboestrol (DES) injection. Data are means $\pm$ SEM from two experiments, each with nine animals per treatment group. *Values significantly different from controls ($P < 0.01$).

Fig. 4. Protein content of follicular fluid supernatant (FFS) from small, medium and large rat follicles after two injections of diethylstilboestrol (DES; 2 mg per rat) at 0 and 24 h followed by saline (■) or pentobarbital (▨) treatment at 30 h. The animals were killed 48 h after the second DES injection. Data are means $\pm$ SEM from four experiments, each with pooled ovaries from 10–12 rats. Comparisons were made for each follicle size between treatments and protein content increased in medium follicles, but decreased in large follicles significantly (* $P < 0.05$) after blockage of gonadotrophins.

Fig. 5. Effects of 150 and 300 μg of follicular fluid supernatant (FFS) from (a) medium and (b) large rat follicles on [³H]thymidine incorporation, after treatment with diethylstilboestrol (DES, 2 mg per rat). The treatment groups were (□) one injection, killed at 24 h; (■) two injections, killed at 24 h; (▨) two injections plus saline, killed at 48 h; and (▩) two injections plus pentobarbital, killed at 48 h. All times of killings depict time since the last injection. Data are means $\pm$ SEM from four experiments. The shaded area represents control $\pm$ SEM values. *Values significantly different from controls ($P < 0.01$). Values with similar superscripts are significantly different from each other (* $P < 0.05$). FFS protein (150 and 300 μg) from medium follicles inhibited incorporation 48 h after the second DES injection, irrespective of whether gonadotrophins were present or blocked. The inhibition seen with 300 μg FFS from medium follicles was significantly greater when gonadotrophins were absent.

resulting in the absence of activity at 48 h after the second injection (36 h after the surge). However, 300 μg of this protein was not stimulatory, probably because, at this higher dose, the concentration of GCMP was sufficient to override the stimulatory effects. It is possible that some of the stimulatory factor was also synthesized in the medium follicles, but its effects were undetectable because of the higher concentration of GCMP in these follicles.

Number of follicles and granulosa cells after pentobarbital blockage of gonadotrophin surges

The number of medium and large follicles (Table 1) increased after two DES injections. This increase did not occur when the

Table 1. Number of rat follicles and granulosa cells after one or two diethylstilboestrol injections, with or without pentobarbital treatment after the second injection

Follicle size	Injections		
	1	2	2 + pentobarbital
Number of follicles per ovary			
Small	146 ± 20	142 ± 7	166 ± 32
Medium	24 ± 3	46 ± 3 ^a	26 ± 4
Large	4 ± 1	8 ± 2 ^a	5 ± 1
Number of granulosa cells per follicle			
Small	310 ± 19	306 ± 11	295 ± 13
Medium	1764 ± 82	2028 ± 106	1886 ± 57
Large	3400 ± 125 ^b	5526 ± 250 ^b	1876 ± 106 ^b

Values are means ± SEM from four experiments, each with 10–12 rats per treatment group.
Values significantly different from each other within each follicle size have the same superscript letters (a, b; *P* < 0.05).

Fig. 7. Effects of microconcentrated follicular fluid supernatant on [³H]thymidine incorporation after separation into three fractions of differing molecular mass. Follicular fluid supernatant was collected after pooling all three sizes of follicles to maximize recovery. Data, expressed as percentage of inhibition or stimulation over controls, are means ± SEM from three experiments, each with pooled ovaries from 10–12 rats (**P* < 0.01).

Fig. 6. Effects of follicular fluid supernatant (FFS) on thymidine incorporation after treatment with diethylstilboestrol (DES) or pregnant mares' serum gonadotrophin (PMSG). Rats were treated with DES (four injections), or PMSG and killed 24 or 48 h later. FFS protein (250 µg) from medium follicles was tested for inhibitory effects on [³H]thymidine incorporation. Each value represents mean ± SEM from three experiments, each with 10 animals per treatment group. Values significantly different from controls are denoted by asterisks (**P* < 0.01; ***P* < 0.05).

gonadotrophins were blocked by pentobarbital treatment of the DES-treated rats. Similarly, the number of granulosa cells increased significantly after two injections, but this increase was prevented by pentobarbital blockage of gonadotrophins. There were no significant differences in number of granulosa cells in the medium or small follicles, suggesting that the gonadotrophins were affecting only the large follicles.

Fig. 8. Dose-dependent effects of rat follicular fluid supernatant fractions (a) < 10 kDa and (b) > 10 kDa on [³H]thymidine incorporation. Data are means ± SEM from three experiments, each with pooled ovaries from 10–12 animals. *Values significantly different from controls (*P* < 0.05).

Fig. 9. SDS-polyacrylamide gel electrophoresis of microconcentrated rat follicular fluid supernatant (FFS) proteins showing migration profiles of standard proteins (5–7 (a) and 1–5 (b)). Lanes 4 and 5 in (b) represent one particular standard with molecular mass 6.5 kDa from the Sigma GF 70 set of standards. Lanes 1–4 (a) and 6–8 (b) are FFS samples having different molecular mass ranges. Diffuse bands of proteins are seen in lanes 3 and 4 in (a) and lane 6 in (b) because of low amounts of protein loaded in each lane.

Effect of PMSG treatment on activity of FFS peptide

The studies with pentobarbital suggested that gonadotrophins had no modulatory role on the actions of the inhibitory peptide. They also suggested that the peptide was induced by oestrogen. FFS from medium follicles from PMSG-treated ovaries was tested for the presence of inhibitory activity at 24 and 48 h after treatment (Expt 4): 300 µg of FFS protein was inhibitory to thymidine incorporation only after 48 h of PMSG treatment (Fig. 6), suggesting that inhibitory activity was present when endogenous oestradiol concentrations were high (Chakravorty *et al.*, 1991a).

Effects of fractionated FFS protein

For these experiments, follicles were isolated and all sizes were pooled for collection and fractionation of FFS protein.

Fig. 10. Effects of rat follicular fluid supernatant proteins with molecular mass range of (a) <10 kDa and (b) >10 kDa on [³H]thymidine incorporation after separation by non-denaturing polyacrylamide gel electrophoresis and elution from the 0.5 cm gel slices. This experiment was repeated and identical results were obtained, but data from only one experiment are shown. The horizontal line represents control values. *Values significantly different from controls (**P* < 0.05).

Proteins with molecular masses <10 kDa were inhibitory, while proteins with molecular masses >30 kDa had significant stimulatory effects (Fig. 7). The proteins with molecular masses 10–30 kDa were inhibitory, but this inhibition was not statistically significant. Different doses of the partially purified FFS protein fractions were tested for their effects on [³H]thymidine incorporation. Doses between 4 and 16 µg of the fraction of molecular mass <10 kDa showed a linear dose-dependent inhibition (Fig. 8a). A dose of 60–90 µg of the >30 kDa fraction was stimulatory (Fig. 8b). Non-specific effects of blood-borne factors in FFS were eliminated by fractionating serum from rats treated with four DES injections into ranges of <10 kDa and >10 kDa, and 100 µg of each fraction was tested for effects on [³H]thymidine incorporation. Both fractions stimulated incorporation rates by 20–30% over controls (data not shown).

PAGE of FFS fractions

Of the three fractions obtained after microconcentration of FFS, the >30 kDa fraction showed several well-defined bands (Fig. 9), but these were not seen in the other <10 kDa fraction. However, this fraction showed diffuse protein bands in the

appropriate molecular mass ranges, confirming accuracy of microconcentration.

Effects of proteins eluted from non-denaturing PAGE

FFS proteins were fractionated into those with molecular mass $<$ and $>$ 10 kDa. Each fraction was further separated electrophoretically by a non-denaturing polyacrylamide gel. The separated proteins were eluted from the gels and tested for effects on [3 H]thymidine incorporation (Fig. 10). Two peaks of inhibitory activity were found in the $<$ 10 kDa fraction, corresponding to 0.9–1.3 kDa and 1.7–2.3 kDa. Two peaks of stimulatory activity were detected with molecular mass $>$ 10 kDa, corresponding to molecular masses of 22–26 and 34–38 kDa.

Discussion

The role of hormones in the regulation of mitosis of granulosa cells during follicle development is unclear. Gonadotrophins affect granulosa cell proliferation only in certain stages of follicles in hamsters (Roy and Greenwald, 1988), whereas, in rats, follicles having diameters $>$ 350–400 μ m are affected maximally by the FSH surge (Hirshfield, 1985). FSH and oestradiol treatment of hypophysectomized rats results in rapid granulosa cell proliferation and follicle growth (Rao *et al.*, 1978), but the stimulatory effects are not sustained with prolonged treatment, suggesting that, even though these hormones are mitogenic, their actions are limited. When the FSH surge is prevented by barbiturates, there are fewer follicles of $>$ 450 μ m in diameter at oestrus, suggesting that, besides recruiting primary follicles, FSH stimulates granulosa cell multiplication in large ($>$ 400 μ m) follicles. The findings of the present study support this concept by showing that gonadotrophin blockage leads to a reduction in the number of granulosa cells in large follicles.

The second established mitogenic stimulus for granulosa cell proliferation is oestrogen. We have reported that oestrogen can be stimulatory as well as inhibitory depending on follicle size and that inhibition of medium follicles was via a peptide (Chakravorty *et al.*, 1991b), which we have named GCMP. The present study confirms the existence of GCMP not only in the DES model, but also in a commonly used, more physiological model: the PMSG-treated immature rat model. In both models, inhibitory activity was found 48 h after the last injection, only after oestrogen concentrations had been high for some time. In addition, a transient stimulatory effect was seen with FFS from large follicles, which was possibly due to the gonadotrophin surge that occurred 12 h after the second DES injection. Since LH has been shown to inhibit follicle growth in hamsters (Roy and Greenwald, 1986b), it is also likely that the gonadotrophins, and not DES, were responsible for modulating the activity of GCMP. We tested this hypothesis by collecting FFS protein from follicles of different sizes after oestrogen treatment, and testing for effects on [3 H]thymidine incorporation, in the presence and absence of gonadotrophins. Hence, the objective of the study was to investigate gonadotrophic and oestrogenic regulation of FFS factors and their characterization.

Our results suggest that the stimulatory peptide was dependent upon gonadotrophins (probably FSH) and that the

production of GCMP was dependent upon oestrogen. This was confirmed by using pentobarbital to block the gonadotrophins. We found that the effect of GCMP predominated when the synthesis of the stimulatory peptide was prevented by blocking gonadotrophins. The stimulatory peptide had an effect only when the more potent GCMP was present at a lower concentration in the large follicles. Pentobarbital caused an increase in protein in medium follicles and a decrease in protein in large follicles. Since such a differential effect of pentobarbital was seen on FFS protein concentrations in follicles of different sizes, it is unlikely that pentobarbital caused a non-specific effect on protein synthesis. However, pentobarbital is known to have a deleterious effect on ovarian physiology, inducing atresia of follicles (Terranova, 1980; Uilenbroek *et al.*, 1980); the effect of pentobarbital on granulosa cells seen in the present study could be a generalized one, further augmenting the oestrogenic effect. We have already expressed reservations about the DES model (Chakravorty *et al.*, 1991a), and, hence, the PMSG model was also used to demonstrate the effects of GCMP. The DES/pentobarbital model was used only to generate enough GCMP for further characterization.

The effects of the peptide were also reflected in numbers of follicles and granulosa cells (Table 1). The increase in number of medium follicles seen at 48 h in controls was probably due to FSH driving the growth of smaller follicles into the medium-sized pool resulting in an increase in the number of medium follicles. This increase did not occur when FSH was blocked. However, the total number of granulosa cells was not affected; we propose that, at this stage, FSH is not the primary mitogenic stimulus in medium follicles. Rather, it is oestrogen, particularly because at this time the concentrations of GCMP were probably not sufficient to retard DNA synthesis and cell proliferation. Hence, there was no decrease in number of granulosa cells in the medium follicles, thereby demonstrating an apparent dissociation between numbers of granulosa cells and follicles in the FSH-deficient animals. Numbers of granulosa cells and large follicles increased in the presence of FSH and significantly decreased when FSH was blocked, suggesting that gonadotrophins were affecting the large follicles maximally and specifically.

Oestrogen and gonadotrophin modulation of follicle growth via the synthesis of GCMP and the stimulatory peptide found in this study could account for the follicle growth patterns reported in rats and hamsters. In rats, Hirshfield and Midgley (1978) have shown that, while follicles of $<$ 390 μ m diameter are present on all days of the cycle, there is a discontinuous movement of follicles of this size range into the pre-Graafian size range. Follicles of 390–500 μ m were totally absent at proestrus, but appeared at oestrus. This could be due to the FSH surge on the afternoon of proestrus, stimulating the production of a growth stimulatory factor which causes follicles of 350–450 μ m to grow. The cessation of growth of follicles of $<$ 390 μ m at proestrus could be due to the presence of GCMP in these medium-sized follicles. According to Hirshfield (1983), the size range of 350–450 μ m is an FSH-regulated turning point in the developmental process. Prior to this stage, intra-ovarian factors such as high oestrogen or androgens may be more important in determining granulosa cell proliferation. According to the findings of Hirshfield (1983), by the time the follicles have reached a diameter of 390 μ m, 'selection' has

already taken place and apparently, at this point, the follicles require high FSH for only a short period. This would explain the transient nature of activity of the gonadotrophin-induced stimulatory peptide seen in the present study. Moreover, the stage of development which is dependent upon high concentrations of FSH corresponds approximately to the time when the follicles are 350–500 µm in diameter (Hirshfield, 1983). The FSH dependency of this class of follicle (classified as large in the present study) could be due to the proposed stimulatory factor.

In hamsters, it has been shown that DNA synthesis following the FSH surge differs with the stage of the follicle development (Roy and Greenwald, 1986a). The proestrus rise in FSH-stimulated DNA synthesis was evident in stages 2–4 and 9 and 10, while stages 5–7 were unaffected. The same pattern was seen when FSH was given exogenously (Roy and Greenwald, 1986b). These findings once again demonstrated that only the early and penultimate stages of granulosa cell mitosis during follicle growth are regulated by FSH, while the intermediate stages are under intragonadal control.

Physical and biochemical characterization of the FFS collected from the different-sized follicles was difficult because of the extremely limited quantities of the samples. However, partial characterization of the two peptides was attempted first by microconcentration and then by PAGE. We found that the inhibitory peptide (GCMP) was localized in the 10 kDa fraction. A similar protein of molecular mass 10 kDa is present in ovine follicular fluid which inhibits [³H]thymidine incorporation in fibroblasts (Carson *et al.*, 1988). The stimulatory effects were found in the molecular mass range of > 30 kDa. Dose-dependent effects were seen with both fractions, but were more pronounced with < 10 kDa proteins, supporting our previous postulation that inhibitory effects of GCMP were more potent and that GCMP was present at a greater concentration, leading to overriding of the effects of the stimulatory peptide.

It has been hypothesized that several angiogenic factors are produced in the ovary in response to gonadotrophins and oestrogens causing hyperaemia of the ovary (Koos and Le Maire, 1983). The possibility that the inhibitory peptide is blood-borne was tested by fractionating serum from rats treated with four DES injections, and effects of the fractions on [³H]thymidine incorporation were tested. Corresponding serum fractions did not exhibit any inhibitory effects, suggesting that the factor was of intra-ovarian origin and not present in an ultrafiltrate of serum.

Experiments using SDS-PAGE were not successful in determining the exact molecular masses of the two peptides. Well-defined bands were not evident, particularly with the < 10 kDa fraction probably because only a small amount of the proteins (25–50 µg) was loaded on to the gel and because the proteins did not withstand denaturing with SDS. However, SDS-PAGE did verify the purity of the different fractions obtained by microconcentration. The two peaks of inhibitory activity could be GCMP (1.8–2.3 kDa) and a degradation product or a monomeric form of GCMP (0.9–1.2 kDa). The two peaks of stimulatory activity, one corresponding to 22–26 kDa and the other to 34–38 kDa are likely to be transforming growth factor-β (a homodimeric 25 kDa protein) and the FSH-dependent stimulatory peptide found in the present study (about 36 kDa). It has been shown that transforming growth factor-β stimulates DNA synthesis in rat granulosa cells

(Dorrington *et al.*, 1988); Knecht *et al.* (1986) have shown that FSH does stimulate the synthesis of proteins of molecular masses 25 and 36 kDa among several other proteins, in granulosa cell cultures. They also found that continued presence of FSH or high concentrations of cAMP were required to maintain the synthesis of these proteins. This could explain the transient production of the stimulatory 30 kDa peptide found 12 h after the gonadotrophin surge in the present study and by loss of activity with time and after blocking the FSH surge.

It has become evident that granulosa cells in large follicles form a heterogeneous population, with distinctive differences between peripheral, antral and periculus cells with regard to enzyme content, morphological characteristics, receptor content and mitogenic properties (Greenwald and Terranova, 1988). We have not attempted to relate the effects of the FFS peptides to the different granulosa cell types, but mitotic properties of the peri-antral granulosa cells show evidence of being influenced by the surrounding follicular fluid. These cells, which are in direct contact with follicular fluid components, proliferate the most, showing extensive labelling (Hirshfield, 1986). Incipient atretic changes are also noted first in these cells, the earliest reflection of which is reduced [³H]thymidine uptake (Hay *et al.*, 1979; Peluso *et al.*, 1980).

Several peptides regulate granulosa cell function (Savion and Gospodarowicz, 1980; Richards *et al.*, 1983). Most of these proteins are detected by their biological properties, but little is known about their hormonal regulation. Each phase of granulosa cell proliferation is said to be associated with the induction of specific proteins in response to separate hormonal cues. Expression of these proteins could be time-dependent and could vary with the stage of follicle development (Knecht *et al.*, 1986). Characterization and identification of these ovarian proteins would result in a better understanding of the processes involved in granulosa cell mitosis – a key feature in follicle growth. The data presented in this study show the presence of both positive and negative autocrine/paracrine regulators of granulosa cell mitosis, which are under the control of different hormones. Both FSH and oestrogens are mitogenic *in vivo*, but neither hormone is effective *in vitro* (Richards, 1979), suggesting that specific factors are induced locally within the gonad by these hormones. A recent report has shown the presence of several mitotic regulators within follicles (Hammond and English, 1987). In the present study, the detection of both stimulatory and inhibitory peptides in follicular fluid supernatant, which are controlled by separate hormones, shows that granulosa cell proliferation is under multifactorial regulation. The process is not simple but is dictated by several regulatory elements (May *et al.*, 1990). The relative concentration of the two peptides reported here may be instrumental in directing the process of granulosa cell proliferation, while other factors, such as the number of granulosa cells in the follicle and the concentration of such steroids as androgens, may be, indirectly, contributing to the process by creating an environment conducive to either peptide action. We suggest that medium follicle growth is regulated primarily by the oestrogen-induced inhibitory peptide that we have named 'granulosa cell mitostatic peptide (GCMP)'. This peptide is potent enough to make cessation of mitosis a rule rather than an exception in medium follicles and, hence, the majority of these follicles are directed towards the atretic pathway. Only medium follicles that have a lower concentration of GCMP and, hence,

possess a full complement of granulosa cells (McNatty *et al.*, 1979) are capable of further growth into large follicles. These large follicles are controlled primarily by gonadotrophins, particularly FSH via the synthesis of stimulatory peptide. Hence, our study demonstrates the presence of two opposing factors acting together to strike a delicate balance in regulating the extent of granulosa cell proliferation and thereby determining the ultimate fate of the follicles.

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References

- Adashi EY, Resnick CE, Brodie AMH, Svoboda ME and Van Wyk JJ (1985) Somatomedin-c mediated potentiation of FSH-induced aromatase activity of cultured rat granulosa cells *Endocrinology* **117** 2313–2320
- Bogovich K, Richards JS and Reichert LE (1981) Obligatory role of luteinizing hormone (LH) in the initiation of preovulatory follicular growth in the pregnant rat: specific effects of human chorionic gonadotrophin and follicle stimulating hormone on LH receptor and steroidogenesis in theca, granulosa and luteal cells *Endocrinology* **109** 860–867
- Carson RS, Robertson DM and Findlay JK (1988) Ovine follicular fluid inhibits thymidine incorporation by 3T3 fibroblasts *in vitro* *Journal of Reproduction and Fertility* **82** 447–455
- Chakravorty A, Mahesh VB and Mills TM (1991a) Regulation of follicular development by diethylstilboestrol in ovaries of immature rats. *Journal of Reproduction and Fertility* **92** 307–321
- Chakravorty A, Mahesh VB and Mills TM (1991b) Inhibition by diethylstilboestrol of proliferative potential of follicles of different sizes in immature rat ovaries *Journal of Reproduction and Fertility* **92** 323–332
- Dorrington JH, Chuma AV and Bendell JJ (1988) Transforming growth factor- β and follicle stimulating hormone promote rat granulosa cell proliferation *Endocrinology* **123** 353–359
- Gospodarowicz D and Bialecki H (1979) Fibroblast and epidermal growth factors are mitogenic agents for cultured granulosa cells of rodent, porcine and human origin *Endocrinology* **104** 757–761
- Greenwald GS and Terranova PF (1988) Follicular selection and its control. In *The Physiology of Reproduction* Vol. 1 pp 387–446 Eds E Knobil and JD Neill. Raven Press, New York
- Hammond JM and English HF (1987) Regulation of deoxyribonucleic acid synthesis in cultured porcine granulosa cells by growth factors and hormones *Endocrinology* **120** 1039–1046
- Hay ME, Moor RM, Cran DG and Dott HM (1979) Regeneration of atretic sheep ovarian follicles *Journal of Reproduction and Fertility* **55** 195–207
- Hirshfield AN (1983) Compensatory ovarian hypertrophy in the long-term hemicastrate rat: size distribution of growing and atretic follicles *Biology of Reproduction* **28** 271–278
- Hirshfield AN (1985) Comparison of granulosa cell proliferation in small follicles of hypophysectomized, prepubertal and mature rats *Biology of Reproduction* **32** 979–987
- Hirshfield AN (1986) Patterns of [3 H]thymidine incorporation differ in immature rats and mature cycling rats *Biology of Reproduction* **34** 229–235
- Hirshfield AN and Midgley AR, Jr (1978) Morphometric analysis of follicular development in the rat *Biology of Reproduction* **19** 597–605
- Knecht M, Shinohara D and Catt KJ (1986) Biosynthesis of cellular and secreted proteins during follicle stimulating hormone-induced granulosa cell differentiation *Endocrinology* **119** 1388–1396
- Koos RD and LeMaire WJ (1983) Factors that may regulate the growth and regression of blood vessels in the ovary *Seminars in Reproductive Endocrinology* **1** 295–307
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of Bacteriophage T4 *Nature* **227** 680–685
- Lobb DK, Skinner MK and Dorrington JD (1988) Rat thecal/interstitial cells produce a mitogenic activity that promotes the growth of granulosa cells *Molecular and Cellular Endocrinology* **55** 209–215
- Lowry OH, Rosebrough HF, Farr HL and Randall RF (1951) Protein measurement with the Folin phenol reagent *Journal of Biological Chemistry* **143** 257–269
- McNatty KP, Smith D, Makris A, Osathanondh R and Ryan KJ (1979) The microenvironment of the human antral follicle: interrelationships among the steroid levels in the antral fluid, the population of granulosa cells, and the status of the oocyte *Journal of Clinical Endocrinology and Metabolism* **49** 851–860
- Maizel JV (1971) Polyacrylamide gel electrophoresis of viral proteins. In *Methods in Virology* pp 179–246 Eds K Maramorosch and H Koprowski. Academic Press, NY
- May JV, Frost JP and Bridge AJ (1990) Regulation of granulosa cell proliferation: facilitative roles of platelet-derived growth factor and low density lipoproteins *Endocrinology* **126** 2896–2905
- O'Connor JL, Wolfe RR, Allen MB and Mahesh VB (1982) Effects of neonatal androgenization on the LHRH response of dispersed pituitary cell cultures *Archives of Andrology* **8** 29–35
- Peluso JJ, England-Charlesworth C, Bolender DL and Steger RW (1980) Ultrastructural alterations associated with the initiation of follicular atresia *Cell and Tissue Research* **211** 105–115
- Rao IM and Mahesh VB (1986) Role of progesterone in the modulation of the preovulatory surge of gonadotropins and ovulation in the pregnant mares serum gonadotropin primed immature rat and the adult rat *Biology of Reproduction* **35** 1154–1161
- Rao MC, Midgley AR, Jr and Richards JS (1978) Hormonal regulation of ovarian cellular proliferation *Cell* **14** 71–78
- Richards JS (1979) Hormonal control of ovarian follicular development: a 1978 perspective *Recent Progress in Hormone Research* **35** 343–373
- Richards JS (1980) Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation *Physiological Reviews* **60** 51–73
- Richards JS, Sehgal N and Tash JS (1983) Changes in content and cAMP dependent phosphorylation of specific proteins in granulosa cells of preantral and preovulatory ovarian follicles and in corpora lutea *Journal of Biological Chemistry* **258** 5227–5232
- Roy SK and Greenwald GS (1986a) A quantitative analysis of *in-vitro* incorporation of [3 H]thymidine into hamster follicles during the oestrous cycle *Journal of Reproduction and Fertility* **77** 143–152
- Roy SK and Greenwald GS (1986b) The effects of FSH and LH on incorporation of [3 H]thymidine into follicular DNA *Journal of Reproduction and Fertility* **78** 201–209
- Roy SK and Greenwald GS (1988) The effects of follicle stimulating hormone, luteinizing hormone and prolactin on follicular deoxyribonucleic acid synthesis in the hamster *Endocrinology* **122** 952–958
- Savio N and Gospodarowicz D (1980) Pattern of cellular peptide synthesis by cultured bovine granulosa cells *Endocrinology* **107** 1798–1806
- Terranova PF (1980) Effects of phenobarbital-induced ovulatory delay on the follicular population and serum levels of steroids and gonadotrophins in the hamster: a model for atresia *Biology of Reproduction* **23** 92–99
- Uilenbroek JTJ, Woutersen PJA and van der Schoot P (1980) Atresia of pre-ovulatory follicles: gonadotrophin binding and steroidogenic activity *Biology of Reproduction* **23** 219–229