Hyaluronan Synthesis by Mouse Cumulus Cells Is Regulated by Interactions between Follicle-stimulating Hormone (or Epidermal Growth Factor) and a Soluble Oocyte Factor (or Transforming Growth Factor β1)*

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Expansion of the cumulus cell-oocyte complex (COC) in the preovulatory mammalian follicle requires a transient induction of hyaluronan (HA) synthesis by the cumulus cells. We studied the interactions of known factors that regulate this process by isolating compact COCs from mice and inducing their expansion in vitro. Maximum HA synthesis requires either follicle-stimulating hormone (FSH) or epidermal growth factor (EGF) in combination with either a soluble factor(s) produced by the oocyte or transforming growth factor β1. FSH (or EGF) exerts its effects during the first 2 h of incubation, before HA synthesis actually begins. The oocyte factor(s) or transforming growth factor β1) exerts its effects from 2 h onwards and must be continuously present throughout the subsequent ~10 h to achieve a maximum level of HA synthesis. FSH stimulates intracellular cAMP synthesis, which correlates with net HA production up to ~14 fmol/COC at 5 ng/ml FSH; however, higher concentrations of FSH increase cAMP levels ~10-fold higher with no additional effect on HA synthesis. EGF at saturating concentrations for HA synthesis does not stimulate cAMP above basal levels. Tyrosine kinase inhibitors genistein and tyrphostin AG18 nearly abolish the HA synthesis response to EGF and inhibit the response to FSH by ~60%, suggesting that a tyrosine kinase activity is involved for both factors, whereas FSH also operates partially through another signaling pathway. Actinomycin D abolishes HA synthesis if added at the beginning of culture and reduces HA synthesis by ~50% if added between 6–12 h when HA synthesis is normally maximal. The results suggest that regulation of HA synthesis is primarily controlled at the transcriptional level.

In the mammalian preovulatory follicle, the cumulus oophorus expands dramatically when the cumulus cells (CCs), consisting of a few layers of granulosa cells closely surrounding the oocyte, are induced to synthesize large amounts of hyaluronan (HA) (1). The secreted HA interacts with specific matrix components, thereby forming a highly hydrated and viscoelastic matrix in the intercellular spaces, which results in an ~20-fold increase in volume of the cumulus cell-oocyte complex (COC) (2–4). Recent findings show that CCs synthesize a dermatan sulfate (DS) proteoglycan with high molecular weight that also accumulates in the extracellular matrix, most likely through specific binding to HA (5). The expansion of the COC may facilitate the extrusion of the oocyte through the ruptured follicle wall during ovulation and assist its capture by the oviductal fimbriae and entry into the oviduct (6, 7). Moreover, HA and DS proteoglycans may contribute to the success of oocyte fertilization by stabilizing the structure of the egg zona pellucida (8) and by stimulating sperm activation (9 and motility (10, 11).

Presently, it is not known if COC expansion in vivo is induced directly by gonadotropins or is mediated indirectly by the mural granulosa cells (12). However, the time course of mouse COC expansion and HA synthesis induced in vitro by follicle-stimulating hormone (FSH) is nearly the same as that in vivo, and net production of HA per cell is essentially the same (1). Studies in vitro have also shown that CCs dissected from mouse COCs and cultured without oocytes do not produce significantly higher amounts of HA when stimulated with FSH but do so when oocytes or oocyte-conditioned medium are also present (13). Thus, synthesis of HA by mouse CCs in vitro requires the combined action of FSH and a soluble factor(s) produced by the oocyte. Conversely, FSH induces CCs to increase the synthesis of DS independent of the presence of oocytes (13). A transient increase of intracellular cAMP is triggered by FSH treatment (14), and dbcAMP, a synthetic analogue of cAMP, induces similar effects on both HA and DS synthesis (15), suggesting that FSH action is mediated by this cyclic nucleotide.

Epidermal growth factor (EGF) can substitute for FSH, and transforming growth factor β1 (TGFβ1) can substitute for the oocyte factor(s) in promoting these CC functions (16). Like the oocyte factor(s), TGFβ1 synergizes with FSH (or EGF) to increase HA production, and EGF, like FSH or dbcAMP, requires the presence of the oocyte factor(s) (or TGFβ1) to induce HA synthesis while inducing DS synthesis even in their absence (16). It is not known to what extent FSH and EGF share
common intracellular signaling pathways for inducing HA and DS synthesis by CCs. EGF operates primarily through tyrosine kinase pathways and is generally considered to exert its effects independent of cAMP pathways. However, a slight but significant increase of intracellular levels of cAMP has been observed in EGF-stimulated mouse COCs (17). On the other hand, it has been suggested that a protein tyrosine kinase activity is involved in the cAMP-dependent signal transduction pathway of gonadotropins in promoting differentiation (18) and luteinization of granulosa cells, as well as in events required for ovulation (20, 21).

The aims of the present study were to investigate at which intracellular level the oocyte factor(s) (or TGFβ1 and FSH or EGF) interact to control HA and DS synthesis and to define the roles of cAMP and tyrosine kinase activity in the signal transduction pathways elicited by FSH and EGF that are involved in the activation of these functions in COCs.

EXPERIMENTAL PROCEDURES

Materials—Female Swiss CD-1 mice were obtained from Charles River. Pregnant mares’ serum gonadotropin (PMSG) was from Intervet (Boxmeer, Holland). Papain, dbcAMP, t-glutamine, genistein, dimethylsulfoxide (type 0X), and 3-isobutyl-1-methylxanthine were purchased from Sigma. Eagle’s minimum essential medium with Earle’s salt, Hepes, and fetal bovine serum (FBS) were from Life Technologies, Inc. Bovine serum albumin was from Miles (Milano, Italy). Gentamicin sulfate was from Whittaker Bioproducts (Walkersville, MA). Chondroitin ABC (Proteus vulgaris) and chondroitin sulfate disaccharides (ΔDI-6S, ΔDI-4S, and ΔDI-6S) were from Seikagaku Kogyo (Tokyo, Japan). β-[3H(N)]Glucosamine (30–40 Ci/mmol) and [35S]sulfate (3 Ci/mmol) were added to the culture medium. In the FSH, EGF, and dbcAMP time course experiments, culture media were collected at different times, and the cultures were washed three times with 20 μl of basal medium containing radiolabeled precursors at identical concentrations as in the initial medium and then incubated for the remaining time in basal medium containing the same concentrations of radiolabeled precursors. The same procedure was used in the TGFβ1 time course experiments except that FSH was present with the radiolabel precursors in the culture medium used for washing and subsequent culturing.

All manipulation steps of extraction were done under dimethylpolysiloxane. After labeling, 20 μl of a papain solution (750 mIU) was added, and the cultures were incubated for 1 h at 65°C. The extraction was completed by adding 40 μl of guanidine HCl containing 4% (w/v) Triton X-100 and 40 μl of 4% guanidine HCl containing 4% (w/v) Triton X-100. Each extract (~120 μl) was transferred to a tube and was stored frozen until further analysis.

Quantitation of HA and DS—Each extract was heated at 100°C for 3 min to inactivate the papain. Bovine serum albumin and HA (100 μg of each) were added to improve recovery of radiolabeled macromolecules during subsequent gel filtration procedures. Each extract was diluted to 500 μl by adding 0.1 m Tris, 0.1 m sodium acetate, pH 7.3, followed by elution on a column of Sephadex G-50 (2 ml bed volume) equilibrated with 0.1 m Tris, 0.1 m sodium acetate, 0.5% Triton X-100, pH 7.3. This step removes the unincorporated radioisotopes and exchanges the guanidine HCl for a buffer suitable for chondroitinase digestion. The macromolecules in the excluded volume fraction were digested for 1 h by adding 20 μl (0.1 unit) of chondroitinase ABC dissolved in 0.1 m Tris, 0.1 m sodium acetate, pH 7.3. Approximately one-third of each digested sample was chromatographed on a column of Sephadex G-50 (4 ml bed volume), equilibrated, and eluted with the same buffer used for the first elution. The excluded and included fractions were counted for radioactivity to determine the proportions of the radiolabeled macromolecules digested by the enzyme. The remaining portion of each sample was analyzed by high pressure liquid chromatography (HPLC) to determine the relative amounts of HA and DS disaccharides (22). For this purpose, each sample was treated with Bio-beads to remove Triton X-100 and then with 100% ethanol (sample: ethanol in a ratio 1:4) to precipitate undigested macromolecules. After centrifugation at 12,000 rpm for 10 min the supernatant was collected and concentrated to a final volume of ~20 μl under vacuum on a Speed Vac concentrator (Savant). The concentrated sample was applied to the HPLC column after the addition of ΔDI-6S, ΔDI-4S, and ΔDI-6S disaccharide standards (5 μg of each) as internal standards. The ΔDI-HA standard in each sample was derived from the enzymatic digestion of the carrier HA. Each sample was eluted on a column of Partisil PAC equilibrated with acetoni-trile/0.1 M tris(hydroxymethyl)aminomethane buffer in a ratio of 22:78. The aqueous buffer contained 0.5 m Tris, 0.1 m boric acid, pH 8. Fractions of 0.7 ml were collected, and radioactivities in each were determined with a Beckman LS 3801 counter. Differentiation of 35S and H activities was done by calculating 35S spill-over into the H channel using 35S standards prepared for each set of samples. The mass of HA and DS synthesized during the labeling period (in hexosamine equivalents) was determined as described previously (23) by calculating the specific activity of the UDP-N-acetylhexosamine pools from the ratio of H to 35S in the ΔDI-4S and multiplying the total 3H dpm digested by chondroitinase (Sephadex G-50 analysis) by the proportion of H in ΔDI-HA (Partisil PAC analysis).

cAMP Assay—Intracellular cAMP levels were measured by a radioimmunoassay (24). Briefly, COCs were incubated in a 20-μl culture drop in the presence of FSH or EGF at the concentrations specified in the text. After 1 h of incubation, the media were removed, and COCs were then washed twice with 50 μl of Eagle’s minimum essential medium buffered with Hepes containing 1 mM 3-isobutyl-1-methylxanthine in order to inhibit phosphodiesterase activity and prevent cAMP hydrolysis. Intracellular cAMP was extracted by adding 150 μl of ice-cold 15% (vol/vol) trichloroacetic acid to the culture drop, and subsequently the extracts were centrifuged at 12,000 rpm at 4°C for 10 min, and the supernatants were collected and extracted twice with 5 vol of water saturated with diethylther. Samples were acetylated before the assay following the procedure of Harper and Brooker (25). The radioimmunoassay had a sensitivity of 2–4 fmol of cAMP, an intra-assay coefficient of variation of 5.0%, and an interassay coefficient of variation of 10.3%.
RESULTS

Dose Dependence of FSH and EGF, Individually or in Combination, on HA and DS Synthesis—COCs isolated from PMSG-primed mice were cultured in the presence of increasing concentrations of FSH or EGF or combinations of both for 18 h, a period of time required for full expansion in vitro and for maximal HA and DS accumulation in the COC matrix. [3H]Glucosamine and [35S]sulfate were used as labeling precursors, and net synthesis of HA and DS was determined as described under “Experimental Procedures.” Control cultures were incubated without either FSH or EGF. The bracket bars indicate the S.E. for three cultures at each point. Hcn, hexosamine.

values were subtracted from all determinations. Between 4 and 80 COCs were used in each determination.

Statistics—All data shown are means of at least duplicate determinations. The statistical significance of differences between two groups of data was evaluated by the Student’s t test; differences were considered significant when P values were smaller than 0.05.

Dose Dependence of FSH and EGF, Individually or in Combination, on HA and DS Synthesis—COCs isolated from PMSG-primed mice were cultured in the presence of increasing concentrations of FSH or EGF or combinations of both for 18 h, a period of time required for full expansion in vitro and for maximal HA and DS accumulation in the COC matrix. [3H]Glucosamine and [35S]sulfate were used as labeling precursors, and net synthesis of HA and DS was determined as described under “Experimental Procedures.” The results (Fig. 1) show that EGF can stimulate HA and DS synthesis to the same maximum level as that achieved by FSH. Maximal stimulation occurred at 1 ng/ml, as previously observed (15). Treatment of COCs with both factors combined at optimal doses did not promote any further increase of HA and DS synthesis. However, an additive effect was observed with combinations at suboptimal doses (Fig. 1, dashed curves).

The high sensitivity of COCs to EGF stimulation raised the possibility that the observed effect with EGF might be due to a minor contamination of the hormone preparation with EGF. This was ruled out by the observation that a polyclonal EGF-neutralizing antibody had no effect on FSH stimulation of HA and DS synthesis. A near maximum stimulation of HA synthesis was reached when the cultures contained 20 oocytes (Fig. 2, solid circle), a ratio of cells to oocytes equivalent to that in the original COCs. Further, the maximum response was essentially equivalent to that for intact COCs. Increasing concentrations of TGFβ1 also increased HA synthesis with no effect on DS synthesis. A near maximum stimulation of HA synthesis occurred at 1 ng/ml, but the maximum level achieved was only ~60% that achieved in co-cultures with oocytes. The combination of oocytes and TGFβ1 gave additive responses at suboptimal levels of each (Fig. 2, dashed curves) but did not achieve a significantly higher value than that with oocytes alone at optimal ratios.

Temporal Effects of FSH, dbcAMP, and EGF on HA and DS Synthesis—The length of time that COCs must be exposed to FSH, dbcAMP, or EGF to induce maximal synthesis of HA and DS was determined. Optimal doses of FSH (5 ng/ml), dbcAMP (1 mM), or EGF (50 pg/ml) were added at the beginning of the cultures and then removed at different times. All cultures were incubated for the subsequent time in medium without the factors and stopped at 18 h for HA and DS determination. Radiolabeled precursors were present throughout the 18-h incubation. The results (Fig. 3) indicate that each factor was progressively more effective in inducing HA and DS synthesis with increasing exposure time up to ~2 h. Longer exposure did
not increase the net production of HA and DS. Thus, these factors exert their maximal effects before HA synthesis actually begins at \( \sim 2.5\) h (15).

**Temporal Effects of the Oocyte Factor(s) and TGF\( \beta \) on HA and DS Synthesis**—The temporal effects of the oocyte factor(s) on HA synthesis were studied by adding 5 ng/ml FSH to cultures with 20 intact COCs at zero time and incubating them in the absence of FBS to facilitate removal of the oocytes at later times as described under “Experimental Procedures.” Net HA synthesis is not altered by the presence or the absence of FBS (4). For TGF\( \beta \) cultures, oocytes were first removed from 20 COCs, and the CCs were subsequently incubated in the presence of FSH and with 5 ng/ml TGF\( \beta \) for different lengths of time. The times at which the oocytes were removed or at which the medium was replaced with medium with FSH but without TGF\( \beta \) are shown on the x axis of Fig. 4. All cultures were incubated for 18 h after the initial addition of FSH, with radioisotopes present throughout the 18 h. The amounts of HA synthesized in the cultures increased continuously with increased time of exposure to the oocytes or to TGF\( \beta \) through 12 h, by which time HA synthesis is almost complete in intact COCs incubated in similar conditions (15). This indicates that continuous exposure of the CCs to the oocyte factor(s) or to TGF\( \beta \) is necessary to achieve maximal HA synthesis. As in the previous experiments (Fig. 2), neither factor affected DS synthesis.

A similar experiment was done in which CC cultures were first incubated in the presence of FSH for different periods of time before adding oocytes, or TGF\( \beta \), to the medium as indicated in the diagram at the top of Fig. 5. When either oocytes or TGF\( \beta \) were added at 2 h after exposure to FSH, a nearly maximal stimulation of HA synthesis was achieved (Fig. 5). However, net synthesis of HA rapidly decreased when the oocytes or TGF\( \beta \) were added at yet later times. Only \(-50\%\) of the maximum was achieved when the factors were added at 6 h, a value similar to that observed when the factors were removed at 6 h (Fig. 4). These results again indicate that maximal achievable synthesis of HA requires the continuous presence of either the oocyte factor(s) or TGF\( \beta \); their removal leads to a rapid decrease in synthesis, and their initial absence fails to initiate high levels of synthesis. As for earlier experiments, neither factor altered DS synthesis.

**Effect of Actinomycin D on HA and DS Synthesis**—Cultures of intact COCs incubated in the presence of 5 ng/ml FSH and 5 ng/ml TGF\( \beta \) were treated with 1 \( \mu \)g/ml actinomycin D. The TGF\( \beta \) was added to supplement the oocyte factor(s) in case actinomycin D treatment prevented synthesis or secretion of the oocyte factor(s). The cultures were incubated for 6 or 12 h; labeled between 0 and 6, 0 and 12, or 6 and 12 h; and treated with actinomycin D between 0 and 6 or 6 and 12 h as indicated at the bottom of Fig. 6. The concentration of actinomycin D was not cytotoxic as indicated by trypan blue exclusion by COCs after each exposure time, nor did this concentration prevent resumption of meiosis by the enclosed oocyte after removal (data not shown). The presence of actinomycin D from the beginning (0–6 h) totally prevents HA synthesis (Fig. 6), indicating that transcription of mRNA for new protein(s), including most likely an HA synthase, is required to initiate HA synthesis. Once HA synthesis has reached a high level at 6 h (15), the addition of actinomycin D decreases net synthesis of HA between 6–12 h to \(-50\%\) of the amount synthesized by the positive controls (FSH + TGF\( \beta \)) during the same time period (arrows, Fig. 6). This suggests that HA synthesis continues for a period of time (\(-3\) h) until the critical mRNA species synthesized before the actinomycin D block and the protein(s) transcribed from them are depleted. The results suggest that the net synthesis of HA is primarily under transcriptional control and that the critical mRNA species have relatively short half-lives.
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TABLE I

| Treatment | cAMP (pmol/COC) | HA (pmol hexN/COC) | DS (pmol hexN/COC) |
|-----------|----------------|--------------------|-------------------|
| Basal     | 0.2 ± 0.0       | 1.0 ± 0.1          | 0.5 ± 0.1         |
| FSH (ng/ml)| 4.2 ± 0.4       | 2.5 ± 0.1          | 0.8 ± 0.1         |
| 2.0       | 8.5 ± 1.0       | 5.5 ± 1.6          | 1.3 ± 0.1         |
| 5.0       | 14.1 ± 0.1      | 8.4 ± 1.5          | 1.5 ± 0.0         |
| 30.0      | 78.5 ± 7.5      | 8.6 ± 0.7          | 1.4 ± 0.2         |
| 100.0     | 163.0 ± 22.0    | 9.6 ± 1.0          | 1.4 ± 0.0         |
| EGF (ng/ml)| 1.0 ± 0.1       | 3.3 ± 1.0          | 4.3 ± 1.0         |

*The ± symbols indicate the S.E. values from three cultures for each treatment.

Effect of FSH and EGF on Intracellular cAMP Levels—

The amounts of HA and DS synthesized during 18 h of culture were determined as described under "Experimental Procedures." Values are given as percentages of the values for the cultures exposed to FSH (solid bars), with FSH and TGFb, (open bars), or with FSH, TGFb, and actinomycin D (cross-hatched bars). The cultures were labeled between 0 and 6, 0 and 12, or 6 and 12 h and treated with actinomycin D between 0 and 6 or 6 and 12 h as indicated at the bottom of the figure. The dashed line indicates the amount of HA synthesized in the 0–12-h cultures before the addition of actinomycin D. The arrows indicate the relative amounts of HA synthesized in the cultures after the addition of actinomycin D at 6 h. The bracket bars indicate the range for two cultures at each point. See the text for details. HexN, hexosamine.

Effects of Tyrosine Kinase Inhibitors on FSH and EGF Induction of HA and DS Synthesis—Two inhibitors of tyrosine kinases, genistein and tyrphostin AG18, were studied to determine whether tyrosine kinase-mediated phosphorylation is involved in the regulation of HA and DS synthesis by FSH and EGF. COCs were stimulated with 5 ng/ml FSH or with 50 ng/ml EGF in the presence of increasing concentrations of these inhibitors. Genistein inhibited EGF-induced synthesis of HA and DS by COCs in a dose-dependent manner, with 20 μM yielding −50% less HA and DS than controls and 40–80 μM inhibiting synthesis above basal level nearly completely (Fig. 7). Morpho-

Effect of actinomycin D on HA and DS synthesis by intact COCs—Cultures of COCs were incubated in basal medium (solid bars), with FSH and TGFb, (open bars), or with FSH, TGFb, and actinomycin D (cross-hatched bars). The cultures were labeled between 0 and 6, 0 and 12, or 6 and 12 h and treated with actinomycin D between 0 and 6 or 6 and 12 h as indicated at the bottom of the figure. The dashed line indicates the amount of HA synthesized in the 0–12-h cultures before the addition of actinomycin D. The arrows indicate the relative amounts of HA synthesized in the cultures after the addition of actinomycin D at 6 h. The bracket bars indicate the range for two cultures at each point. See the text for details. HexN, hexosamine.

The total amounts of 3H in HA for the 0–12-h labeling period are greater by −20% than the sum of the 0–6- and 6–12-h labeling periods. This reflects the time required for the [3H]glucosamine to equilibrate with the intracellular UDP-N-acetylhexosamine metabolic precursor pool. This will have less of an effect during the 0–6- and 0–12-h labeling periods because equilibration is occurring before HA synthesis has begun, whereas during the 6–12-h labeling period, the [3H]glucosamine equilibrates when HA synthesis is at or near maximal. The effects of actinomycin D on DS synthesis were very similar to those for HA (Fig. 6). In this case, the DS would normally be synthesized covalently bound to core proteins to form proteoglycans, and actinomycin D most likely prevents further transcription of mRNA for these core proteins.

Effect of FSH and EGF on Intracellular cAMP Levels—Previous studies showed that treatment with 1 μg/ml FSH transiently increases intracellular cAMP levels of COCs, with maximal levels reached at 1 h of incubation (14). The following experiments were done to establish whether a correlation exists between cAMP formation and HA and DS production stimulated by FSH. COCs were incubated in basal medium alone or with increasing doses of highly purified FSH. The cultures were stopped either at 1 h of culture to measure intracellular cAMP levels or at 18 h to determine the amount of HA and DS synthesized (Table I). The increase of intracellular cAMP levels induced by FSH was dose-dependent with an ED50 of −30 ng/ml and reached maximal values 50–60-fold higher than that observed in COCs cultured in basal medium. Increases of intracellular cAMP correlated with increases in HA and DS synthesis for FSH concentrations up to 5 ng/ml, when the increase of the cyclic nucleotide was only −6-fold. At higher FSH concentrations, −10-fold still higher levels of cAMP were induced but without a further increase of HA and DS synthesis. Treatment of COCs with 1 ng/ml EGF stimulated maximal production of HA and DS synthesis without increasing the cAMP level significantly over that in control cultures (Table I).

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FIG. 5. HA and DS synthesis by CCs in response to the addition of oocytes or TGFβ1, at different times after initiating cultures with FSH. Cultures of CCs were incubated with FSH alone for the indicated times (dashed lines in the diagram) before adding oocytes (closed circles) or TGFβ1 (open circles) followed by incubation up to 18 h (solid lines in the diagram). The amounts of HA and DS synthesized during 18 h of culture were determined as described under “Experimental Procedures.” Values are given as percentages of the values for the cultures exposed to oocytes or TGFβ1, for the entire 18 h, which were 6.9 ± 0.7 and 4.3 ± 0.4 pmol hexosamine/COC equivalent for cultures with oocytes or TGFβ1, respectively. The bracket bars indicate the S.E. for three cultures at each point.

FIG. 6. Effect of actinomycin D on HA and DS synthesis by intact COCs. Cultures of COCs were incubated in basal medium (solid bars), with FSH and TGFβ1 (open bars), or with FSH, TGFβ1, and actinomycin D (cross-hatched bars). The cultures were labeled between 0 and 6, 0 and 12, or 6 and 12 h and treated with actinomycin D between 0 and 6 or 6 and 12 h as indicated at the bottom of the figure. The dashed line indicates the amount of HA synthesized in the 0–12-h cultures before the addition of actinomycin D. The arrows indicate the relative amounts of HA synthesized in the cultures after the addition of actinomycin D at 6 h. The bracket bars indicate the range for two cultures at each point. See the text for details. HexN, hexosamine.

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FIG. 7. HA and DS synthesis by COCs after treatment with different concentrations of genistein. Cultures of intact COCs were treated with saturating concentrations of FSH (○), EGF (▲), or dbcAMP (▼) in the absence or the presence of increasing concentrations of genistein. The cultures were incubated with genistein for 1 h before adding the stimulators to allow its penetration. Cultures incubated in the absence of any of the factors gave the value indicated by the solid circle (●) (base level control). All cultures were incubated for 18 h before HA and DS determinations. The bracket bars indicate the S.E. for three cultures at each point. HexN, hexosamine.

FIG. 8. HA and DS synthesis by COCs after treatment with different concentrations of tyrphostin AG18. Cultures of intact COCs were treated with saturating concentrations of FSH (○), EGF (▲), or dbcAMP (▼) in the absence or the presence of increasing concentrations of tyrphostin AG18. The cultures were incubated with tyrphostin AG18 for 1 h before adding the stimulators to allow its penetration. Cultures incubated in the absence of any of the factors gave the value indicated by the solid circle (●) (base level control). All cultures were incubated for 18 h before HA and DS determinations. The bracket bars indicate the S.E. for three cultures at each point. HexN, hexosamine.

logical analyses showed that EGF stimulation of cumulus expansion was also completely prevented by the presence of 60 μM genistein; the CCs adhered to the bottom of the culture dish and flattened similarly to unstimulated COCs (data not shown). Little or no inhibition of FSH-induced HA synthesis was observed up to 40 μM genistein. However, reduction of −60% of HA and DS synthesis (Fig. 7) and partial inhibition of cumulus expansion (data not shown) were observed at higher concentrations (80 and 100 μM genistein). Similar inhibitory effects of 100 μM genistein were observed for cultures incubated with 1 mM dbcAMP.

The effect of tyrphostin AG18 was somewhat different (Fig. 8). At doses up to 20 μM, HA and DS synthesis induced by either FSH or EGF were inhibited equally and in a dose-dependent manner. Above 20 μM, HA and DS synthesis were unaltered in the presence of FSH and remained at the plateau value, −40% of the difference between basal and fully stimulated levels, whereas HA synthesis declined further but at a slower rate in the presence of EGF to less than −20% of the difference at 80 μM. At 80 μM tyrphostin AG18, COC expansion in the presence of EGF was nearly completely inhibited but only partially inhibited in the presence of FSH (data not shown). The results with dbcAMP at 80 μM tyrphostin AG18 were similar to those for FSH at the same concentration of inhibitor.

DISCUSSION

Cumulus expansion is the result of a transient increase of HA synthesis by CCs. Studies in vitro have documented that HA synthesis by mouse CCs is only slightly increased by the presence of either the oocyte alone or FSH alone. However, a dramatic increase is observed when the cells are cultured in the presence of both, suggesting a synergistic effect between a soluble factor(s) produced by the oocyte and the hormone (13). CCs begin to synthesize HA 2–3 h after stimulation with FSH in the presence of the oocyte. The rate of synthesis reaches a maximal level at −6 h, which is sustained up to 12 h, and then decreases, ceasing by 18 h when maximum expansion is reached (15). Results of the present study show that FSH and the oocyte factor(s) differ in their temporal patterns of action on HA synthesis. FSH exerts its effect on CCs early and rapidly because HA synthesis and expansion achieve maximum levels even when FSH is removed after 2 h of culture, a time before HA synthesis has actually begun. The presence of the oocyte is not required during this initial inductive phase, because nearly identical results were obtained when the oocytes were either added with FSH at the beginning of culture or 2 h later. However, longer delays before adding oocytes or precocious removal of the oocytes at times before HA synthesis would normally cease yields progressively less HA by FSH-stimulated CCs. These results indicate that the oocyte and hence the soluble factor(s) secreted by the oocytes (13) must be continuously present during the time that HA synthesis is ongoing to achieve maximum HA synthesis.

Several lines of evidence suggest that FSH exerts its influence on HA synthesis via cAMP pathways. Like FSH, dbcAMP in combination with oocytes transiently stimulates HA synthesis (15), and it shows a temporal course of induction similar to that observed with FSH, i.e., needs to be present only during the first 2 h. In addition, COCs become fully responsive when exposed to FSH for a time period during which cAMP accumulates maximally in response to the hormone (14). Further, the initial increases of intracellular cAMP levels induced by increasing concentrations of FSH closely correlate with the net production of HA. However, COC synthesis of HA is already maximal when a level of ~14 fmol of cAMP/COC is reached, which is only ~10% of the maximum increase in cAMP that can be generated by higher levels of FSH. This suggests that a relatively low elevation of this cyclic nucleotide by FSH is sufficient to elicit the full HA metabolic response. One hypothesis to account for this result is that a cAMP-dependent protein...
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c kinase could be localized in close physical proximity to the adenylylate cyclase coupled to the FSH receptor, perhaps by anchoring to specific binding proteins (26). Interestingly, similar cAMP levels (8–12 fmol/COC) are reached in vivo in COCs 2–4 h after human chorionic gonadotropin injection into PMSG-primed mice (27, 28) at a time at which HA synthesis is being initiated (15). Therefore, based on the results in vitro, it seems reasonable to hypothesize a primary role for cAMP in regulating HA metabolic activity and in promoting cumulus expansion in vivo. This is consistent with a previous study that showed that dbcAMP transiently increases HA synthase activity and HA production by rat fibroblasts with a time course similar to that observed for CCs (29).

Simultaneous addition of actinomycin D to FSH-stimulated CCs completely blocked HA synthetic activity, as was also observed for dbcAMP-stimulated fibroblasts (29, 30). This suggests that transcriptional events are involved in the pathway by which cAMP induces HA synthetic activity, most likely involving increased production of mRNA for HA synthase and any other protein that may be required to facilitate HA production. The presence of actinomycin D during the period from 6 to 12 h after induction reduces HA production by ~50%. This suggests that the continued synthesis of appropriate mRNA species is required to sustain the high rates of HA production even after the maximum rate of synthesis is achieved. Approximately 50% of maximum HA production was also obtained when oocytes were added to or removed from cultures at 6 h after induction with FSH. These results suggest that the oocyte factor(s) may regulate FSH action by acting at the transcriptional level.

We have previously shown that TGFβ1, among several growth factors tested, is the only one able to mimic the oocyte factor(s) in stimulating HA synthesis by FSH-treated CCs. However, this growth factor is less effective than the oocyte factor(s) and neutralizing antibodies against TGFβ1 do not inhibit the response to the oocyte factor(s) (16), indicating that these two factors are different. We now report that their effects are additive at suboptimal concentrations and that they show identical temporal patterns of induction. Several distinct gene products of the TGFβ family have been identified that are specific for each tissue and expressed in different phases of cell differentiation. Each one initiates some similar responses and, in general, enhances the accumulation of extracellular matrix by stimulating the synthesis of specific matrix components and by inhibiting matrix degradation (reviewed in Ref. 31). An oocyte factor with properties identical or very similar to those of the HA-inducing oocyte factor (32) has been shown to inhibit urokinase plasminogen activator synthesis by CCs (33) and is also mimicked by TGFβ2. These data suggest that HA and urokinase plasminogen activator synthesis by CCs may be regulated by a single factor produced by the oocyte, one that most likely belongs to the TGFβ family. A good candidate is the growth/differentiation factor 9, a new member of the TGFβ family specifically expressed by the oocytes (34).

The present study and previous observations (16) indicate that the production of HA and DS by cultured COCs in vitro is stimulated by EGF treatment to the same level as by FSH. Dose-response experiments show that COCs are very responsive to this growth factor, with as little as 50 pg/ml EGF causing a maximum response, and that treatment with both factors combined at optimal doses does not promote any further increase of HA or DS synthesis. In addition, EGF shows the same temporal pattern of action as does either FSH or dbcAMP. A slight but significant increase of cAMP has been detected in mouse COCs stimulated with EGF in the presence of phosphodiesterase inhibitor (17). However, when mouse COCs were stimulated with EGF alone at a concentration that is 20-fold higher than the minimum concentration required to induce full stimulation of HA synthesis, no appreciable elevation of cAMP was observed. Similar results have been reported for rat (35) and porcine (36) COCs. Thus, unlike FSH, EGF action is not mediated by cAMP. EGF is known to regulate a complex network of intracellular signaling pathways by binding and activating a receptor associated tyrosine kinase. The observation that genistein and tyrphostin AG18, two specific inhibitors of tyrosine kinases (37–39), completely block EGF stimulation of HA and DS synthesis, is therefore consistent and indicates that tyrosine phosphorylation is an obligatory step in EGF activation of HA synthesis.

The different membrane transduction signals elicited by FSH and EGF may converge on a common pathway leading to the increase in HA and DS synthesis. It has been recently reported that intracellular cAMP elevation can activate mitogen-activated protein kinases, which are part of a tyrosine kinase cascade system of the activated EGF receptor in PC12, COS-7, and B-16 melanoma cell cultures (40–42). It has also been shown that EGF increases tyrosine phosphorylation and activity of mitogen-activated protein kinases by porcine granulosa cell cultures (43). An obligatory tyrosine phosphorylation-dependent step in the gonadotropin-cAMP-dependent signal transduction pathway has been proposed based on the observations that tyrosine kinase inhibitors block the expression of: (a) steroidogenic enzymes and FSH-induced synthesis of luteinizing hormone receptor in differentiating rat granulosa cells (18, 19) and (b) steroidogenic enzymes and prostaglandin endoperoxide synthase-2 induced by luteinizing hormone or FSH in preovulatory rat granulosa cells (20, 21). All these responses occurred without affecting cAMP production. We now show that FSH and dbcAMP stimulation of HA and DS synthesis can be significantly reduced by genistein and tyrphostin AG18, albeit not to the same degree as for EGF-stimulated cultures.

This suggests that tyrosine phosphorylation of an intracellular protein may also be involved in the transduction pathway of FSH downstream from the formation of cAMP. FSH and dbcAMP stimulation of HA and DS synthesis by COCs is not completely abolished by these inhibitors, and at the highest concentrations used, the CCs continue to synthesize HA and DS but at a lower rate. This observation suggests that CAMP generated by FSH stimulation may induce the increase of HA and DS production through multiple pathways, in one of which tyrosine kinase activity is not an obligatory step.

It remains unclear as to which factors are involved in the regulation of HA and DS synthesis leading to cumulus expansion in vivo. An endogenous luteinizing hormone surge or an injection of human chorionic gonadotropin into mice will trigger COC expansion in vivo, whereas the same hormones fail to induce expansion of COCs in vitro. On the other hand, follicular fluid obtained from PMSG-primed mice before human chorionic gonadotropin injection is very active in inducing expansion of isolated COCs (12). Thus, it has been suggested that human chorionic gonadotropin interferes with an inhibitor in the follicle, which prevents a COC response to stimulants present in the follicular fluid. Substantial amounts of FSH are present in the follicular fluid, and EGF/TGFα seems to be locally produced in the murine ovary (Ref. 44 and reviewed in Ref. 45). High EGF-like activity has also been found in porcine follicular fluid (46). Dose response curves reported in the present paper show that FSH and EGF induce maximal HA and DS synthesis at concentrations that are within the physiological range, making

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both molecules good candidates for control of cumulus expansion in vivo. The additive effect of FSH and EGF at suboptimal doses and the similarity in their kinetics of action indicate that they could work in combination in vivo to ensure full expansion.

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