HORMONALLY INDUCED CELL SHAPE CHANGES IN CULTURED RAT OVARIAN GRANULOSA CELLS

THEODORE S. LAWRENCE, ROSEMARY D. GINZBERG, NORTON B. GILULA, and WILLIAM H. BEERS

From The Rockefeller University, New York 10021

ABSTRACT

Cultured rat ovarian granulosa cells undergo a dramatic morphological change when exposed to follicle-stimulating hormone (FSH). Exposure to FSH causes the flattened epithelioid granulosa cells to assume a nearly spherical shape while retaining cytoplasmic processes which contact the substrate as well as adjacent cells. This effect of FSH is preceded by a dose-dependent increase in intracellular cAMP, is potentiated by cyclic nucleotide phosphodiesterase inhibitors, and is mimicked by dibutyryl cAMP. Prostaglandins E_1 or E_2 and cholera enterotoxin also cause the cells to change shape. A subpopulation of the cells responds to luteinizing hormone. These morphological changes, which are blocked by 2,4-dinitrophenol, resemble those produced by treating cultures with cytochalasin B. Electron microscopy shows that the unstimulated, flattened cells contain bundles of microfilaments particularly in the cortical and basal regions. After FSH stimulation, microfilament bundles are not found in the rounded granulosa cell bodies but they are present in the thin cytoplasmic processes. These data suggest that the morphological change results from a cAMP-mediated, energy-dependent mechanism that may involve the alteration of microfilaments in these cells.

KEY WORDS rat ovarian granulosa cells · cell shape change · hormonal stimulation · cell-cell communication · plasminogen activator · cyclic AMP

Changes in cellular morphology occur in a wide range of biological processes including differentiation, transformation, and hormonal stimulation. In many instances, cAMP has been implicated as an effector of these changes. Cultured neuroblastoma cells, which have been used as a model system for differentiation, provide an example of a cAMP-induced change in cell shape. Exposure of cultures to $N^6,O^{2'}$-dibutyryl cAMP (Bt_2cAMP)[1] causes the cells to express a number of differentiated properties including neurite growth and arborization (27, 13). cAMP also appears to be responsible for the phenomenon of "reverse transformation" in Chinese hamster ovary cells. These cells lose their transformed morphology and become fibroblastic in appearance upon exposure to cAMP analogues (14, 28). In the case of hormonally sensitive cells, increased intracellular cAMP levels generated by hormonal stimulation have been reported to trigger cell shape changes. For

[1] *Abbreviations used in this paper:* Bt_2cAMP, $N^6,O^{2'}$-dibutyryl cAMP; DNP, 2,4-dinitrophenol; FSH, ovine follicle-stimulating hormone; LH, ovine luteinizing hormone; MIX, 3-methyl-1-isobutyl-xanthine; PCA, perchloric acid; PMSG, pregnant mare serum gonadotropin; rFSH, rat follicle-stimulating hormone; rLH, rat luteinizing hormone.

instance, bone cells in culture, which are spherical under control conditions, become stellate after treatment with parathyroid hormone (21). Another example is the prostaglandin E_2-stimulated change in the morphology of cultured Schwannoma cells from an irregular shape under control conditions to a rounded morphology after exposure to hormone (32). Y-1 cells, which are derived from an adrenal cortical tumor, are a third cell type that exhibit hormonally stimulated cell shape changes. These cells become rounded when exposed to ACTH, while they are flattened under control conditions (30, 8). In all three of these examples, the effect of the hormone is mimicked by Bt_2cAMP. Additional examples of cAMP-induced cell shape changes can be found in the recent review of Willingham (39).

Data from systems that undergo cAMP-dependent cell shape changes support the concept that flattened cells contain a cytoskeletal array of microfilaments and microtubules (26, 40). Other studies utilizing immunofluorescence (19, 25), and cytoskeletal disrupting agents such as cytochalasin B (38), demonstrate the existence of bundles of actin-containing microfilaments in flattened cells. Rounded cells, in contrast, have fewer filaments, and these are poorly organized. Data from diverse systems indicate that bundles of microfilaments appear when a rounded cell becomes flattened, regardless of the stimulus for the shape transition (6, 2, 41, 37). On the other hand, the cell shape changes observed in other systems appear to be dependent on microtubules (26).

Cultured granulosa cells have previously been used to study the effects of hormone stimulation in vitro. Stimulation with gonadotropins has been reported to cause cytological changes and steroid production correlated with luteinization (7), and ovine follicle-stimulating hormone (FSH) has been shown to stimulate the synthesis and secretion of plasminogen activator by these cells (34, 4).

In this study a striking morphological change is reported that occurs when cultures of rat ovarian granulosa cells are exposed to FSH, ovine luteinizing hormone (LH), E prostaglandins, and other drugs that elevate intracellular cAMP levels. In addition, data are presented concerning the mechanism of this change, and its potential role in ovulation is discussed.

MATERIALS AND METHODS

Materials

Rats of the Sprague-Dawley strain were purchased from Taconic Farms (Germantown, N. Y.). Chemicals and hormones were obtained from the following sources: ovine FSH (S-12) and LH (S-19), rat follicle-stimulating hormone (rFSH) (13), and rat luteinizing hormone (rLH) (14) (National Institute of Arthritis, Metabolism, and Digestive Diseases); prostaglandins E_1, E_2, $F_{1\alpha}$, and $F_{2\alpha}$ (the gift of Dr. John Pike, The Upjohn Company, Kalamazoo, Mich.); A23187 (the gift of Dr. Otto K. Behrens, Eli Lilly Research Laboratories, Indianapolis, Ind.); Bt_2cAMP, cycloheximide, pregnant mare serum gonadotropin (PMSG), colchicine, cytochalasin B and 3':5' cyclic nucleotide phosphodiesterase (Sigma Chemical Co., St. Louis, Mo.); colcemid and tissue culture media (Grand Island Biological Co., Grand Island, N. Y.); plastic petri dishes (BioQuest, BBL & Falcon Products, Becton, Dickinson & Co. (Cockeysville, Md.); vincristine sulfate (Eli Lilly); [^{3}H]leucine (New England Nuclear, Boston, Mass.); [^{3}H]thymidine and cholera enterotoxin (Schwarz/Mann, Div., Becton, Dickinson & Co., Orangeburg, N. Y.); cAMP standard (Collaborative Research, Waltham, Mass.); materials for electron microscopy as described in Kalderon et al. (16). All other chemicals were of reagent grade.

Methods

PREPARATION OF GRANULOSA CELL CULTURES AND LIGHT MICROSCOPY: Granulosa cells were prepared from PMSG-primed rats as previously described (5), except that Liebowitz's L-15, a phosphate-buffered medium, was used instead of medium 199. After the cells were harvested and washed, they were dispersed, counted, and plated into a 35-mm petri dish containing a no. 1 cover slip at a density of 5 $\times$ 10^5 cells/dish in complete culture medium (L-15 supplemented with 10% bovine serum, 100 μg streptomycin/ml and 100 U penicillin/ml. The cultures were incubated at 37°C in water-saturated air, and after 16–24 h the cover slip was placed, cell side down, over a medium-filled well formed by cutting a hole in a glass slide and attaching a second cover slip underneath. The configuration allowed the use, on living cells, of a relatively high resolution objective lens ($\times$40 planapo) and condensor, both of which required a short working distance. Cells were observed and photographed with a Zeiss photomicroscope II.

SCANNING ELECTRON MICROSCOPY: Our initial scanning electron microscope studies of cells grown directly on a plastic substrate indicated that apparent artifacts, such as broken processes and cell membrane fissures, were frequently present after critical point drying. Plating the cells on rat tail collagen gels largely alleviated this problem. The collagen gels were prepared in 35-mm Falcon plastic petri dishes by neutralizing acid collagen extracts with a 2:1 mixture of 10-fold concentrated medium 199 (without bicarbonate) and 0.34 N NaOH (9). The gels were washed for 2–3 h in several changes of phosphate-buffered saline before adding cells. Granulosa cells were plated in medium 199 supplemented with 10% fetal bovine serum at 5 $\times$ 10^5 cells per

gel, and incubated at 37°C for 2–5 days before stimulation. The day before stimulation, the medium was changed to L-15 supplemented with 10% bovine serum. The cells were stimulated with PMSG at a final concentration of 10 μg/ml in the presence of 10^{-4} M 3-methyl-1-isobutyl-xanthine (MIX). 75 min later, the medium was removed and the gels were fixed for 12 min with 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.3), rinsed in Na cacodylate buffer, postfixed with 1% OsO_4 in 0.1 M Na cacodylate buffer, and rinsed again. The gels were dehydrated through graded alcohols. Small pieces of gels were detached from the petri dish, transferred to acetone on a glass cover slip, and critical point dried from liquid CO_2 in a Sorvall critical point drying apparatus (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.). After gold coating (Edwards 306 vacuum coater, Edwards High Vacuum, Inc., Grand Island, N. Y.), the specimens were examined with an ETEC Autoscan microscope.

TRANSMISSION ELECTRON MICROSCOPY: Granulosa cell cultures were plated at a density of 10^6 cells/35-mm petri dish. 1 h after treatment with 10^{-4} M MIX and 10 μg PMSG or FSH/ml, the medium was removed and the cultures were fixed for 12 min with 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.2). After two buffer rinses, the cultures were treated with 1% osmium tetroxide in 28 mM veronal acetate buffer (pH 7.4) for 1 h, and processed for electron microscopy as reported previously (16). Control cultures were processed as above but without the addition of drugs.

ELECTROPHYSIOLOGY: Granulosa cells were plated at a density of 5×10^5 cells/35-mm culture dish in complete L-15 medium. The dish was placed in a temperature-controlled stage at 37 ± 0.5°C. Cells were impaled with 40–100 MΩ micropipette electrodes prepared from omega dot glass (Hilgenberg, Germany). The microelectrodes were filled with 3 M KCl and mounted on Leitz micromanipulators (E. Leitz, Inc., Rockleigh, N. J.). Membrane potentials were recorded with a high impedence negative capacity compensated amplifier (The Rockefeller University, N. Y.) and were displayed on a Tektronix 5103 oscilloscope (Tektronix, Inc., Beaverton, Oreg.). Current was injected through an active bridge circuit.

AUTORADIOGRAPHY: Granulosa cells were plated at 5×10^5 cells/35-mm petri dish in complete culture medium. After 16 h, 10 μCi thymidine/ml (6 Ci/mmol) was added to the cultures. Some cultures simultaneously received 10 μg PMSG/ml. After 24 h, the cultures were washed twice with L-15. Complete culture medium was replaced and the cells were cultured for an additional 24 h. The cultures were then fixed and processed for autoradiography according to the previously described modifications (16) of the method of Pitts and Simms (24), except that Ilford L4 emulsion was used. Nuclei was scored as labeled if they contained an increased density of silver grains compared to the background. Data are expressed as the percent of labeled nuclei. More than 1,000 total nuclei were counted for each condition.

ANALYSIS OF MORPHOLOGICAL CHANGE: Granulosa cells were prepared as described for the electrophysiological studies. After 16–24 h, 5–10 clusters of 50–250 cells each were randomly chosen and their location was marked on the petri dish. The only criteria for selection were that the cluster was well-flattened and relatively free of adherent debris. The cultures were allowed to re-equilibrate at 37°C and after 1 h the desired drugs or hormones were added. For experiments examining the effect of a drug on hormonally induced cell shape changes, the cells were incubated for 10 min with the drug before addition of the hormone. Changes in cell morphology were monitored by phase-contrast microscopy. At the times indicated for each experiment, the medium was removed from individual cultures and they were fixed for 15 min with 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.2), washed twice with the cacodylate buffer, and stained for 2–5 min with 0.1% toluidine blue-O in 50 mM sodium borate. The premarked regions of the culture dishes were photographed at ×150, coded by one of the investigators, and scored blind by at least two individuals. Cells were scored as morphologically changed on the basis of: (*a*) an increased intensity of cytoplasmic staining relative to the flattened cells, and (*b*) the existence of cell-associated processes. The data are expressed as the fraction of the total number of scored cells in each culture (~500 cells/culture) that exhibited altered morphology. The percentage of cells scored as rounded in control cultures (<2%) was subtracted from each value; this value appears to result from adherent cellular debris in the cultures and not from the actual existence of rounded cells. There was considerable variability between experiments in the percentage of cells that change shape in response to a given stimulus. However, if experiments were normalized on the basis of the response to a standard hormonal stimulus (10 μg FSH or PMSG/ml), the values from different experiments were in good agreement. This permitted data from several experiments to be combined.

CAMP DETERMINATIONS: Freshly harvested cells were aliquoted into Falcon plastic culture tubes (3×10^6 cells per tube) in 1 ml of medium 199, supplemented with 10% plasminogen-depleted fetal bovine serum, and gassed with 5% CO_2 in air. After a 5-min equilibration period in a 37°C water bath, the cells were stimulated by adding FSH in a small volume of medium and shaking before returning the tubes to the water bath. At appropriate times the tubes were removed to an ice bath, the cells were pelleted (135 *g*, 4°C, 2 min), the supernate was discarded, and ~0.25 ml of cold 1% perchloric acid (PCA) added. The pellet was dispersed in the PCA, and the samples were placed on ice for at least 10 min. The specimens were centrifuged again to remove cellular debris, and the supernate was decanted and stored frozen. The amount of cAMP in purified supernatant aliquots from these samples was determined

by radioimmunoassay according to the method of Steiner et al. (33) with a commercially prepared cAMP radioimmunoassay kit (Collaborative Research Inc., Waltham, Mass.), using the second antibody method of separation. PCA extracts were purified according to the directions furnished with the radioimmunoassay kit (see also references 22 and 18). Addition of known quantities of cAMP standard to unstimulated cell pellets at the time of PCA addition indicated that 80% of the cAMP present at that stage could be assayed after purification. Preincubation of purified samples with cyclic nucleotide phosphodiesterase resulted in the removal of all detectable cAMP.

PLASMINOGEN ACTIVATOR DETERMINATIONS: To measure intracellular plasminogen activator, cells were plated at a density of 10^6 cells/35-mm petri dish in L-15 medium supplemented with 10% plasminogen-depleted fetal bovine serum. After 3 h the medium was removed and fresh medium with or without FSH was added. At the indicated times after exposure to FSH, the cultures were washed twice with 0.1 M Tris·HCl (pH 8.1), and then 0.1 M Tris·HCl (pH 8.1) containing 0.1% Triton X-100 was added to each dish. The cells were scraped from the dish, and the resulting suspension was frozen immediately. Upon thawing, the suspension was assayed as described by Unkeless et al. (36).

Extracellular plasminogen activator was assayed as described by Strickland and Beers (34). Briefly, 5×10^4 cells were plated in L-15 supplemented with 10% plasminogen-depleted fetal bovine serum into multiwell dishes that had been coated with [^{125}I]fibrinogen. After 3 h the medium was removed, the cultures were washed in L-15, and the indicated drugs were added. For both intracellular and extracellular assays, data are expressed as the fraction of available substrate that was solubilized.

ANALYSIS OF LEUCINE INCORPORATION INTO MACROMOLECULES: Granulosa cells were plated at a density of 10^6 cells/35-mm petri dish. After 4 h, 30 μCi of [^{3}H]leucine (115 Ci/mmol)/ml was added to complete culture medium in the presence or absence of the substances indicated in the text. The cultures were then incubated for 1 h, the medium was removed, and the cells were washed twice with L-15. After the final wash, ice-cold 5% TCA was added to the dishes which were allowed to stand for an additional 30 min on ice. The cells were then scraped from the dish, and the unincorporated label was removed by filtration (Millipore Corp., Bedford, Mass.; 0.45 μm). After extensive washing with cold TCA, the filters were counted in a liquid scintillation spectrometer. The results are presented as counts per minute incorporated into nonfilterable material.

RESULTS

Description of the Hormone-Induced Morphological Change

Granulosa cells in culture form clusters of flattened cells with a typical epithelioid morphology (Fig. 1 *A*). Within 1 h after exposure of the culture to FSH, the cells become rounded but retain narrow cytoplasmic extensions or processes which contact the substrate as well as neighboring cells (Fig. 1 *B*). Images such as those seen in Fig. 1 and time-lapse cinematography show that these cellular processes are not newly formed cytoplasmic extensions; rather they reflect the retention of regions of contact after the remainder of the cell has contracted toward the center. 2–3 h after the cells have contracted, they again flatten and are morphologically indistinguishable from unstimulated cells. A qualitatively similar response occurs regardless of variations in the substrate (glass, plastic, or collagen gels), growth medium (L-15 or 199), or time in culture. The oldest cultures tested (2 wk) still contain cells capable of changing shape in response to hormone, but the proportion of stimulated cells is decreased.

Examination of granulosa cell cultures by scanning electron microscopy gives a more detailed picture of the morphological transformation induced by FSH. The unstimulated cells appear to be very flat with broad regions of intercellular contact, and microvillar processes can be found on the apical surface (Fig. 2*A*). After exposure to the gonadotropin, the cell bodies assume a nearly spherical shape. The increased intercellular space resulting from this morphological transition highlights the processes that maintain contact between cells. The processes often have multiple branches and in some cases they contact several cells. Microvilli are generally absent on the apical surfaces of these cells (Fig. 2 *B*).

Electron microscope studies of thin sections show that flattened, unstimulated cells often possess bundles of microfilaments. These microfilaments are usually found in the cytoplasm adjacent to the plasma membrane, especially near the culture dish (Fig. 3). These microfilaments are actinlike in appearance (~7 nm in diameter) unlike the larger intermediate filaments (~10 nm in diameter) that also are present in these cells. Cells which have changed shape upon exposure to FSH exhibit a markedly different organization of microfilaments.Although the cell body often contains some microfilaments, these do not appear to be as numerous as in control cells, and microfilament bundles such as those seen in Fig. 3 *B* are rarely found. The processes, on the other hand, often contain microfilaments. In larger processes, microfilaments are usually located beneath the plasma membrane (Fig. 4 *A*), while thinner proc-

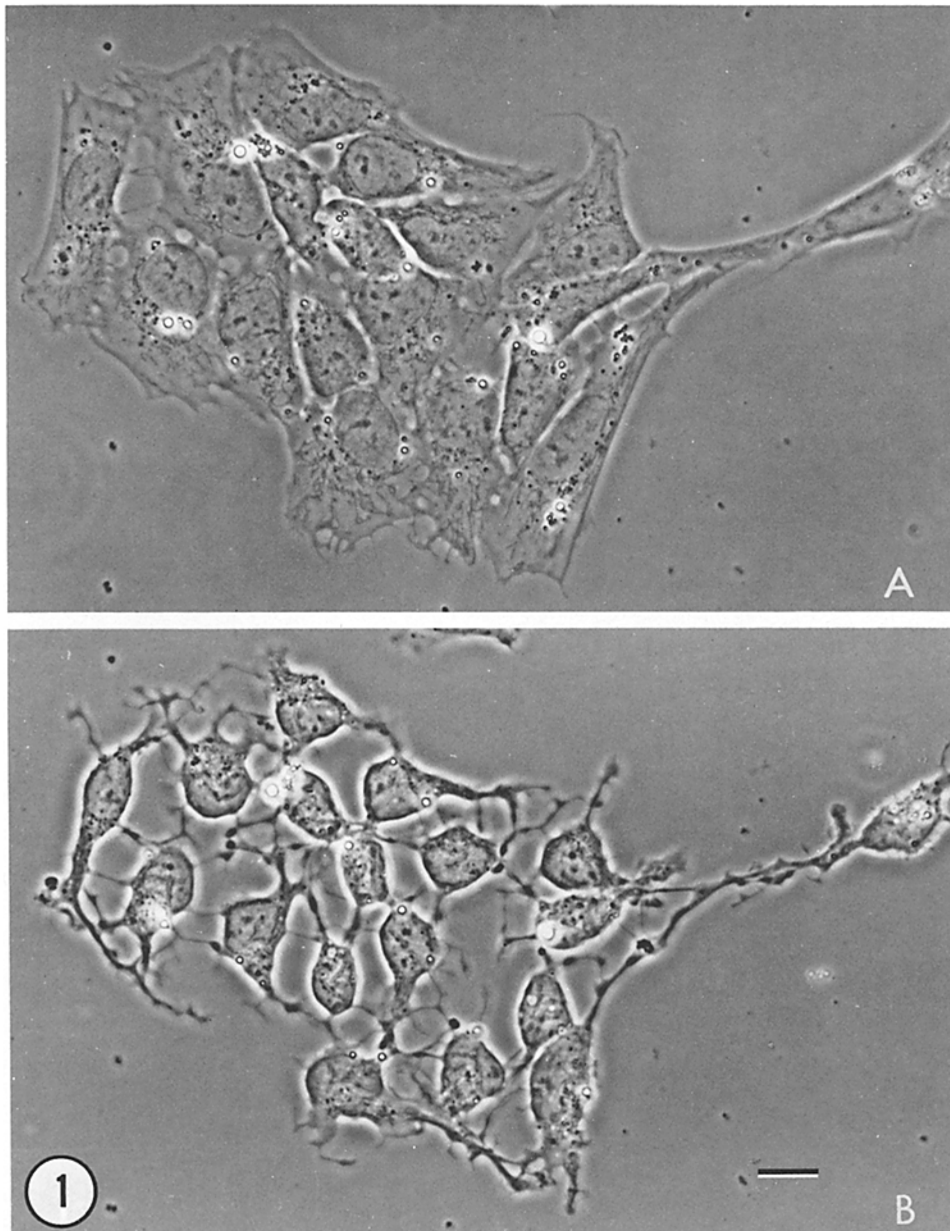

FIGURE 1 Phase-contrast microscopy of the same group of granulosa cells before and after exposure to FSH. Under control conditions (*A*) the cells are flattened. After exposure for 1 h to 10 μg FSH/ml and 10^{-4} M MIX, the same cells become rounded, leaving processes attached to the substrate and to neighboring cells (*B*). Bar, 10 μm. (*A* and *B*) × 800.

esses can be filled with microfilaments (Fig. 4 *B*). Microtubules and intermediate filaments are also common elements in the processes of stimulated granulosa cells (Fig. 4 *A* and *C*).

Thin-section electron microscopy of these cultures also reveals the existence of specialized cell contacts, such as desmosomes and gap junctions. These are often found in the areas of broad intercellular contact between control cells, and in the regions of contact between processes (Fig. 4 *C*) or between a process and the somatic region of a cell in stimulated cultures. The presence of gap

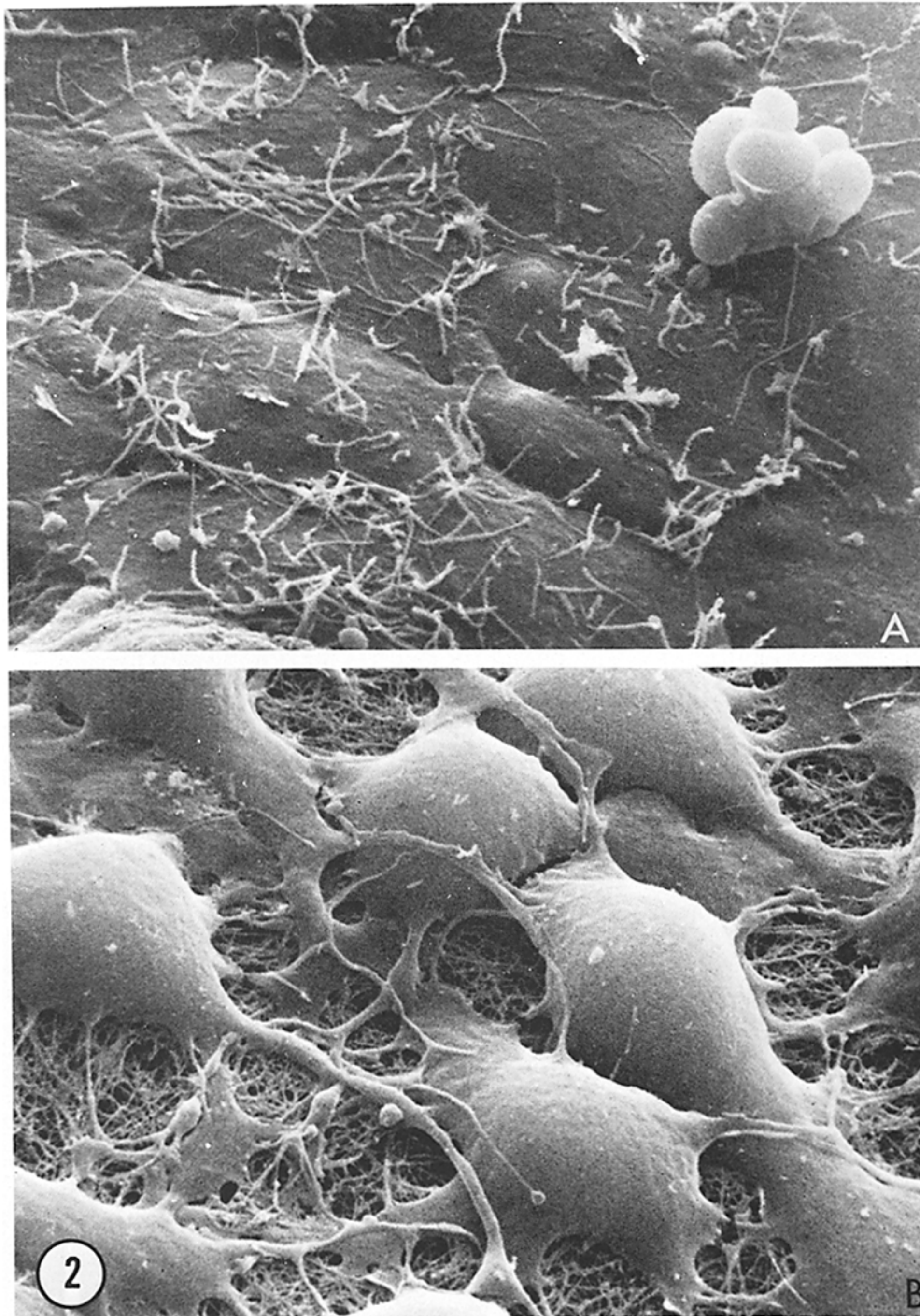

FIGURE 2 Scanning electron microscopy of control and FSH-stimulated cells. (*A*) Under control conditions the flattened cells display microvilli on their apical surface. (*B*) Exposure to 10 μg PMSG/ml and 10^{-4} M MIX causes the cells to lose their microvilli and to become rounded with branching processes. A process from one cell can make multiple contacts with several other cells and processes. (*A* and *B*) × 2,000.

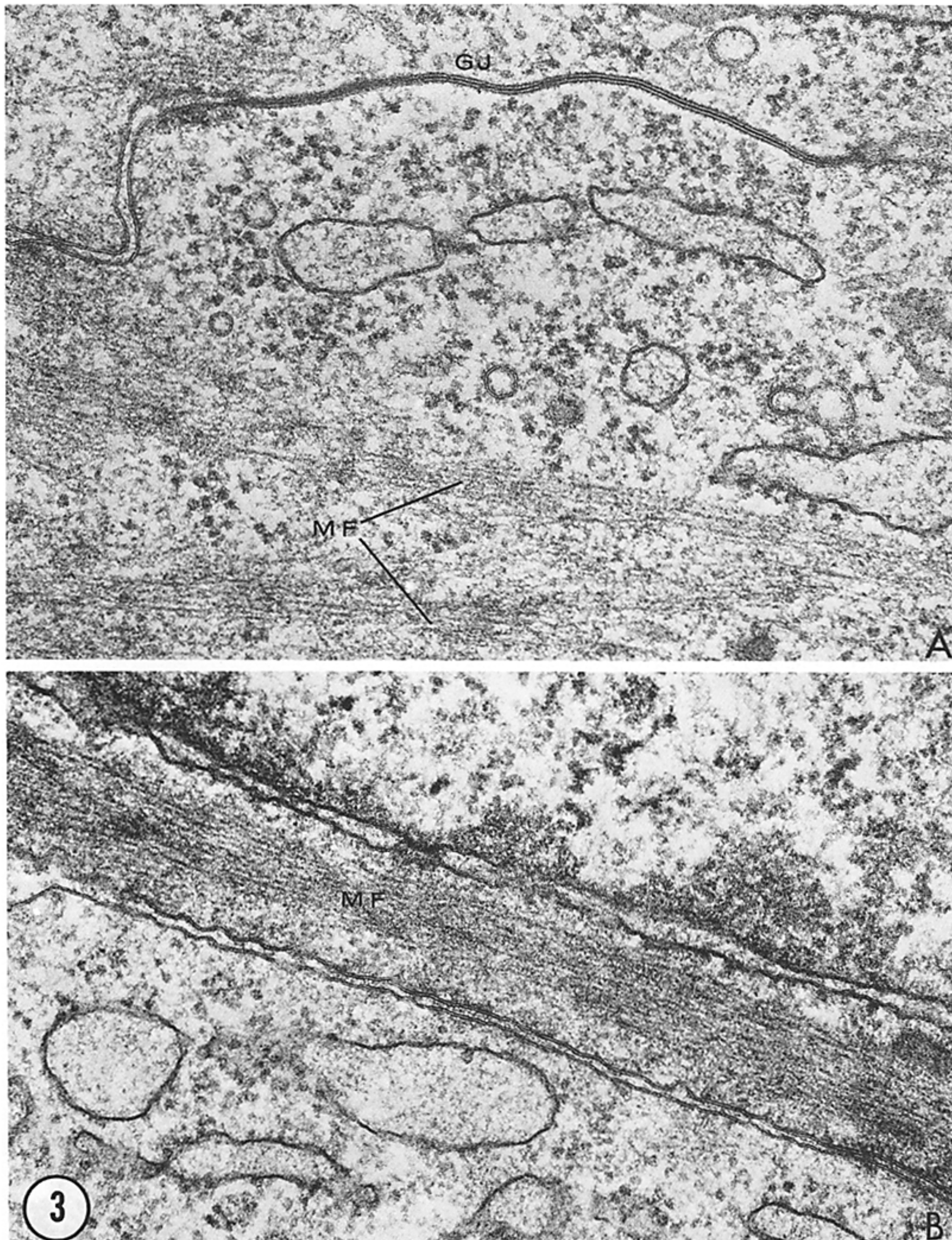

FIGURE 3 Transmission electron microscopy of granulosa cells under control conditions. Both Fig. 3*A* and *B* show areas of contact between two cells. In the upper part of Fig. 3*A*, a prominent gap junction (*GJ*) joins the two cells. The two bundles of microfilaments (*MF*) that are present in the lower third of this image appear to converge at the right. In Fig. 3*B*, a cortical bundle of microfilaments almost fills the cytoplasm between the nucleus and the plasma membrane of the cell in the upper right. Both figures show cells sectioned parallel to the plane of the culture dish. (*A*) × 67,000; (*B*) × 51,000.

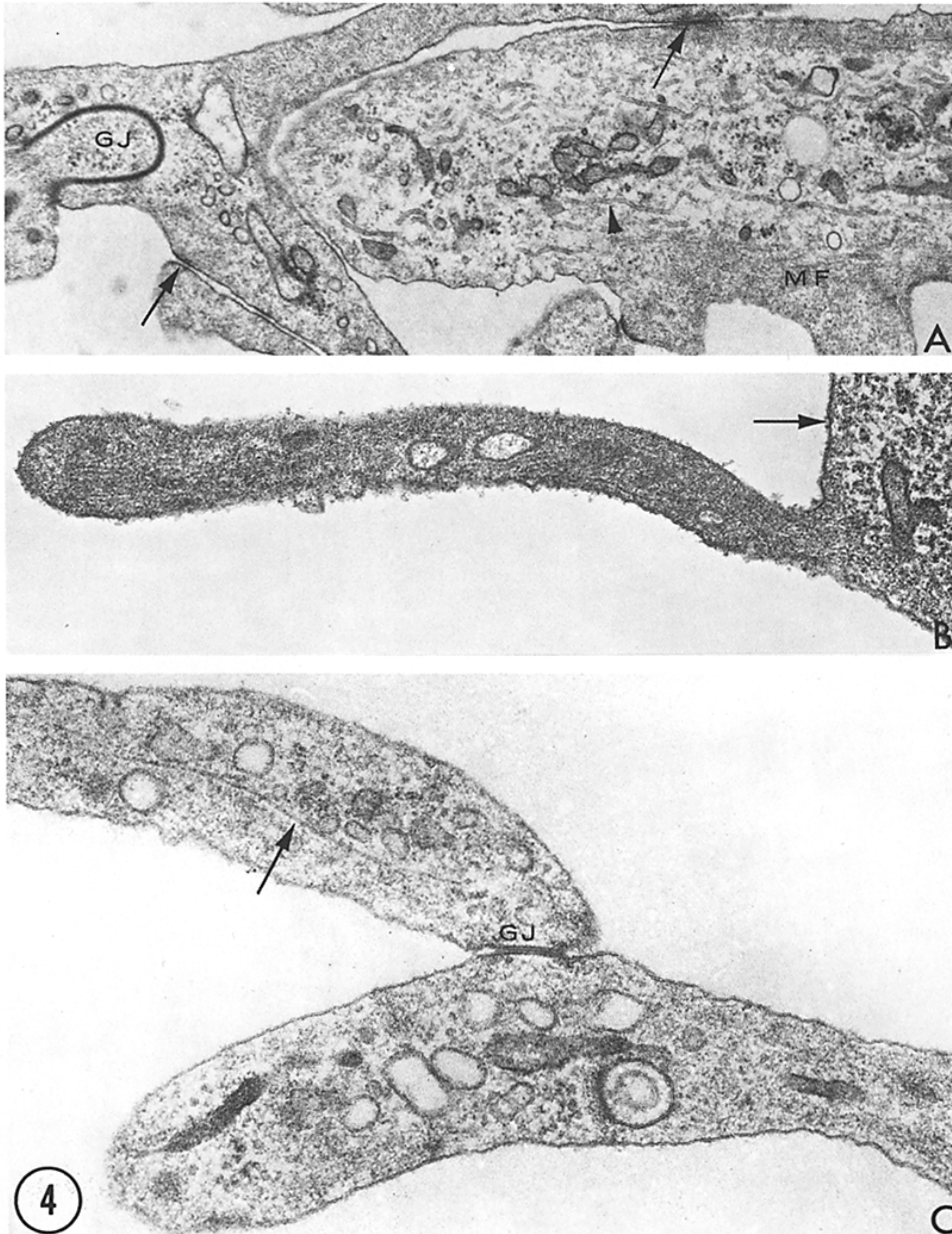

FIGURE 4 Transmission electron microscopy of FSH-stimulated granulosa cells. (*A*) The large process on the right contains subplasmalemmal microfilaments (*MF*) as well as centrally located microtubules (arrowhead). One of the two desmosomal contacts made by this process is indicated (arrow). A second specialized cell junction, the gap junction (*GJ*), is seen between two processes on the left. One of these processes has also established a desmosomal contact (arrow) with another process. (*B*) This cellular process is almost completely filled with microfilaments. The subplasmalemmal region of the cell body on the right contains no filaments (arrow). (*C*) A gap junction (*GJ*) is seen in the region of close apposition between two processes. One process contains intermediate filaments (arrow). All figures show cells sectioned parallel to the plane of the culture dish. (*A*) × 25,000; (*B*) × 35,000; (*C*) × 49,000.

junctions suggests that granulosa cells should be metabolically and ionically coupled (12). In fact, granulosa cells are ionically coupled under control conditions (Fig. 5 A) as well as during exposure to FSH (Fig. 5 B), indicating that this form of intercellular communication is not disrupted during hormonal stimulation. These gap junctions and desmosomes may provide sites of cell-to-cell attachment that remain after hormonal stimulation has caused the rest of the cell to retract.

The microscope analyses indicate that, during the process of rounding, the nucleus remains intact, an observation that suggests that the rapid, gonadotropin-induced morphological change is not associated with mitosis in these cultures. Additional evidence has been obtained from autoradiographic studies that gonadotropin treatment does not induce cell division. These studies show that the fraction of cells that incorporate [^{3}H]thymidine in a 24-h period is unchanged by the presence of gonadotropin (6.8% under control conditions; 8.4% in the presence of 10 μg PMSG/ml).

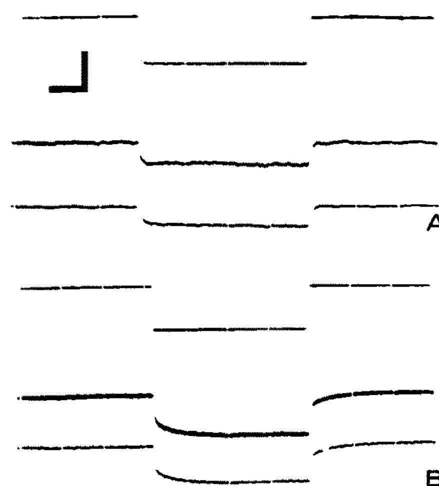

FIGURE 5 Ionic coupling between granulosa cells. For both sets of records, the top trace shows the current injected, the middle trace records the intracellular potential of the cell into which current is injected, and the bottom trace displays the intracellular potential of the adjacent interacting cell. Calibration bars for both sets of records are 0.5 nA and 10 mV for the vertical, and 50 ms for the horizontal. Granulosa cells are ionically coupled under control conditions (*A*) as well as 1 h after stimulation with 10 μg PMSG/ml and 10^{-4} M MIX (*B*).

Evaluation of the Procedure used to Quantitate Morphological Changes

To examine quantitatively the effects of gonadotropins and drugs on granulosa cell morphology, the procedure described in Methods was used. This technique proved to be a reliable quantitative measure of the cellular response. Between any two of the observers who conducted these analyses, a correlation coefficient (r) of >0.93 ($P <$ 0.001) was obtained for any set of 20 pictures. The consistency of a single observer was determined by including the same photograph for scoring on different occasions. Repetitive scorings did not differ by >11%. Although in most cases there was a clear distinction between cells that had changed shape and those that had not, there were some cells in each sample that exhibited intermediate morphology after stimulation. In scoring these cells, some observers recorded consistently higher values than others. However, the greatest difference between observers was seldom >15%.

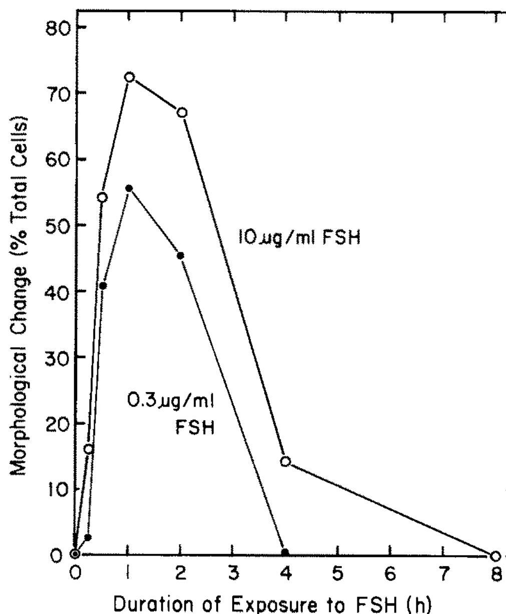

FIGURE 6 Time-course of the FSH-induced cell shape change. Granulosa cell cultures were exposed to either 10 μg FSH/ml (○) or 0.3 μg FSH/ml (●), and they were fixed after the indicated times. The cultures were then processed and scored according to the procedure described in Methods.

Specificity of the FSH-Induced Response

The FSH-stimulated shape change of granulosa cells is both time- and dose-dependent. Maximal rounding occurs ~1 h after exposure of the cells to gonadotropin, and by 4 h most of the cells have flattened again (Fig. 6), at which time they are

morphologically indistinguishable from those observed before the addition of the hormone. All of the cells in a population do not undergo cell rounding, even at the highest concentration of gonadotropin. At the time of maximal response, most clusters contain both rounded and flattened cells.

The dose-response curve of the morphological change in response to FSH and LH is presented in Fig. 7. As seen in the figure, this preparation of FSH is ~30-fold more potent than an equivalently purified preparation of LH. This is similar to the response pattern when plasminogen activator production is monitored after stimulation with these gonadotropin preparations (34).

Most of the response to LH seen in Fig. 7 can be accounted for by FSH which is known to contaminate this preparation (~1 part in 30). However, this cannot explain the response of low magnitude to 0.1 μg/ml LH, a concentration containing insufficient contaminating FSH to cause any detectable cell shape change. This observation suggests that a proportion of the cells responds specifically to LH. To determine better the specificity of the response, experiments were conducted with highly purified preparations of rat gonadotropins. The effect of rFSH is considerably greater than that of rLH, but in the range of 10–100 ng/ml a stimulation of smaller amplitude is seen with rLH (Fig. 8). This preparation contains <1 part FSH in 5,000, and thus it appears that a subpopulation of cells is capable of responding to LH.

Evidence for cAMP Mediation of Cell Shape Change

The effect of FSH on cell shape change is augmented when the cyclic nucleotide phosphodiesterase inhibitor, theophylline, is included in the culture medium (Fig. 6), suggesting that the response may be mediated by cyclic nucleotides. This is supported by the finding that direct exposure of the cultures to membrane-permeable derivatives of cAMP, such as Bt_2cAMP, results in a dose-dependent increase in the percentage of stimulated cells (Fig. 9). This effect of Bt_2cAMP is not due to the presence of butyrate, a breakdown product of Bt_2cAMP, because 1 mM butyrate alone has no detectable influence on morphology. Theophylline also enhances the response of the cells to the nucleotide. Measurements of intracellular cAMP levels (Figs. 10 and 11) provide additional evidence for the participation of cAMP

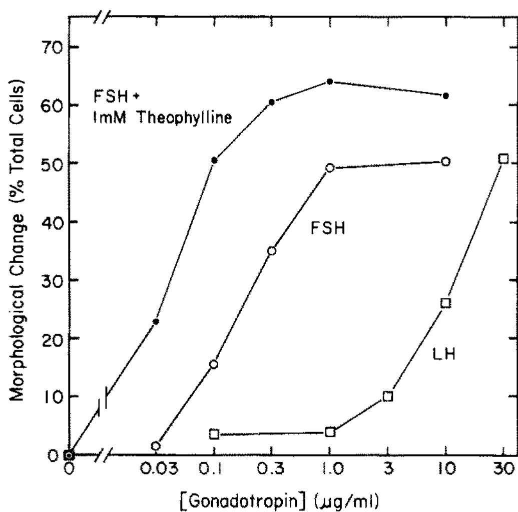

FIGURE 7 Specificity, dose dependence, and theophylline potentiation of gonadotropin-induced morphological change. Granulosa cell cultures were exposed to the indicated concentrations of LH (□) or FSH, the latter in the presence (●) or absence (○) of 1 mM theophylline. After 1 h the cultures were fixed and scored. Data from several experiments are represented.

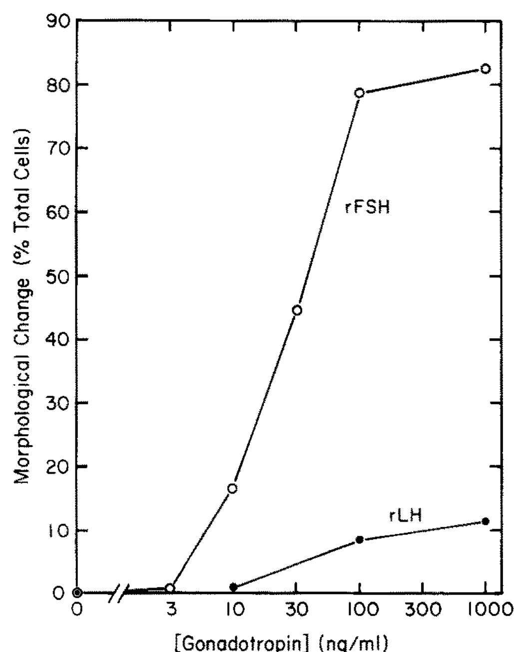

FIGURE 8 Specificity of the morphological response to rat gonadotropins. Cultures of granulosa cells were exposed to the indicated concentrations of rat FSH or rat LH. After 1 h the cultures were fixed and scored.

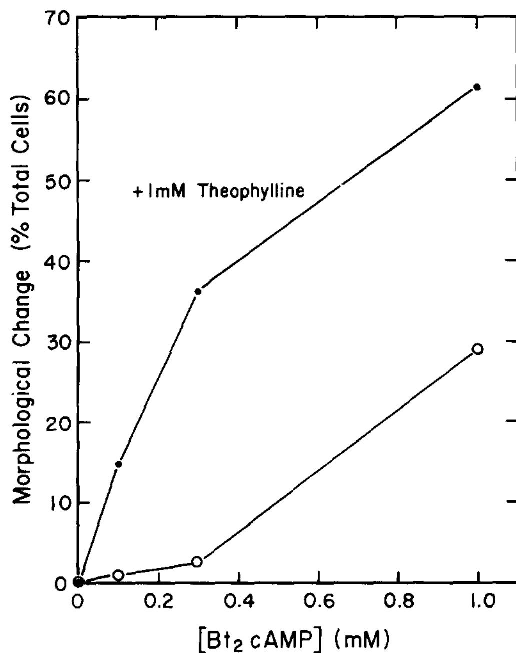

FIGURE 9 Dose dependence of the cell shape change induced by Bt_2cAMP. Granulosa cell cultures were stimulated by Bt_2cAMP alone (○) or in the presence of 1 mM theophylline (●). After 1 h the cultures were fixed and scored.

in the hormonally induced cell shape change. After the exposure of granulosa cells to FSH, there is a rapid rise in the intracellular concentration of cAMP (Fig. 10), which reaches a maximum within 30–60 min at a level 15- to 20-fold higher than background. Increases of two- to fivefold are observed within the first 2 min of exposure to hormone. After peak levels are achieved, the concentration of nucleotide falls to control levels in ~5–6 h. The dose dependency of the FSH-induced rise in cAMP levels (Fig. 11) is similar to the response pattern for cell shape change (Fig. 5). This pattern is also seen if FSH-stimulated plasminogen activator production is monitored (34).

Effect of Cholera Enterotoxin and Prostaglandins on Cell Morphology

Cholera enterotoxin, which stimulates cAMP synthesis in cells that contain adenyl cyclase, causes a shape change in granulosa cells (Fig. 12) which is identical to that elicited by FSH stimulation. However, with the toxin there is a 30-min lag in the onset of the morphological changes after stimulation. A similar lag period has been observed in cholera enterotoxin-stimulated cAMP-dependent protein kinase in cultured ovarian cells (3) as well as other cell systems (10, 20). This toxin also stimulates plasminogen activator synthesis in granulosa cells (Pesek and Beers, unpub-

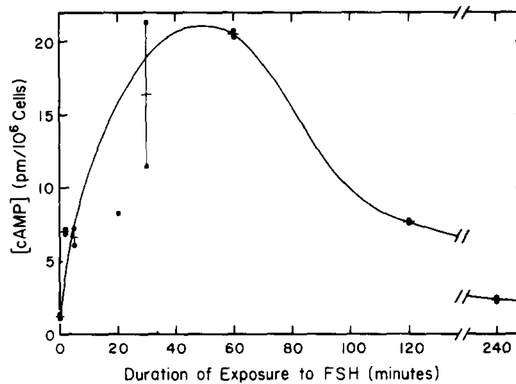

FIGURE 10 Time-course of cAMP increase in response to FSH. Granulosa cells (3×10^6), suspended in 1 ml of medium, were exposed to 10 μg FSH/ml. At the times indicated, the tubes were placed on ice and assayed for cAMP as described in Methods. Duplicate cell preparations were assayed. Each of the duplicates is presented here (●) and the mean is indicated by the horizontal bar.

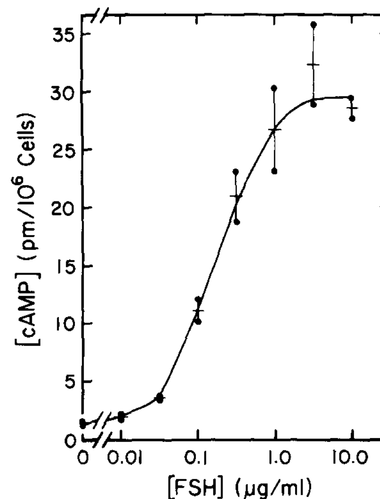

FIGURE 11 Dose dependence of the FSH-induced increase in cAMP. Granulosa cells, prepared as in Fig. 10, were exposed to the indicated concentrations of FSH. After 1 h the tubes were placed on ice and assayed for cAMP as described in Methods. Duplicate cell preparations were assayed for each concentration; the data are expressed as in Fig. 10.

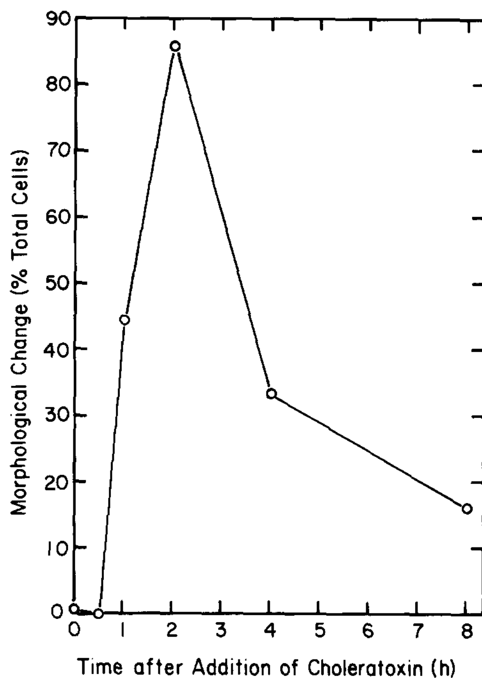

FIGURE 12 Time-course of the effect of cholera enterotoxin on cell shape. Cultures of granulosa cells were exposed to 100 ng/ml of cholera enterotoxin, and then fixed at the indicated times. This concentration of toxin causes maximum stimulation of cell rounding (data not shown).

lished observations).

In a previous study it was shown that, in addition to FSH, prostaglandins of the E series were able to stimulate the production of plasminogen activator by granulosa cells, presumably via a cAMP-dependent pathway (34). Prostaglandin E_1 also causes granulosa cells to change shape (Fig. 13), as does prostaglandin E_2 (data not shown). Prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$, however, do not stimulate morphological changes in these cells.

Temporal Sequence of cAMP Synthesis, Cell Shape Change, and Plasminogen Activator Production

Fig. 14 shows the time-course of the appearance of plasminogen activator in cultured granulosa cells after exposure to FSH. Also plotted in the figure are the increases in cAMP and cell shape. Intracellular plasminogen activator levels begin to rise $\sim 1/2$ h after exposure of the cultures to FSH, at which time cAMP levels are near maximum. Plasminogen activator levels continue to rise until about the 4th h after stimulation when intracellular cAMP has returned to near basal levels. The time-course of the morphological change more closely follows the increase in cAMP; however, the morphological alteration persists for 1–2 h after the nucleotide levels begin to decrease.

Studies of the Mechanisms Involved in Granulosa Cell Rounding

To assess which aspects of granulosa cell metabolism and structure might be involved in the hormone-dependent morphological changes, we have conducted the studies listed below:

(a) Effect of alterations in Ca^{++} levels: Under serum-free conditions, cell rounding is insensitive to both the absence of, and abnormally high levels of, Ca^{++} (0–10 mM). In the presence of the calcium ionophore A23187 (10 μM), rounding is likewise unaffected at Ca^{++} concentrations from 0 to 1 mM. The combined effect of the ionophore and 10 mM Ca^{++} is toxic to the cells.

(b) Effect of agents that depolymerize microtu-

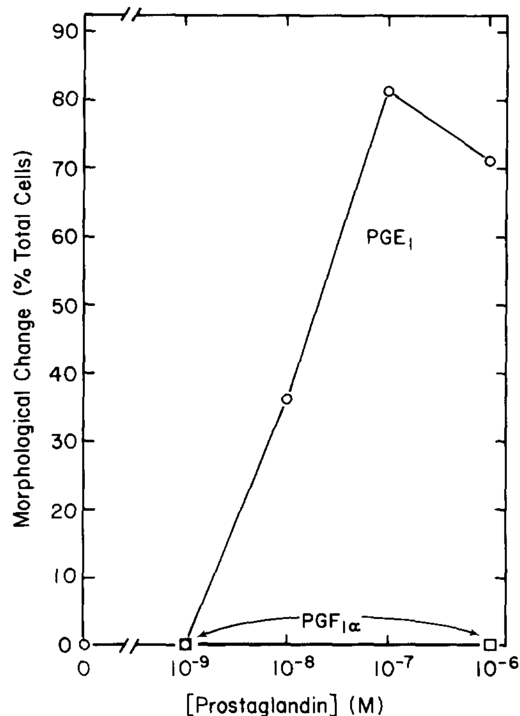

FIGURE 13 Dose dependence of the morphological response to prostaglandins. Cultures of granulosa cells were exposed to the indicated concentrations of PGE_1 (O) and $PGF_{1\alpha}$ (□). After 1 h the cultures were fixed and scored.

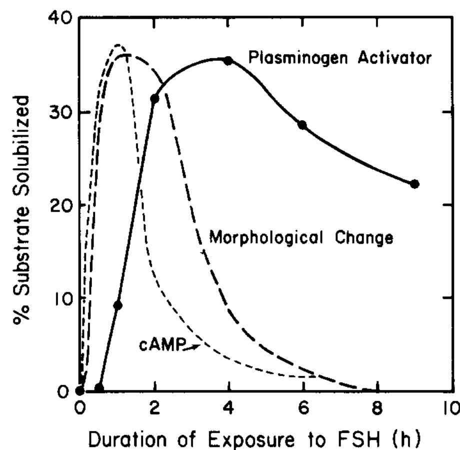

FIGURE 14 Comparison of the time-course of the FSH-induced increase in cAMP, cell rounding, and plasminogen activator. The curves for cAMP (- -) and morphological change (—) are replotted from Fig. 10 and Fig. 6, respectively. Both have been scaled so that the maximum responses are of approximately the same magnitude as the peak levels of plasminogen activator. Values for the plasminogen activator curve (●) were obtained by exposing granulosa cell cultures to FSH and assaying the cultures for intracellular enzyme after the indicated times as described in Methods.

bules: Colchicine (at concentrations up to 4 μg/ml), colcemid (up to 5 μg/ml), and vincristine sulfate (up to 9 μg/ml) in the incubation medium do not alter the response of the cells to FSH. Although these agents are ineffective in inhibiting the response, studies using an antitubulin antibody in an indirect immunofluorescence procedure demonstrate that colcemid disrupts the organization of the microtubules in the granulosa cell cytoplasm (Lawrence and Gilula, unpublished observations).

(c) Effect of cytochalasin B: At concentrations in the range of 1–10 μg/ml, cytochalasin B causes granulosa cells to undergo a shape change similar to that observed during FSH stimulation. The response to this drug follows a time-course similar to that seen with gonadotropin, and is reversed upon removal of the drug. However, the shape changes induced by cytochalasin B include the appearance of cytoplasmic blebs (Fig. 15) which are not characteristic of the FSH-stimulated cells shown in Fig. 1 B.

(d) Effect of inhibitors of protein synthesis and oxidative phosphorylation: FSH-induced cell shape changes are inhibited by 2,4-dinitrophenol (DNP) but not by cycloheximide, indicating that cell shape change is an energy-dependent process that does not require protein synthesis. The effects of DNP on plasminogen activator synthesis and protein synthesis are identical (see Table I). The

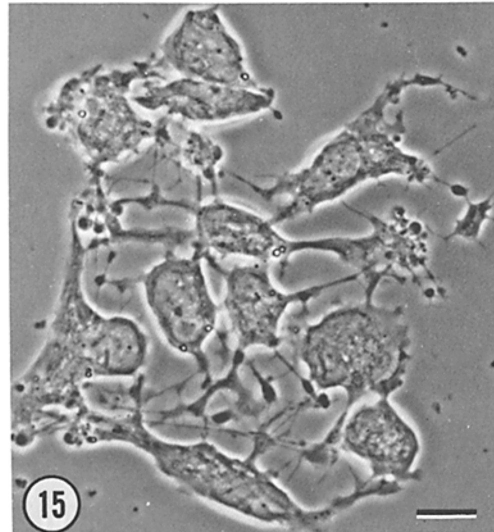

FIGURE 15 Effect of cytochalasin B on cell shape. Granulosa cells were prepared for light microscopy as described in Methods. The cultures were then exposed to 10 μg cytochalasin B/ml in 1% DMSO for 1 h. 1% DMSO had no effect on cellular morphology. Bar, 10 μm. × 800.

TABLE I

Drug	[^{3}H]leucine incorporation	Plasminogen activator activity	Morphological change
	cpm	*% Substrate solubilized*	*% Cells changed shape*
Control	3,341	0	0
FSH	3,606	68	82
DNP	88	0	0
DNP + FSH	342	6	7
Cycloheximide	172	0	0
Cycloheximide + FSH	179	1	93

Effect of DNP and cycloheximide on cell shape change, plasminogen activator secretion, and incorporation of [^{3}H]leucine into macromolecules. For the cell shape change experiments, granulosa cell cultures were exposed to the indicated drugs. After 1 h the cultures were processed and scored. Extracellular plasminogen activator and [^{3}H]leucine incorporation were determined according to the procedure described in Methods. Where indicated, FSH and cycloheximide were present at a concentration of 10 μg/ml, and 1 mM DNP was used.

inhibitory effect of DNP is completely reversed after its removal.

DISCUSSION

In this paper we have described a hormonally induced change in the morphology of cultured granulosa cells. This change can be quantified in a highly reproducible fashion and has been characterized pharmacologically. The morphological transformation is independent of the external calcium concentration, and does not seem to depend on protein synthesis or microtubular assembly. It is, however, an energy-requiring process that appears to involve changes in the organization of cytoplasmic microfilaments. A major proportion of the cells (50-90%) respond to FSH, PMSG, Bt_2cAMP, prostaglandins E_1 or E_2, and cholera enterotoxin by transiently assuming a spherical shape while maintaining processes attached to the substrate and to neighboring cells. A subpopulation of the cells (10-15%) respond to LH in the same manner. The FSH-induced change in shape appears to be mediated by cAMP. This conclusion is based on the following findings: (*a*) the effect of FSH is potentiated by cyclic nucleotide phosphodiesterase inhibitors; (*b*) the hormone-induced change is mimicked by exposure to Bt_2cAMP; and (*c*) FSH, at concentrations that lead to cell shape changes, causes an increase in intracellular cAMP levels that precedes the change in cell shape. Similar concentrations of gonadotropin have been reported to increase cAMP levels in porcine granulosa cells (17). Previous studies have demonstrated that findings *a* and *b* also apply to the FSH-induced production of plasminogen activator by cultured granulosa cells (34). Evidence presented here shows that both the FSH-stimulated shape change and the increase in cAMP levels precede the synthesis of plasminogen activator. The cAMP measurements strongly support the conclusion that the production of plasminogen activator, as well as cell shape change, is mediated by cAMP.

Although the gonadotropin and drug dose dependence of the morphological response resembles that of plasminogen activator synthesis, these two responses exhibit several properties that are distinctly different.

(*a*) It appears that highly purified LH can cause a subpopulation of the cells to respond morphologically; this is not the case for plasminogen activator (4). (*b*) Studies of plasminogen activator production by granulosa cells, using an assay system that detects plasminogen activator production by single cells, indicate that no more than 20-30% of the cells respond to gonadotropin (5). The morphological change occurs in a much larger proportion of the cells. (*c*) Protein synthesis is not required for the cells to undergo the morphological change; it is necessary for plasminogen activator production. (*d*) Cytochalasin B can induce cell rounding. This drug does not cause granulosa cells to produce plasminogen activator; nor does it inhibit FSH-dependent plasminogen activator production of these cells (Beers, unpublished observations).

A number of the experiments reported here were conducted in an attempt to understand the mechanism underlying the morphological changes. On the basis of studies of the effect of cycloheximide, vincristine, colchicine, and colcemid, it appears that neither protein synthesis nor microtubular assembly is necessary for hormonally induced changes in granulosa cell shape. Studies on the effect of alterations of the concentration of extracellular Ca^{++} and of a Ca^{++} ionophore indicate that this phenomenon is also independent of external Ca^{++} levels, although we cannot rule out the possibility that internal Ca^{++} stores may be involved. Cytochalasin B, which disorganizes cytoplasmic microfilaments (31, 38), does cause a morphological change similar to that seen in response to FSH. DNP which blocks oxidative phosphorylation also blocks hormonally induced cell shape changes.

Electron microscope studies supplement these data. The subplasmalemmal bundles of microfilaments found in control cells appear to be decreased in both size and number upon stimulation by FSH. In other cell systems (see Introduction), decreases in the number of microfilaments have also been correlated with the transition from a flattened to a rounded morphology. On the other hand, dense bundles of microfilaments are present in the stimulated cell processes. The data presented in this study are consistent with the hypothesis that increased internal levels of cAMP lead to an energy-dependent alteration of granulosa cell microfilaments, resulting in the transition from a flattened to a spherical shape.

The physiological role of the hormone-induced morphologic change is not clear. Long-term changes in cell shape have been shown to influence the proliferation of cultured endothelial cells (11). However, the transient cell rounding reported here does not signal a round of cell division

in these cells; nor is it associated with DNA synthesis, as assessed by autoradiographic [^{3}H]thymidine incorporation studies. The morphological change does not interrupt communication between cells. In addition, the relationship between cell shape change and plasminogen activator production remains unclear. The possibility that plasminogen activator production causes cells to change shape could be suggested on the basis of studies on virally transformed cells (15). However, this hypothesis can be excluded in this system on the basis of points *c* and *d* above, as well as the fact that the morphological response precedes the production of the enzyme. Although it seems likely that these two cellular functions are related only in that they share cAMP as a mediator, it is possible that morphological change is a necessary precondition for plasminogen activator production. On the other hand, in ACTH-stimulated Y-1 cells, hormone-induced cell shape changes and steroidogenesis can be completely dissociated (8). Thus the elucidation of the relationship between these multiple responses to the same hormone must await further investigation.

The morphological change which occurs after in vitro exposure of the cells to physiological levels of gonadotropin appears to be the result of increased levels of intracellular cAMP. This nucleotide has been shown to increase in ovarian follicles upon hormonal stimulation (1, 35). Although the morphological changes which we have observed may be accentuated by or totally dependent on the nearly two-dimensional form that these cells assume in culture, the alteration of certain elements of the granulosa cell cytoskeleton could occur in vivo as well. Disruption of the cytoskeleton has been previously correlated with decreased substratum adhesiveness in cultured cells (41, 40, 29). The existence of such a phenomenon in vivo might, when coordinated with the intrafollicular appearance of a protease such as plasmin, facilitate the dislocation of granulosa cells from the follicle wall near the time of ovulation (23).

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