

CELL-TO-CELL COMMUNICATION AND OVULATION

A Study of the Cumulus-Oocyte Complex

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ABSTRACT

Cell-to-cell communication was characterized in cumulus-oocyte complexes from rat ovarian follicles before and after ovulation. Numerous, small gap junctional contacts were present between cumulus cells and oocytes before ovulation. The gap junctions are formed on the oocyte surface by cumulus cell processes that traverse the zona pellucida and contact the oolemma. The entire cumulus mass was also connected by gap junctions via cumulus-cumulus interactions. In the hours preceding ovulation, the frequency of gap junctional contacts between cumulus cells and the oocyte was reduced, and the cumulus was disorganized. Electrophysiological measurements indicated that bidirectional ionic coupling was present between the cumulus and oocyte before ovulation. In addition, iontophoretically injected fluorescein dye was transferred between the oocyte and cumulus cells. Examination of the extent of ionic coupling in cumulus-oocyte specimens before and after ovulation revealed that ionic coupling between the cumulus and oocyte progressively decreased as the time of ovulation approached. In postovulatory specimens, no coupling was detected. Although some proteolytic mechanism may be involved in the disintegration of the cumulus-oocyte complex, neither the cumulus cells nor the oocyte produced detectable levels of plasminogen activator, a protease which is synthesized by membrana granulosa cells.

In summary, cell communication is a characteristic feature of the cumulus-oocyte complex, and this communication is terminated near the time of ovulation. This temporal pattern of the termination of communication between the cumulus and the oocyte may indicate that communication provides a mechanism for regulating the maturation of the oocyte during follicular development before ovulation.

KEY WORDS cell communication · gap junctions · ionic (electrotonic) coupling · dye transfer · cumulus-oocyte complex · ovulation

The avascular compartment of the mammalian ovarian follicle contains several different types of cells whose metabolism and function must be precisely controlled during the hours preceding

ovulation. Since a number of known hormone-dependent metabolic and morphologic changes occur during this rather brief time period (4), the follicle represents a unique system to study the regulation of heterologous cell interactions during development and differentiation.

The major population of cells within the follicular antrum are epithelial granulosa cells. Within

this population, there is a definite heterogeneity with respect to morphology and hormone binding. For example, receptors for human chorionic gonadotropin are present on granulosa cells in the peripheral region of the follicle whereas the granulosa cells near the antral cavity and the oocyte have few detectable receptors for the hormone (1). In addition, some of the granulosa cells are physically associated with the follicle wall, some with the oocyte, some with other granulosa cells, and some with the antral fluid.

The granulosa cells that surround the mammalian oocyte are known as the cumulus oophorus. The innermost layer of cells in the cumulus, the corona radiata, sends cytoplasmic processes through the intervening zona pellucida to contact the oolemma (9, 20, 26, 31). There have been several reports that gap junctions are present in the regions of contact between these heterologous cells (cumulus oocyte) (2, 3, 28). Since gap junctions have been implicated as a structural pathway for cell-to-cell communication (7, 16, 17, 23),¹ it has been tempting to speculate that the presence of these junctional elements may indicate that there is a significant exchange of molecules between the cumulus cells and oocyte during follicular development. This possibility may be of considerable importance in terms of oocyte metabolism and maturation since it has been suggested that cumulus cells are responsible for providing several trophic or metabolic factors to the preovulatory oocyte (8, 12, 15, 22). Recently, it has been demonstrated that [³H]uridine can be incorporated into oocyte RNA in intact mouse cumulus-oocyte preparations, whereas it is not incorporated in identical preparations where the cumulus has been removed (30). A similar cumulus cell dependency for growth of mouse oocytes has also been reported (13).

Before the time of ovulation, the number of cumulus cell processes to the oocyte decreases (19, 20, 26), and there is a hormonally induced elevated concentration of plasminogen activator in the follicular fluid that is produced by the granulosa cells (6, 27). After ovulation few, if any, cumulus-oocyte processes are evident. Thus, eventually, the oocyte loses all physical contact with its associated cumulus cells.

The experiments described in this paper were

¹ In this paper, the terms cell-to-cell communication and intercellular communication are used to refer to the process of transfer of cytoplasmic substances between cells via a low-resistance junctional mechanism.

undertaken in order to determine the structural and physiological basis for cell-to-cell communication in the rat cumulus oophorus-oocyte complex. In addition, temporal characteristics of communication have been examined during the stages of follicular development that lead to the ovulatory event. A preliminary report of these studies has been presented previously (14).

MATERIALS AND METHODS

Materials

Immature female Sprague-Dawley rats (26 days old) were obtained from Holtzman Co. (Madison, Wis.). Other materials utilized in this study include: pregnant mare serum gonadotropin (PMSG); human chorionic gonadotropin (HCG); poly-L-lysine, mol wt 70,000 (Sigma Chemical Co., St. Louis, Mo.); medium 199, and penicillin-streptomycin (Grand Island Biological Co., Grand Island, N. Y.); fetal bovine serum (Reheis Chemical Co., Chicago, Ill.); Falcon culture dishes (BioQuest, BBL & Falcon Products, Cockeysville, Md.); disodium fluorescein and osmium tetroxide (4% aqueous) (Polysciences, Inc., Warrington, Pa.); glutaraldehyde solution (50% wt/wt, biological grade), paraformaldehyde, and uranyl acetate (Fisher Scientific Co., Pittsburgh, Pa.); Epon 812; tannic acid, analytical grade (Mallinckrodt, Inc., St. Louis, Mo.); and omega dot tubing (Hilgenberg Glass, Germany).

Preparation of Cumulus-Oocyte Complexes and Granulosa Cells

Female Sprague-Dawley rats, 26 days old, were injected subcutaneously with 5 IU of PMSG in 0.1 ml of 0.9% NaCl. After 48 h, the animals were administered 25 IU of HCG. With this regimen, five to six follicles per ovary will ovulate 10–12 h after the HCG injection. Preovulatory follicles are distinguishable from other graafian follicles in the ovary on the basis of size and coloration approx. 4 h after the HCG injection.

Cumuli and granulosa cells were prepared from individual follicles at various stages of development by the following procedure. The rats were decapitated and the ovaries were dissected free of adhering tissue in the presence of medium 199 with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Follicles were punctured with a 26-g needle and their contents expressed into the medium. The preparation was then examined at 10–20 × magnification, and cumuli were removed with a fine pipette. These were transferred to fresh medium, washed by repeated transfers and then deposited in a tissue culture vessel containing medium 199. The remaining granulosa cells were then collected by centrifugation, washed and plated at the desired density in [¹²⁵I]fibrin-coated culture vessels (27) and analyzed for plasminogen activator as described below. Postovulatory cumuli were isolated from oviducts 22–

29 h after the HCG injection (10–17 h after ovulation) by the following method. The ovary and oviduct were surgically removed from the animal and placed in medium 199. The oviduct was then excised from the ovary and placed in fresh medium. In the extended oviduct, a bolus of 5–6 cumuli could be located and visualized with the aid of a dissecting microscope at 10–20 \times . Once the cumuli were located, the oviduct was cut immediately distal to the bolus, and the bolus was then expelled into the medium by oviductal peristalsis. The postovulatory cumuli were then recovered from the culture dish and utilized for the physiological or biochemical determinations.

Light Microscopy

Light micrographs of follicles from appropriately treated animals were obtained by cutting 1 μ m thick sections of Epon-embedded specimens that were fixed and processed for electron microscopy. The 1 μ m thick sections were mounted on glass slides and stained with toluidine blue. All photomicrographs were taken with bright field optics in a Zeiss Photomicroscope II.

Electron Microscopy

THIN SECTIONS: Ovarian samples from appropriately primed animals were fixed with a combination of 4% glutaraldehyde (vol/vol) and 0.5% paraformaldehyde (wt/vol) in 0.1 M cacodylate buffer (pH 7.3) for 2 h at room temperature. At this time, the follicles were dissected as intact tissue fragments for subsequent processing. Care was taken to avoid rupturing the follicles. Regions of oviduct that contained a bolus of postovulatory material were selected with a dissection microscope and processed as intact tissue segments. After initial aldehyde fixation, the samples were treated for 1 h with 1% (vol/vol) osmium tetroxide in veronal-acetate buffer, followed by buffer rinses. Then the samples were treated en bloc with 1% tannic acid in 0.05 M sodium cacodylate buffer (pH 7.0) for 45 min, washed with 1% sodium sulfate in 0.1 M sodium cacodylate buffer (pH 7.3) for 10 min, and then treated with uranyl acetate in veronal-acetate buffer for 1 h. The samples were dehydrated in a graded series of ethanol (70%, 95%, 100%), and embedded in Epon 812. Thin sections were cut with a diamond knife on a Sorvall Porter-Blum MT2-B Ultramicrotome and mounted on copper grids. The sections were stained with uranyl acetate and lead citrate before examination in a Philips 300 transmission electron microscope at 80 kV.

FREEZE-FRACTURE: Appropriate follicles were dissected from intact ovaries that had been fixed for 2 h at room temperature in a mixture of 4% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). The samples were then treated for 2–4 h at room temperature with 25% glycerol in 0.1 M cacodylate buffer before rapid freezing in Freon 22 and storage in liquid nitrogen. The samples were freeze-

fractured and replicated with a carbon-platinum mixture in a Balzers BM 360 apparatus at -115°C . The replicas were cleaned in bleach and distilled water before mounting on 300–400-mesh grids. All electron microscope observations were made with a Philips 300. The freeze-fracture images have been mounted so that the shadow direction is from the bottom to the top of the micrographs.

Physiology

Microelectrode impalements were significantly facilitated by attaching the cumulus-oophorus complexes to culture dishes that had been pretreated with poly-L-lysine. Optimal attachment results were obtained by treating the culture dishes overnight with 0.1% (wt/vol) poly-L-lysine. This treatment was followed by rinsing with distilled water, and then adding medium 199 before the addition of the biological samples.

ELECTROPHYSIOLOGICAL RECORDING: Dishes containing cumuli were placed on the stage of an inverted microscope (Leitz Diavert) and recording was carried out at room temperature. Preparations were used for a maximum of 3 h after the rat was killed. Microelectrodes, made from omega dot tubing, were bent on a nicrome wire to approx. 45° and filled with 2.8 M KCl to give resistances of 50–70 M Ω . The microelectrodes were connected through Ag/AgCl wires to the input of capacity-compensated DC amplifiers. One microelectrode served to record membrane potential and the other electrode was used to inject pulses of current through a bridge circuit. The indifferent electrode was a 0.5-mm capillary tube filled with Ca, Mg-free PBS dissolved in 2–3% agar. This tube connected a medium-filled reservoir containing a Ag/AgCl lead with the culture dish. The bath was held at virtual ground by connecting the indifferent electrode to the summing junction of a Philbrick P24 operational amplifier (Teledyne Philbrick, Dedham, Mass.) used to monitor current intensity. Current pulses for stimulation were provided by Tektronix pulse generators (Tektronix, Inc., Beaverton, Ore.). Membrane potentials and current intensity were displayed on a Tektronix 5111 storage oscilloscope and photographed with a kymograph camera. The image of the cells to be penetrated was projected through the microscope and photographed on 35 mm film. A minimum resting membrane potential of -10 mV was required before a cell was used for the coupling studied.

DYE INJECTIONS: For the dye transfer experiments, electrodes (approx. 10 M Ω when filled with 2.8 M KCl) were filled with 5% (wt/vol) disodium fluorescein, dissolved in distilled water or 0.1 M KCl. Negative current pulses ($7-10 \times 10^{-9}$ A) of approx. 0.5 s duration were injected at a frequency of 1/s. Fluorescein was visualized through bright-field optics using a 100-W tungsten halogen lamp, a combination of BG 12, 23, and 38 excitation filters, a KP 490 cut-off filter and a KP 530 barrier filter, and recorded on Tri-X film processed in Acufine.

Plasminogen Activator Determinations

Cumuli or granulosa cells were analyzed for plasminogen activator by a previously described method (27). The cells or cumuli were plated in medium 199 containing 10% plasminogen-depleted fetal bovine serum on a layer of [¹²⁵I]fibrin which had been previously formed on the bottom of the culture vessel. After 12 h, the medium was removed, and after the cultures were washed twice with medium 199, fresh medium 199 containing 10% acid-treated fetal bovine serum was added. Aliquots of the medium were then removed and analyzed for soluble [¹²⁵I]fibrin peptides in a Packard autogammacounter (Packard Instrument Co., Inc., Downers Grove, Ill.) at desired times.

Plasminogen-depleted (21) and acid-treated (low protease inhibitor) (18) fetal bovine sera were prepared as previously described.

Plasminogen was recovered from the lysine-sepharose columns used to plasminogen-deplete the serum by the method of Deutsch and Mertz (11). Conversion of this zymogen to plasmin by urokinase was achieved as previously described (5).

RESULTS

There are a number of hormonally controlled stages in follicular development that are associated with major cytological changes in the cumulus-oocyte complex. Acute alterations in the complex, as well as in the maturation stages of the oocyte, take place in preovulatory follicles just before follicular rupture at ovulation. In this study, specific stages of follicular development were examined in order to determine (a) whether cell-to-cell communication exists within the oocyte complex, and (b) whether there are any changes in communication that might be related to the events of oocyte maturation and ovulation.

Light Microscopy

The following stages of cumulus-oocyte development were examined in this study: (a) Specimens were prepared from rats 48 h after PMSG injection. At this time, a large number of antral follicles are present in the ovary, but ovulation has not occurred. The cumulus-oocyte complex in one of these follicles is shown in Fig. 1A. (b) Rats were administered an ovulatory regimen of PMSG and HCG. Shortly before ovulation, two classes of large follicles are evident. The first, those destined to ovulate, can be distinguished on the basis of their pink color and the fact that they protrude well above the surface of the ovary. The cumulus-oocyte complex from one of these pre-

ovulatory follicles is shown in Fig. 1C. The second, and by far the larger class, consists of large antral follicles that do not ovulate. Fig. 1B is a micrograph of a cumulus-oocyte complex from such a follicle, which was prepared from the same ovary at the identical time as the preovulatory follicle in Fig. 1C. (c) Finally, postovulatory samples were obtained from the oviduct approximately 10 h after ovulation (Fig. 1D).

In antral follicles that are not preovulatory (Fig. 1A and B), the cumulus-oocyte complex is characterized by a compact region of stratified cuboidal epithelial cells (cumulus oophorus) that are separated from the oocyte by a clear region, the zona pellucida. The striations in the zona pellucida are due to the cytoplasmic processes that connect the cells of the innermost cumulus layer, the corona radiata, with the oocyte.

In late preovulatory follicles (Fig. 1C) dramatic changes are evident in the complex. The cumulus is less compact, and it is frequently reduced to a single layer of cells, the corona radiata. The cumulus mass is essentially reduced to a population of dissociated cells. The corona radiata is interrupted by regions of missing cells, and the cumulus cells exhibit short thick cytoplasmic processes or blebs. In addition, there are few, if any, detectable striations across the zona pellucida. By comparing the appearance of complexes in follicles that are not preovulatory, either before (Fig. 1B) or after (Fig. 1A) HCG administration, with the late preovulatory sample (Fig. 1C), it would appear that the structural disintegration of the cumulus is a direct correlate of ovulation, and not a general response to elevated gonadotropin levels.

The postovulatory oocyte is still surrounded by a zona pellucida and cells from the cumulus. However, the structural disintegration of the cumulus oophorus is even more pronounced at this time than it was near the time of ovulation. Some cells of the corona radiata remain attached to the zona; however, most of the dissociated cells are retained in the vicinity of the oocyte because of the extracellular matrix that is responsible for generating a "bolus" of oocytes in the oviduct.

Electron Microscopy

The cumulus-oocyte complex in follicles that are not preovulatory is characterized by a large number of cytoplasmic processes that can be detected in the zona pellucida (Fig. 2). These

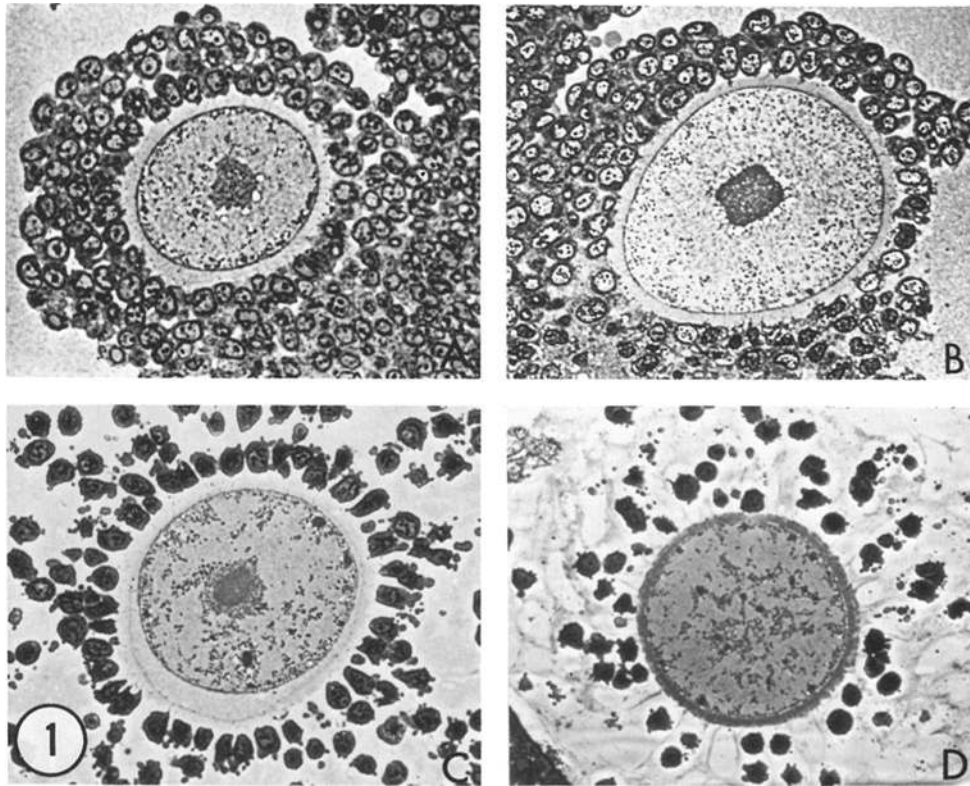


FIGURE 1 Light micrographs of the cumulus-oocyte complex at various stages of development. (A) Appearance of a complex in a follicle from a rat that had been treated with PMSG 48 h earlier. The multilayered cumulus cell region is separated from the oocyte by the zona pellucida. (B) Complex from a nonpreovulatory follicle. The experimental animal had been administered an ovulatory regimen of PMSG and HCG. This sample was taken 10 h after HCG injection, which is near the time that ovulation can be expected to occur. Nonpreovulatory samples, such as this one, were examined routinely as internal controls since their morphological and physiological properties closely resembled the ones from large follicles found in PMSG-treated animals (A). (C) Complex from a preovulatory follicle prepared from the same ovary as that described in (B). The specimen was designated preovulatory on the basis of its size and color. The cumulus is reduced to a single layer of cells that appear to be separated from each other in some regions. (D) Postovulatory specimen obtained from the oviduct about 10 h after ovulation. Some cumulus cells are still loosely associated with the oocyte at this time. (A-D) $\times 420$.

processes are extensions of the epithelial cumulus cells. They traverse the zona pellucida and contact the surface membrane of the oocyte, the oolemma. At low magnification, the cumulus cell processes, which resemble presynaptic boutons, can be readily distinguished at the oocyte surface since they are more dense than the oocyte cytoplasm. The regions of heterologous cell interaction appear to be randomly distributed over the entire surface of the oocyte, and the oocyte microvilli are displaced to accommodate the contacting processes (Figs. 2 and 8).

At the sites of interaction between the cumulus

processes and the oolemma, two prominent types of cell contact are present: One of these is a desmosome; the other is a gap junction. In thin sections, the desmosome which closely resembles a fascia adhaerens is frequently seen as a bipartite structure composed of oocyte and cumulus cell process elements (Figs. 3 and 4). Its presence is indicated by an electron density on the cytoplasmic surfaces of the membranes, and the absence of an electron-dense matrix in the intervening intercellular space. Although there is no distinct internal membrane differentiation that is characteristic of these desmosomes, a loose aggregate of

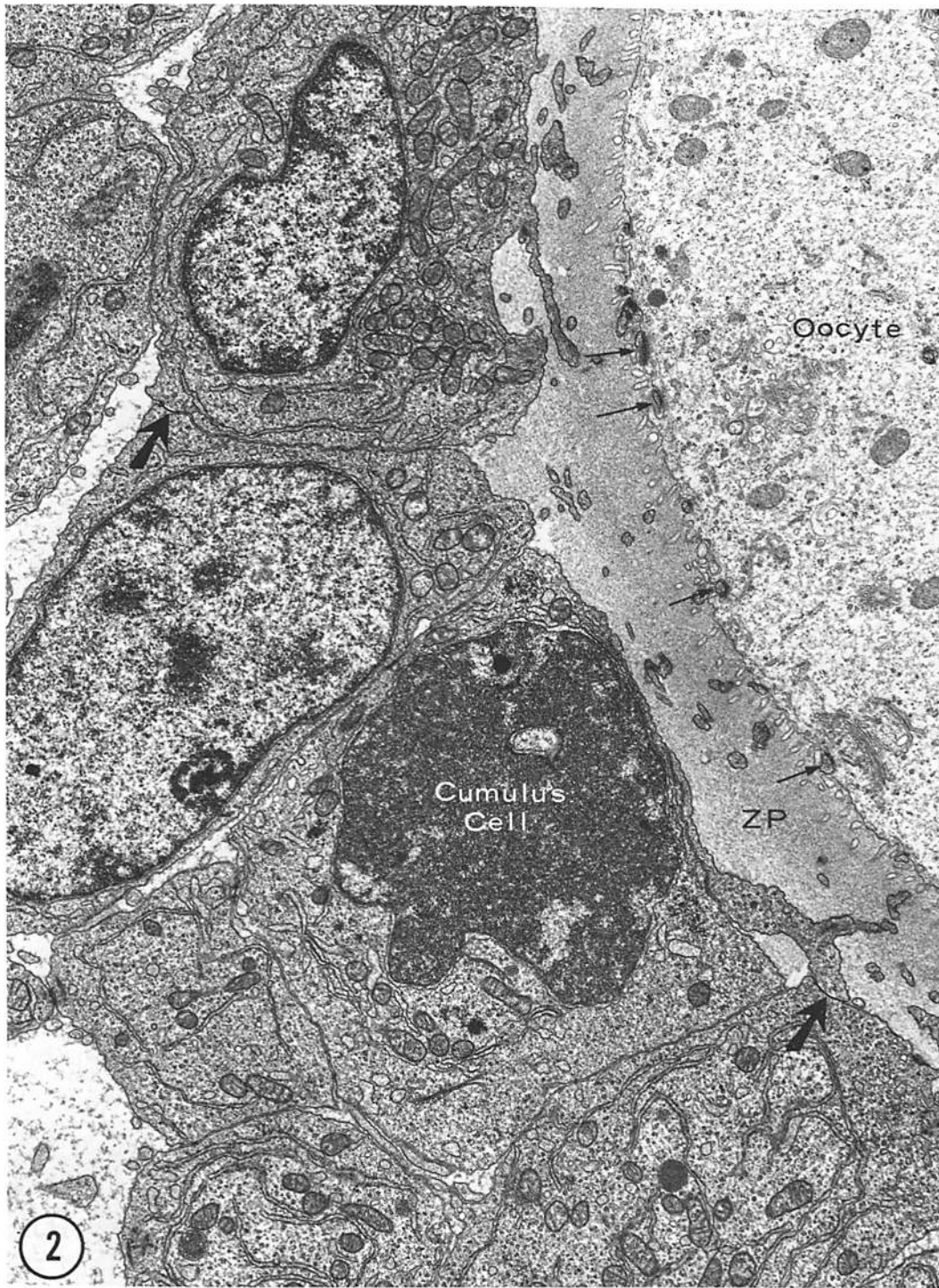
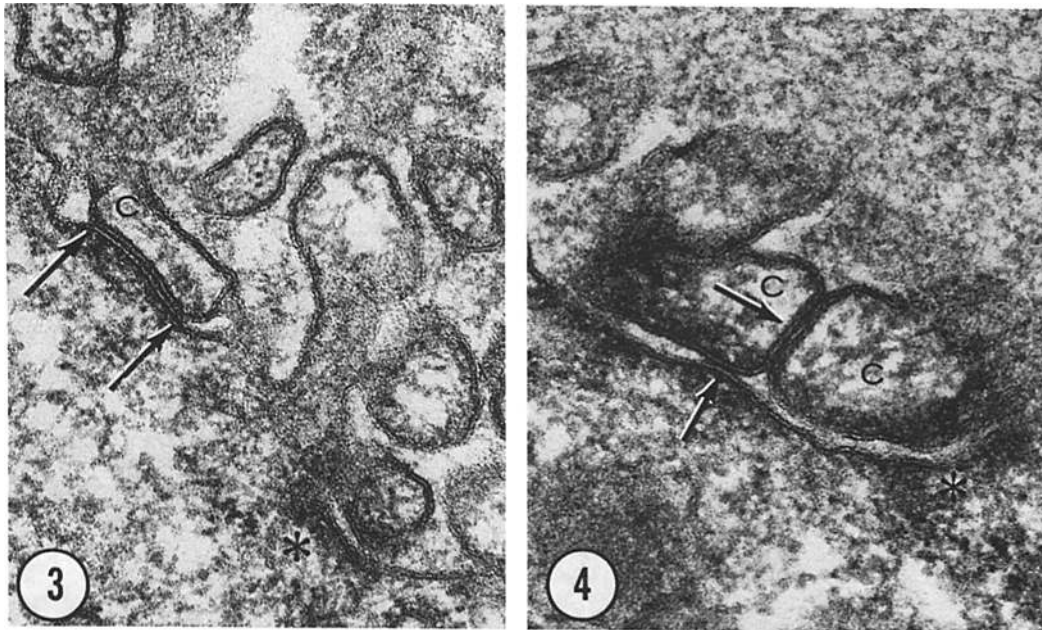


FIGURE 2 Thin section of the cumulus-oocyte complex in a large nonpreovulatory follicle from a rat treated with PMSG and HCG. The specimen was taken 10 h after the HCG injection. Cells of the innermost cumulus layer, the corona radiata, send processes through the zona pellucida (ZP) to form contacts on the surface of the oocyte (small arrows). In this image, the cumulus processes are easily distinguished from the short microvilli on the oocyte surface by the difference in staining density. The cumulus cells are also joined to each other by gap junctions that can be identified even at low magnification (large arrows). $\times 10,000$.



FIGURES 3-4 Thin-sections of the contact between cumulus cell processes and the oocyte plasma membrane.

FIGURE 3 A gap junction is present as a continuous close apposition of the cumulus cell membrane (C) and the oolemma (two arrows). The cytoplasmic surface of the oolemma junctional membrane contains an accumulation of dense material that is not present in the same region of the cumulus cell process. A characteristic desmosomal contact is also present on the oocyte surface (*). $\times 108,000$.

FIGURE 4 A "local circuit" of gap junctional connectivity between the oocyte and two cumulus cell processes (arrows). A typical gap junction is clearly resolved at the point of interaction between the oolemma and one of the cumulus cell processes; the two membranes are separated by a 2-3 nm space or "gap." There are frequent accumulations of dense material on the cytoplasmic membrane surfaces in the regions of gap junctional contact and desmosomal contact (*). These sections were prepared from specimens equivalent to those described in Fig. 2. $\times 125,070$.

large intramembrane particles is frequently present on the outer membrane half (E fracture-face) in the desmosomal regions (Fig. 9). Thus far, it has not been possible to identify a similar arrangement on the inner membrane half (P fracture-face). The desmosomal elements usually coexist with gap junctions in regions of contact, and they may provide a means of stabilizing the interacting elements.

The gap junctional contacts can be resolved in thin sections as composite structures that are comprised of the unit membranes of the oolemma and the cumulus cell process with an intervening 2-4 nm "gap" (Figs. 3 and 4). The gap junctions vary in size, but they are always macular or punctate structures that are formed by direct interaction of a cumulus cell process and the oocyte surface. The surface microvilli of the oocyte are usually dis-

placed from the regions of contact, and no gap junctional contacts have been observed between the oocyte microvilli and the cumulus cell processes. However, gap junctional contacts are frequently present between adjacent cumulus cell processes (Fig. 4).

In freeze-fracture replicas, the form and distribution of the gap junctional contacts are evident. The gap junctions between the heterologous cells vary in size from few (2-3) to more than 100 particles, and they are present as linear aggregates, polygonal aggregates, and pleiomorphic strands (Figs. 5-7). The inner membrane half (P fracture-face) of the oocyte and cumulus junctional membranes contains an aggregate of intramembrane particles that are homogenous in size (about 8-9 nm) (Figs. 5-7). The outer membrane half (E fracture-face) contains a complementary

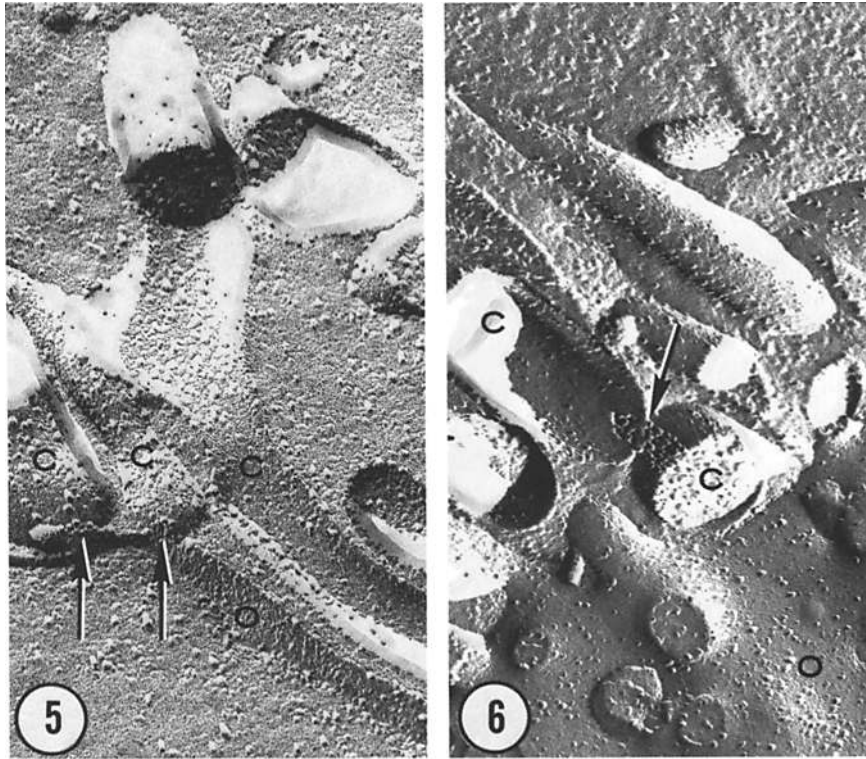


FIGURE 5 Freeze-fracture of the cumulus-oocyte junctional contacts in a nonpreovulatory follicle 10 h after HCG administration. The cumulus processes (*C*) have penetrated the zona pellucida (*ZP*) to interact both with one another as well as with the oolemma. In this instance, the fracture process has exposed the outer membrane half (*E* fracture face) of the oolemma (*O*) and the inner membrane half (*P* fracture face) of the cumulus processes that are in junctional contact with the oocyte. There are two gap junctional contacts between cumulus processes and the oolemma that are indicated in this image (arrows). $\times 97,200$.

FIGURE 6 Freeze-fracture image from a nonpreovulatory follicle showing cumulus processes (*C*) and the site of a cumulus-oocyte gap junction (arrow). The inner membrane half (*P* fracture face) of the oolemma (*O*) has been exposed in this image. $\times 81,000$.

arrangement of pits or depressions (Figs 8 and 9). Extensive freeze-fracture images of the oolemma provide an illustration of the diversity in form of cumulus-oocyte interactions (Fig. 8). In images of the oolemma *E* fracture-face, bulges in regions devoid of microvilli reveal the location of cumulus cell processes that have contacted the oolemma. Almost invariably, gap junctional contacts can be detected in each of these regions of heterologous cell interaction. Thus, in follicles that are not preovulatory a large number of small gap junctional contacts exist between the oocyte and the cumulus cells.

Gap junctions also exist between cumulus cells. These contacts occur in two locations: (*a*) between cumulus cell processes in the region of the zona

pellucida and the oocyte surface (Figs. 4 and 6); and (*b*) between cell processes and somatic portions of the cumulus cells (Figs. 10 and 11). In general, the gap junctions between processes in the oocyte region are small (Fig. 6), while those in the cumulus area are larger macular structures (Figs. 10 and 11).

In late preovulatory specimens, the number of cumulus cell processes are significantly reduced. Gap junctional contacts are still detectable in the complex, but they also are reduced in number. In addition, the remaining cumulus cells are present in a loose arrangement with bulbous cytoplasmic extensions (Fig. 12). This typical cumulus cell morphology is retained in the postovulatory specimens (Fig. 1 *D*).

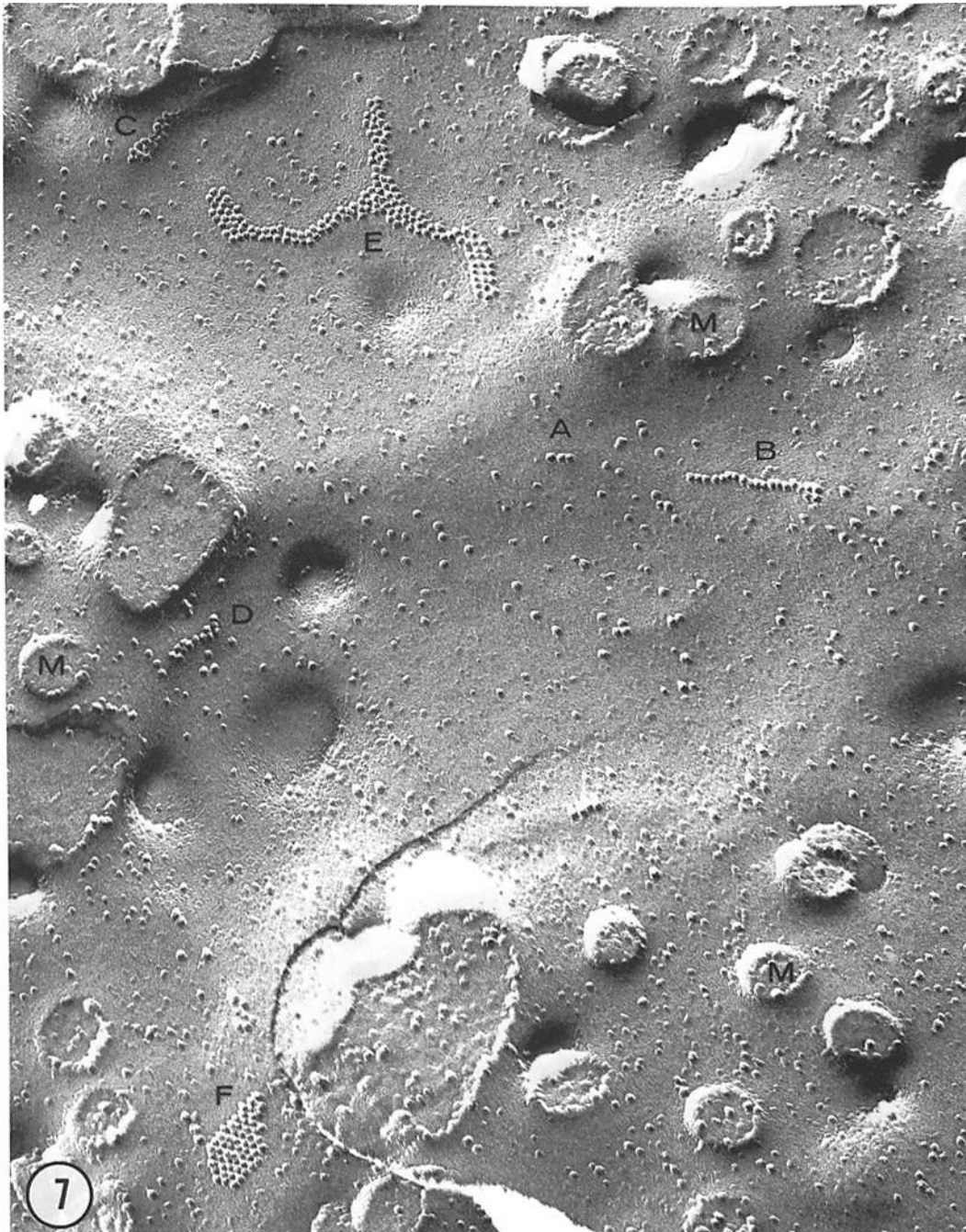


FIGURE 7 Freeze-fracture appearance of the structural pleiomorphism that is present at the site of cumulus-oocyte gap junctions in a nonpreovulatory follicle. On the inner membrane fracture face, the oolemma is characterized by numerous microvilli (*M*) that are completely absent in the regions of gap junctional contact (*A-F*). This image contains a representative sample of the various gap junctional particle arrangements that have been observed. The junctions may exist as a linear aggregate of two to three particles (*A*), a linear aggregate of more than a dozen particles (*B*), a small elongated cluster of particles (*C* and *D*), a y-shaped polygonal aggregate (*E*), and a geometrical arrangement of polygonally packed particles (*F*). $\times 129,600$.

Physiological Observations

With the observation that gap junctions are present at a high frequency between cells of the corona radiata and the oocyte, it was of interest to determine whether communication in the form of ionic coupling and dye transfer could be demonstrated between these cells. Furthermore, since the processes that connect the two cell types appear to be the only available channels for intercellular communication, and since these processes are degraded near the time of ovulation, one could predict that communication should be disrupted as ovulation approaches. In order to demonstrate intercellular communication in this system and to describe its modulation, the electrophysiological studies described below were conducted.

IONIC COUPLING: Microelectrodes were readily inserted into the oocyte and cumulus cells, and resting membrane potentials of -50 to -60 mV were obtained on initial penetration. With time, the resting membrane potentials declined, especially after repositioning the microelectrodes into cumulus cells. Under our experimental conditions, the mean resting potential of the oocytes was -39 ± 3.1 mV. As seen in Fig. 13A, the injection of a current pulse into the oocyte produced an electrotonic potential recorded in the cumulus cell, indicating that the two cells were ionically coupled. Injection of a pulse of current into the cumulus cell produced a potential of similar amplitude in the oocyte, indicating that the ionic coupling was bidirectional. Similar bidirectional coupling could be demonstrated between cumulus cells on opposite sides of the complex (Figs. 13B and D).

DYE TRANSFER: Cells that communicate ionically may also transfer molecules with molecular weights, up to 1,200 daltons (25). In our studies, the fluorescent dye, sodium fluorescein (323 daltons), was used to determine whether this form of communication exists in the cumulus-oocyte system. When fluorescein was iontophoretically injected into the oocyte (Fig. 14A-D and E-H), it subsequently could be detected in the corona radiata and then throughout the cumulus oophorus. Upon insertion of the fluorescein electrode into the oocyte, the dye rapidly filled a circle, which corresponded in outline to the oocyte (Fig. 14B and F). After a delay, dye was observed in some of the granulosa cells of the corona radiata separated from the oocyte by an unfilled area corresponding to the zona pellucida (Fig. 14C and G). It appears from samples such as

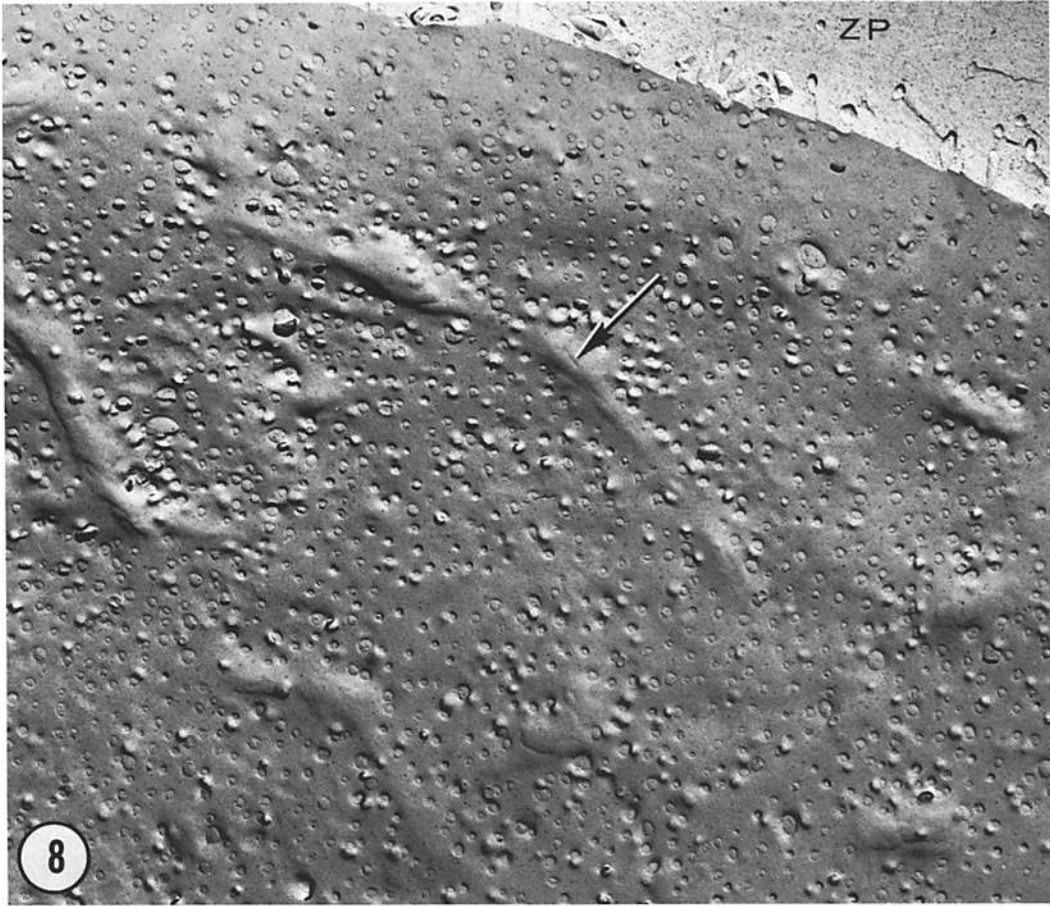
those in Fig. 14C and D that some of the cumulus cells receive dye directly from the oocyte while others may receive dye indirectly via adjacent cumulus cells. With different optics or longer injection times, dye was visualized in granulosa cells peripheral to the corona radiata (Fig. 14D and H). If fluorescein was injected directly into cumulus cells, the dye filled a number of cells (Fig. 14J and K) and ultimately (Fig. 14L) was observed in a large area around the oocyte. In these experiments, it was difficult to determine the extent to which fluorescein was transferred to the oocyte because of the large difference in volume of the oocyte and cumulus cells, and the three-dimensional arrangement of cumulus cells around the oocyte.

TEMPORAL PATTERN OF IONIC COUPLING IN PREOVULATORY FOLLICLES: As stated above, on the basis of morphological evidence it appeared that communication between the cumulus and the oocyte must be interrupted at some time near, or shortly after, ovulation. In order to test this possibility, the following experiment was conducted. Cumulus-oocyte preparations from PMSG-primed rats were examined at various times after the administration of an ovulatory dose of HCG. The immature rats used in these studies ovulated 10-12 h after the gonadotropin was injected. As seen in Table I and Fig. 15, after the HCG injection there was a progressive decrease in the fraction of preovulatory cumulus-oocyte preparations that were ionically coupled. At the time of the HCG injection, 100% of the cumuli were coupled. In the preovulatory population, the fraction decreased to 23% near the time of ovulation and 0% after ovulation. In contrast, cumuli from nonpreovulatory follicles remained coupled throughout the course of the experiment.

As seen in Table I, the transfer resistance in the preovulatory specimens dropped in the hours preceding ovulation. This is consistent with a decrease in the frequency of ionic coupling. It is also interesting that the membrane potential of the oocyte decreased during this same time period from -39 ± 3.1 mV ($n = 22$) 10-12 h before ovulation to -21 ± 2.5 mV ($n = 12$) in postovulatory specimens. A mean resting membrane potential of -14 mV has previously been reported for postovulatory mouse oocytes (24).

Plasminogen Activator Analysis

Granulosa cells secrete plasminogen activator during the hours preceding ovulation (6, 27). This



enzyme converts plasminogen, which is present in follicular fluid to the protease plasmin, a trypsin-like enzyme which might be capable of disrupting the organization of the cumulus and thus play a role in the breakdown in communication that we have reported here.

In order to test this possibility, cumulus-oocyte preparations from preovulatory and nonpreovulatory follicles were analyzed for secreted plasminogen activator. As seen in Table II, neither class produced detectable amounts of the enzyme.

While this result decreases the likelihood that plasminogen activator is directly responsible for the interruption of communication in the cumulus, it is interesting in its own right since it reveals a clear biochemical difference between granulosa cells of the cumulus that are associated with the oocyte (cumulus granulosa) and those associated with the follicle wall (membrana granulosa).

Since the above observation does not completely rule out the possibility of a role for the plasminogen activator-plasmin system in the proc-



FIGURE 10 Gap junctional interaction between corona radiata cells of the cumulus in a nonpreovulatory follicle (thin section). $\times 81,000$.

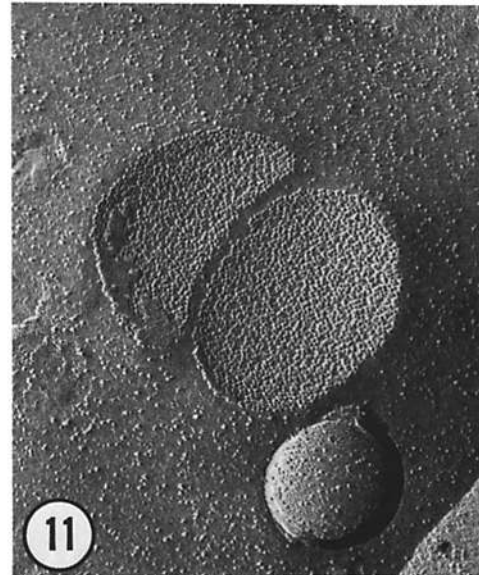


FIGURE 11 Gap junctional interaction between corona radiata cells of the cumulus (freeze-fracture) in a nonpreovulatory follicle. $\times 43,740$.

FIGURE 8 An extensive view of the outer membrane fracture face of the oolemma in a nonpreovulatory follicle. At low magnification, the numerous round profiles indicating the presence of microvilli are the most obvious feature of the fracture face. The microvilli can also be detected as surface structures extending into the zona pellucida (ZP, upper right). The microvilli are conspicuously interrupted by regions of cumulus-oocyte interactions. These regions appear as convex protrusions on this fracture face as a result of the cumulus processes indenting the oocyte surface. At this magnification, gap junctional elements can be detected in the regions of the boutonlike contacts (arrow) $\times 15,280$.

FIGURE 9 Freeze-fracture membrane specializations in a nonpreovulatory follicle associated with the complementary outer membrane half (E fracture face) of the oolemma. A banklike plaque of complementary gap junctional pits is present on this fracture face (arrows) between the clusters of microvilli (M). A loose arrangement of large particles (*) is frequently detected on this fracture face in close proximity to the gap junctional elements; these particles may be related to the desmosomal contacts that are observed in the thin-section images. $\times 81,000$.

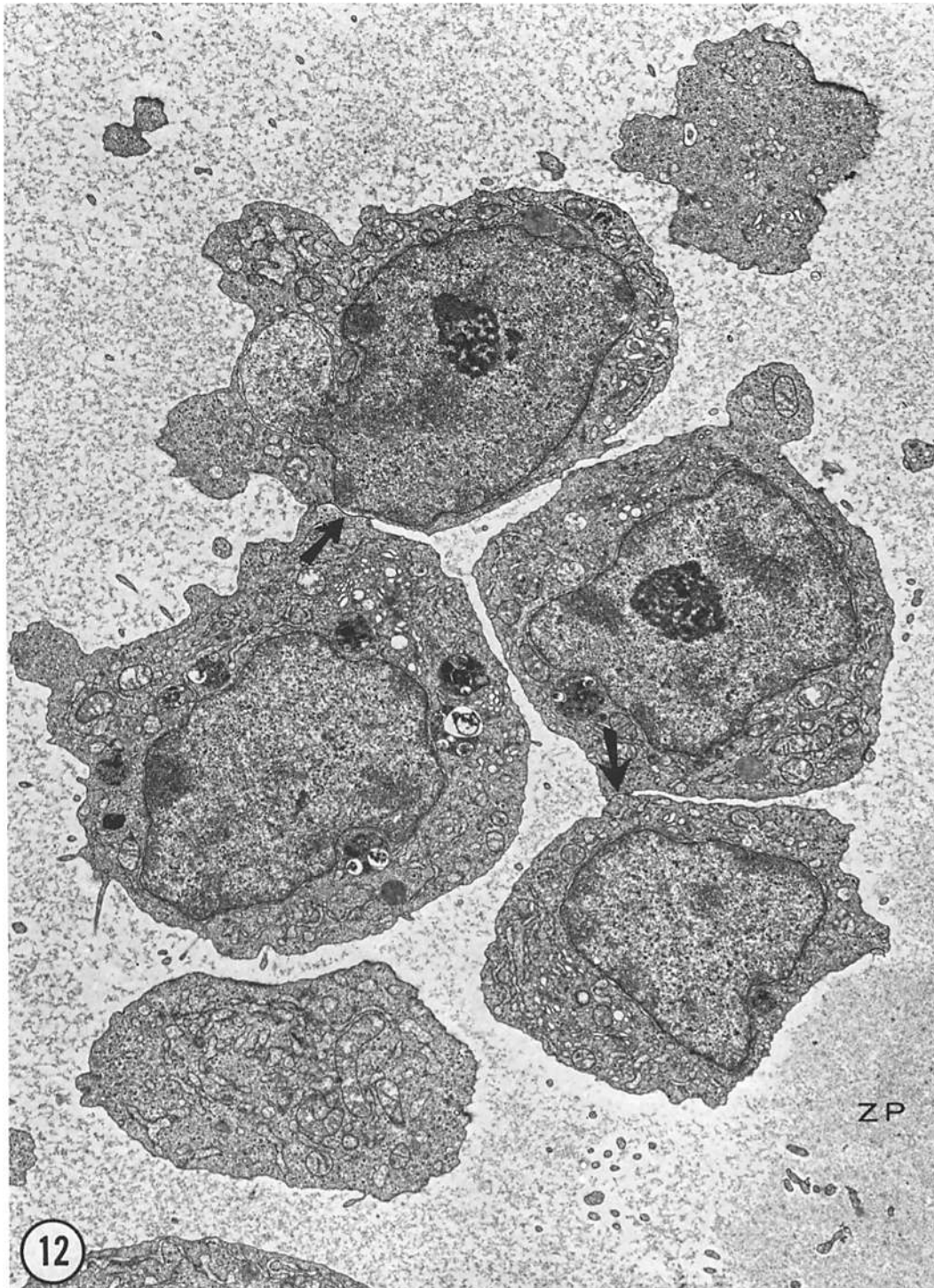


FIGURE 12 Thin-section appearance of the cumulus cells in a preovulatory follicle just before ovulation. The cells of the corona radiata are still close to the zona pellucida (ZP), but they are no longer a tightly packed layer of cuboidal epithelial cells. Some gap junctions are still present between the cells (arrows), the number of cell processes is greatly reduced, and the cells contain many bulbous cytoplasmic extensions on their surfaces. $\times 7,460$.

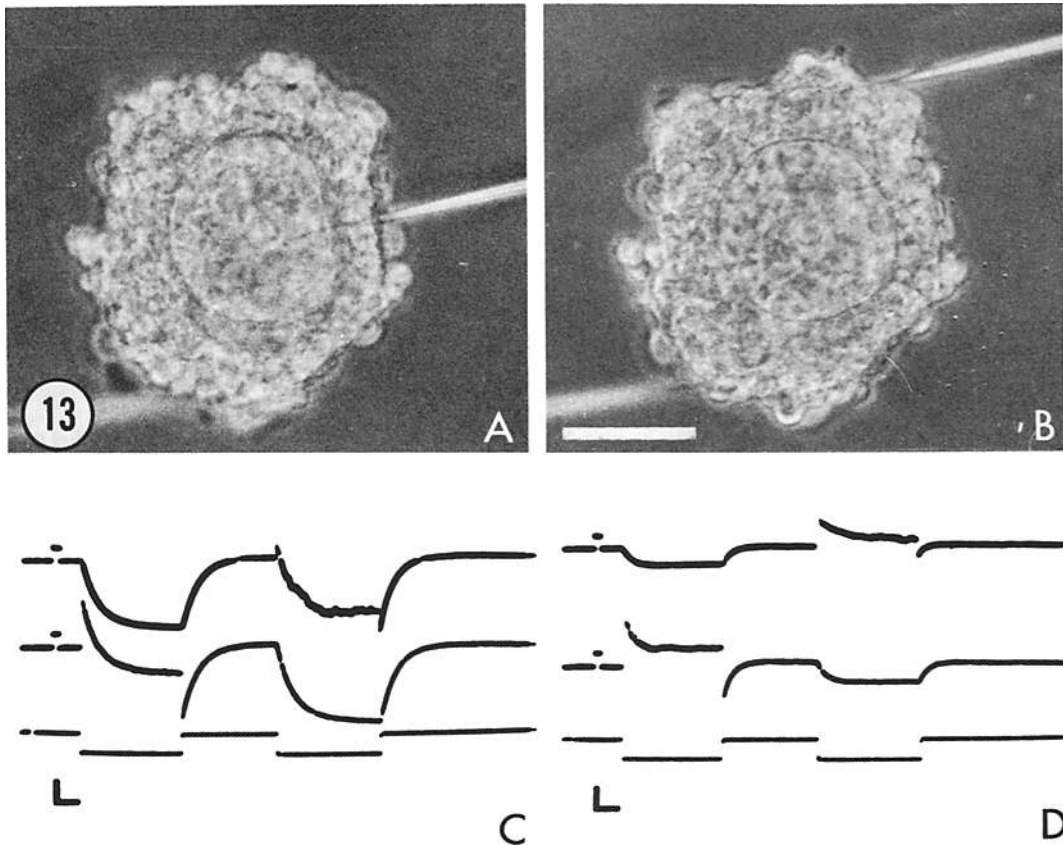


FIGURE 13 Ionic coupling between nonpreovulatory oocyte and cumulus cells (*A* and *C*) and between cumulus cells (*B* and *D*). (*A*) The photomicrograph shows an isolated cumulus with microelectrodes inserted into the oocyte and a cumulus cell. (*B*) The same cumulus preparation as used in (*A*) now with the microelectrodes inserted into two cumulus cells. (*C*) A rectangular pulse of current (lower trace) has been injected into the oocyte, resulting in a bridge deflection (middle trace) and an electrotonic potential recorded in the cumulus cell (upper trace), indicative of ionic coupling. A second pulse of current injected into the cumulus cell resulted in an electrotonic potential (middle trace) of similar magnitude in the oocyte, indicating bidirectional ionic coupling. The resting potentials were -56 mV in the oocyte and -56 mV in the cumulus cell. (*D*) A current pulse injected into the cumulus cell located at the top of (*B*) produced an electrotonic potential recorded in the other impaled cumulus cell. A second pulse delivered to the other cumulus cell in the lower left of (*B*) produced a potential recorded in the cell at the top of (*B*). Resting potentials were -38 mV and -40 mV. In both (*C*) and (*D*), the calibration pulse is 5 mV; vertical calibration line is 2.5×10^{-9} A; horizontal line is 100 ms. The bar in (*B*) represents 50 micrometers for both photomicrographs.

ess of cumulus-oocyte uncoupling, cumulus preparations from PMSG-primed animals were incubated for 24 h in the presence of 100 $\mu\text{g}/\text{ml}$ plasmin. After this treatment, the peripheral cumulus cells were less tightly packed; however, ionic coupling was still detected between oocyte and cumulus cells in every sample that was analyzed. Thus, this finding suggests that plasmin itself is not responsible for the observed breakdown in communication.

DISCUSSION

In this paper, we have shown that intercellular communication exists between cells of the cumulus oophorous and the oocyte. The cells are ionically coupled, and fluorescein dye is readily transferred between them. The channels for this communication appear to be the processes that emanate from the cells of the corona radiata and traverse the zona pellucida. The processes interact with the oolemma, and gap junctions are present

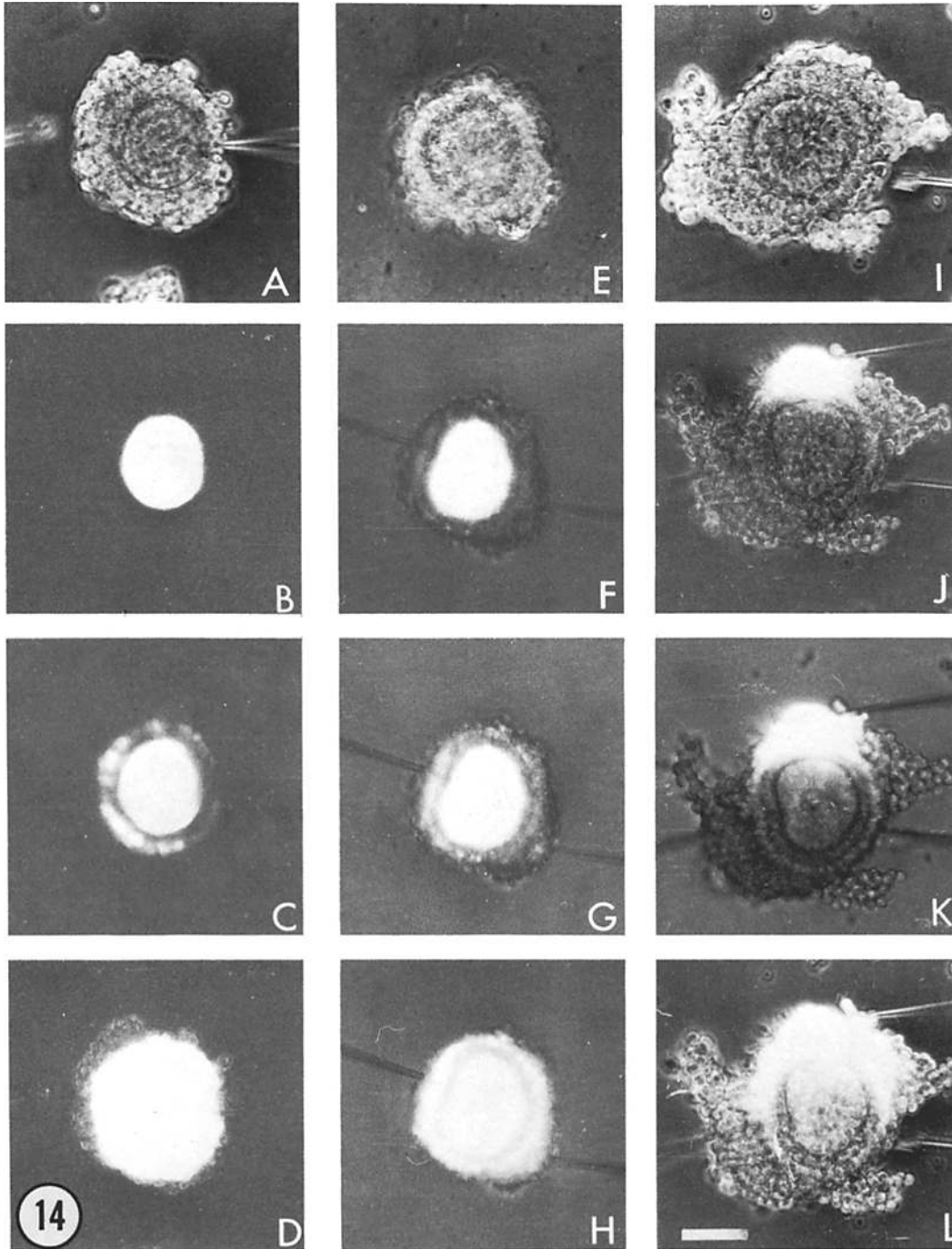


FIGURE 14 Movement of iontophoretically injected fluorescein in the nonpreovulatory cumulus-oocyte complex. Photomicrographs of three different cumuli are shown. In the left panel (A-D), the cumulus-oocyte was photographed with phase optics (A), then the oocyte was injected with fluorescein and subsequently photographed after 16 min (B), 48 min (C), and 51 min (D). Only the oocyte is visualized in (B), but in (C) dye has moved into cumulus cells adjacent to the oocyte without obscuring the zona pellucida. Many cumulus cells show fluorescence in (D); a longer exposure was used in (D) than (C) to permit the detection of cells with relatively little fluorescein. Movement in the second cumulus oocyte is shown in the middle panel (E-H) with photographs taken after 5 min (F), 14 min (G), and 25 min (H). In the third cumulus oocyte shown in the right panel (I-L), dye was injected into a cumulus cell, and it spread quickly into adjacent cumulus cells after 2 min (J), 9 min (K), and into more distant cells after 16 min (L). Cumulus cells located on top of the oocyte are filled with fluorescein and give the impression that dye has filled part of the oocyte. The line in (L) represents 50 micrometers for all the photomicrographs.

TABLE I
Temporal Correlation of Ovulation with the Extent of Cumulus-Oocyte Ionic Coupling

After HCG administration	No. cumuli tested	No. cumuli coupled	Fraction coupled	Transfer resistance mean \pm SE
<i>h</i>			%	M Ω
0	26	26	100	3.01 \pm 0.69
2	23	22	96	2.73 \pm 0.89
5 (PF)	14	9	64	1.33 \pm 0.43
5 (NPF)	12	12	100	2.29 \pm 0.66
7 (PF)	17	6	35	0.50 \pm 0.12
7 (NPF)	11	11	100	2.12 \pm 0.63
10 (PF)	17	4	23	0.89 \pm 0.54
10 (NPF)	8	8	100	1.67 \pm 0.44
24 (Postovulatory)	11	0	0	—

Cumulus-oocyte preparations were examined for ionic coupling (as described in Fig. 13) at the indicated times after the animals received an ovulatory dose of HCG. At the 0- and 2-h determinations it was not possible to distinguish between preovulatory and nonpreovulatory follicles, but from 5 to 10 h the two classes of follicles were easily distinguished on the basis of size and coloration. Under the experimental conditions used in these studies, the animals ovulate 10–12 h after the administration of PMSG. The postovulatory specimens were harvested from the oviduct. The data listed in this table are shown graphically in Fig. 15.

PF equals preovulatory.
NPF equals non-preovulatory.

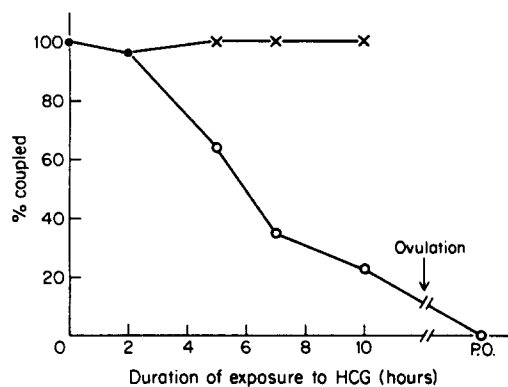


FIGURE 15 The ionic coupling data listed in Table I are shown graphically in this figure. (X—X) nonpreovulatory; and (O—O) preovulatory. P.O.: postovulatory.

in these regions of contact; a finding which is in agreement with that of Anderson and Albertini (3). As the time of ovulation approaches, the fraction of preovulatory cumuli in communication with the oocyte decreases. Once the oocyte is in the oviduct, communication appears to be completely terminated. The interruption of communication only occurs in preovulatory follicles and apparently results from disintegration or retraction of the corona cell processes rather than a complete ablation of all junctions on the surface of the oocyte.

TABLE II
Plasminogen Activator Production by Cells of the Membrana Granulosa and Cumulus Oophorus

Cell type assayed	Substrate solubilized
	%
Granulosa (PF)	29.2
Granulosa (NPF)	2.3
Cumulus (PF)	0.9
Cumulus (NPF)	0.5

Cells of the membrana granulosa (granulosa) and cumulus-oocyte complexes were prepared from preovulatory and nonpreovulatory follicles and assayed for extracellular plasminogen activator on [¹²⁵I]fibrin-coated culture dishes as described in Materials and Methods. Each determination was made with approx. 5,000 cells of each type. The cells were allowed to attach for 12 h and then assayed. The results are presented as the fraction of available substrate hydrolyzed/hour.

PF equals preovulatory.
NPF equals nonpreovulatory.

The biochemical mechanisms involved in the breakdown of communication remain unknown. Since plasminogen activator levels are elevated at the time of ovulation, and since this enzyme generates the protease plasmin within the follicle, we have examined the effect of plasmin on cumulus-oocyte preparations. Our results suggest that plasmin does not, by itself, cause communication to be interrupted.

It is of some interest to consider the role of communication between the cumulus and the oocyte and the significance of its interruption around the time of ovulation. The report that much more [³H]uridine is incorporated into oocytes when they are in contact with the cumulus (30) suggests that the cumulus may be extremely important for oocyte metabolism. Furthermore, specific factors influencing oocyte maturation might also be transmitted to the oocyte by intercellular communication. For example, it has been reported (10, 13) that oocytes increase in size as long as they are still attached to the cumulus; growth is inhibited if the cumulus is detached or removed.

The significance of an interruption of communication near the time of ovulation is not clear. Oocytes removed from antral follicles undergo spontaneous resumption of meiosis. Normally this occurs only after the preovulatory LH surge *in vivo*. Whether the relief of inhibition is purely a result of the oocyte being removed from an environment containing a meiosis inhibitory factor, such as that described by Tsafiri et al. (29), or is also due to a termination of intercellular communication with the cumulus must be examined further.

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REFERENCES

1. AMSTERDAM, A., Y. KOCH, M. E. LIEBERMAN, and H. R. LINDNER. 1975. Distribution of binding sites for human chorionic gonadotropin in the preovulatory follicle of the rat. *J. Cell Biol.* **67**:894-900.
2. AMSTERDAM, A., R. JOSEPHS, M. E. LIEBERMAN, and H. R. LINDNER. 1976. Organization of intramembrane particles in freeze-cleaved gap junctions of rat graafian follicles: optical-diffraction analysis. *J. Cell Sci.* **21**:93-105.
3. ANDERSON, E., and D. F. ALBERTINI. 1976. Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. *J. Cell Biol.* **71**:680-686.
4. BAKER, T. G. 1972. Oogenesis and Ovulation. *In* *Reproduction in Mammals*. Vol. I. Germ Cells and Fertilization. C. R. Austin and R. V. Short, editors. Cambridge University Press, London. 14-45.
5. BEERS, W. H. 1975. Follicular plasminogen and plasminogen activator and the effect of plasmin on ovarian follicle wall. *Cell.* **6**:379-386.
6. BEERS, W. H., S. STRICKLAND, and E. REICH. 1975. Ovarian plasminogen activator: relationship to ovulation and hormonal regulation. *Cell.* **6**:387-394.
7. BENNETT, M. V. L. 1977. Electrical transmission: a functional analysis and comparison to chemical transmission. *In* *Handbook of Physiology*. I. The Nervous System. Section I. Cellular Biology of Neurons. E. R. Kandel, editor. The Williams & Wilkins Company, Baltimore. 357-416.
8. BIGGERS, J. D., D. G. WHITTINGHAM, and R. P. DONAHUE. 1967. The pattern of energy metabolism in the mouse oocyte and zygote. *Proc. Natl. Acad. Sci. U. S. A.* **58**:560-567.
9. BRÖRKMAN, N. 1962. A study of the ultrastructure of the granulosa cells of the rat ovary. *Acta Anat.* **51**:125-147.
10. BLANDAU, R. J., E. WARWICK, and R. E. RUMERY. 1965. *In vitro* cultivation of fetal mouse ovaries. *Fertil. Steril.* **16**:705-715.
11. DEUTSCH, D. G., and E. T. MERTZ. 1970. Plasminogen: purification from human plasma by affinity chromatography. *Science (Wash. D. C.)*. **170**:1095-1096.
12. DONAHUE, R. P., and S. STERN. 1968. Follicular cell support of oocyte maturation: production of pyruvate *in vitro*. *J. Reprod. Fertil.* **17**:395-398.
13. EPPIG, J. J. 1977. Mouse oocyte development *in vitro* with various culture systems. *Dev. Biol.* **60**:371-388.
14. EPSTEIN, M. L., W. H. BEERS, and N. B. GILULA. 1976. Cell communication between the rat cumulus oophorus and the oocyte. *J. Cell Biol.* **70**(2, Pt.2):302a. (Abstr.).
15. ERICKSON, G. F., and R. A. SORENSEN. 1974. *In vitro* maturation of mouse oocytes isolated from late, middle, and pre-antral graafian follicles. *J. Exp. Zool.* **190**:123-127.
16. GILULA, N. B. 1977. Gap junctions and cell communication. *In* *International Cell Biology*. B. R. Brinkley and K. R. Porter, editors. The Rockefeller University Press, New York. 61-70.
17. GILULA, N. B., O. R. REEVES, and A. STEINBACH. 1972. Metabolic coupling, ionic coupling, and cell contacts. *Nature (Lond.)*. **235**:262-265.
18. JONES, P., W. BENEDICT, S. STRICKLAND, and E. REICH. 1975. Fibrin overlay methods for the detection of single transformed cells and colonies of

- transformed cells. *Cell*. **5**:323-329.
19. KRAICER, P. F., D. M. PHILLIPS, R. SANCHEZ, and S. J. SEGAL. 1976. Scanning electron microscope analysis of the cumulus cell-oocyte interaction in the rat. *J. Cell Biol.* **70**(2, Pt. 2):201 a (*Abstr.*).
 20. ODOR, L. D. 1960. Electron microscopic studies on ovarian oocytes and unfertilized tubal ova in the rat. *J. Biophys. Biochem. Cytol.* **7**:567-574.
 21. OSSOWSKI, L., J. P. QUIGLEY, G. M. KELLERMAN, and E. REICH. 1973. Fibrinolysis associated with oncogenic transformation. *J. Exp. Med.* **138**:1056-1064.
 22. PINCUS, G., and E. V. ENZMANN. 1935. The comparative behavior of mammalian eggs *in vivo* and *in vitro*. I. The activation of ovarian eggs. *J. Exp. Med.* **62**:665-675.
 23. PITTS, J. D. 1977. Direct communication between animal cells. In *International Cell Biology*. B. R. Brinkley and K. R. Porter, editors. The Rockefeller University Press, New York. 43-49.
 24. POWERS, R. D., and J. T. TUPPER. 1974. Some electrophysiological and permeability properties of the mouse egg. *Dev. Biol.* **38**:320-331.
 25. SIMPSON, I., B. ROSE, and W. R. LOEWENSTEIN. 1977. Size limit of molecules permeating the junctional membrane channels. *Science (Wash. D. C.)*. **195**:294-296.
 26. SOTELO, J. R., and K. R. PORTER. 1959. An electron microscope study of the rat ovum. *J. Biophys. Biochem. Cytol.* **5**:327-341.
 27. STRICKLAND, S., and W. H. BEERS. 1976. Studies on the role of plasminogen activator in ovulation: *in vitro* response of granulosa cells to gonadotropins, cyclic nucleotides, and prostaglandins. *J. Biol. Chem.* **251**:5694-5702.
 28. SZÖLLÖSI, D. 1975. Ultrastructural aspects of oocyte maturation and fertilization in mammals. In *La fécondation*. C. Thibault, editor. Masson et Cie, Paris. 13-35.
 29. TSAFRIRI, A., S. H. POMERANTZ, and C. CHANNING. 1976. Inhibition of oocyte maturation by porcine follicular fluid: partial characterization of the inhibitor. *Biol. Reprod.* **14**:511-516.
 30. WASSARMAN, P. M., and G. E. LETOURNEAU. 1976. RNA synthesis in fully grown mouse oocytes. *Nature (Lond.)*. **261**:73-74.
 31. ZAMBONI, L. 1974. Fine morphology of the follicle wall and follicle cell-oocyte association. *Biol. Reprod.* **10**:125-149.