QUANTITATION OF NEXUS JUNCTIONS IN THE GRANULOSA CELL LAYER OF RABBIT OVARIAN FOLLICLES DURING OVULATION

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The granulosa cell layer of a mature graafian follicle is integrated by a network of membrane junctions potentially capable of mediating cell-to-cell communication. These highly differentiated regions of plasma membranes of adjoining cells have been identified as nexuses or gap junctions (1, 2, 7, 8). The specific role of these nexuses in follicular activity is uncertain.

We have shown that segments of the granulosa nexuses frequently invaginate into either of the adjoining cells and become isolated cytoplasmic entities (5). This interiorization of nexuses may cause a reduction in intercellular junctions and thereby loosen the granulosa cells at the time of ovulation (5). Bjersing and Cajander (4) reported a significant increase in the relative frequency of interiorized nexuses per granulosa cell near the time of ovulation in ovarian follicles of rabbits treated with human chorionic gonadotropin. Thus, their results were consistent with the hypothesis that interiorization of nexuses reduces cell-to-cell cohesion during ovulation.

This report presents additional data on the frequency of interiorization of nexuses at specific time intervals associated with coitus and ovulation. It includes relevant information about changes in the size of granulosa cells and the abundance of surface nexuses within the granulosa cell layer during the ovulation process.

The term "surface nexuses" will be used in reference to the junctions located on cell cross-section peripheries. The term "interiorized nexuses" will refer to the portions of junctions that have become isolated within individual granulosa cells. These terms, which have been used by Albertini et al. (3), appear to have the appropriate connotations.

MATERIALS AND METHODS

New Zealand rabbits were housed in an animal facility with a 14 h: 10 h, light:dark cycle and were fed Purina rabbit chow (Ralston Purina Co., St. Louis, Mo.) and water ad lib. 91 follicles from 27 sexually mature rabbits were prepared for electron microscopy. Follicles ~0.1 mm in diameter (early antral) and ~1.0 mm in diameter (mature, ovulatory) were selected for experimental tissue. Control follicles were taken from unmated, estrous animals, while the experimental groups were taken at 8, 9, 9½, and 12 h postcoitus (ovulation normally occurs between 9½-10 h postcoitus).

Rabbits were anesthetized intravenously with sodium pentobarbital, and bilateral laparotomies were performed. The whole ovaries were immediately placed in 5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), and the follicle apices were excised. After 60 min, the tissue was rinsed briefly in 0.1 M cacodylate buffer and then immersed in 1% OsO4 in 0.1 M cacodylate buffer for 30 min. After dehydration in ethanol and propylene oxide, the tissue was embedded in Spurr's Epon mixture. Sections, cut on a Porter-Blum MT-2 ultramicrotome (DuPont Instruments, Sorvall Operations, Newtown, Conn.) and mounted on 75/300-mesh copper grids, were stained with 5% aqueous uranyl acetate for 30 min and Reynolds' lead citrate for 30 min. The tissue was examined on a Hitachi HU-11E electron microscope.

During microscope examination the number of interiorized nexuses per cell was counted in at least 200 cells from each group at the specified time intervals. Micrographs of randomly selected areas of these tissues were made such that each experimental group contained at least 30 cells. Total cellular area, nuclear area, and total length of surface nexuses were measured in each cell. Cytoplasmic area was determined by calculating the dif-
ference between total cellular area and the nuclear area, both measured with a Keuffel & Esser compensating polar planimeter (Keuffel & Esser Co., Morristown, N. J.). Cell circumferences were calculated by finding the circumference of a circle equal in area to the total cellular area. Each cell that was measured had a complete plasma membrane, and a large cross section of the nucleus was visible in the micrograph. All measurements were averaged on a per follicle basis. Any follicles which appeared atretic were excluded from the tabulations.

RESULTS

Granulosa Cells in Mature Follicles

Mature follicles (1.0-1.2 mm diam) were examined at specific intervals before and after follicular rupture to quantitate nexuses in the granulosa cell layer during the ovulatory process (Fig. 1). The general morphology of mature granulosa cells has been described previously (5). Within the same follicle, the granulosa cells may appear cuboidal, oval, or sometimes columnar. The average circumference of the cells was 28.19 ± 3.30 μm. The quantitative data on the nexuses are presented in Tables I and II. The standard deviations reflect the large variations that were observed from follicle to follicle.

Granulosa Cells in Early Antral Follicles

Small follicles (0.1-0.2 mm diam) in the early stages of antrum formation were examined to quantitate the distribution of nexuses in the granulosa cells of immature follicles (Fig. 2). These granulosa cells were generally more cuboidal than the cells in mature follicles, and they were slightly smaller, with an average circumference of 23.88 ± 5.06 μm. The quantitative data appear in Tables I and II.

Surface Nexuses

As a mature follicle approaches rupture, there is an appreciable decrease in the percent of the plasma membranes of the granulosa cells that form nexuses with adjoining cells (Table I). This decrease appears to result more from a reduction in the number of surface nexuses per cell rather than from a reduction in the size of individual nexuses.

There is a slight decrease in the average cell circumference at the time of ovulation, but the cells begin to enlarge after ovulation as luteinization progresses.

During ovulation there is no appreciable change in the number of surface nexuses in early antral follicles (Table I). In these immature follicles, the nexuses comprise a slightly lower percent of the plasma membrane in comparison to the granulosa cells in mature follicles of unmated rabbits.

The tabulated values of surface nexus length necessarily underestimate actual values since the plane of cross section would rarely bisect the junction. In addition, cells devoid of visible surface nexuses and the greater abundance of very small nexuses attenuate average values.

Interiorized Nexuses

Approaching the time of rupture, there is a slight reduction in the concentration of interiorized nexuses in the granulosa cells of mature follicles (Table II). The concentration decreases even further as the cells begin to enlarge during the first hours of luteinization.

There are fewer interiorized nexuses in the granulosa cells of early antral follicles, compared to the cells of mature follicles (Table II). However, the frequency of nexus interiorization in early antral follicles increases substantially near the time of ovulation and subsequently appears to decrease during early luteinization.

DISCUSSION

Loewenstein (6) has pointed out that the capacity to form junctional complexes "seems to be a nearly universal feature of cells," implying that the coupled cell system rather than the single cell is the unit in many functional respects. Usually within minutes after establishment of cell-to-cell contact, membrane modifications occur which result in the formation of channels that connect the

Figure 1 Granulosa cells in a mature ovarian follicle. The length of a surface nexus between the two prominent cells in the micrograph is marked by two bars. The arrows identify two conspicuous interiorized nexuses. × 12,300.

Figure 2 Granulosa cells in an early antral follicle. The length of a surface nexus is marked by bars. Two interiorized nexuses are identified by arrows. × 12,300.
In the granulosa cell layer the most apparent membrane junctions have the structural characteristics of a nexus or gap junction (8). These junctions are common throughout the granulosa and are frequently incorporated into the cytoplasm of individual granulosa cells by an endocytic process (5).

Very little is known about the specific functions of either the surface nexuses or the interiorized nexuses in the granulosa. Originally, we suggested (5) that the junctional complexes may be important for the transport of nutrients within the granulosa layer, and in the conduction of changes in the membrane potential of the granulosa syncytium. We are not aware of any evidence that is incongruent with these proposed metabolic and electrical functions of the nexus junctions in the granulosa.

In addition, we pointed out that the junctions may provide some cell-to-cell cohesion and contribute to the structural integrity of the granulosa layer during formation of an antrum (5). In considering this structural feature, we concluded that the interiorization of the nexuses might reduce the cell-to-cell contacts and thereby loosen the granulosa cells and the ovum during ovulation. If this hypothesis were correct, then it should be possible to detect a significant decrease in the number of surface nexuses during ovulation and a concomitant increase in the concentration of interiorized nexuses.

### Table I

| Follicle size | Hours postcoitus | Cells/follicles/rabbits | Avg. no. surface nexuses/cell | Avg length of individual nexus | Ave total length of nexuses/cell | Ave cell circumference | Plasma membrane composed of nexuses |
|---------------|------------------|-------------------------|-------------------------------|-------------------------------|-----------------------------|----------------------|----------------------------------|
| Mature 1.0-1.2 mm diam | Unmated | 47/5/8 | 1.97 ± 0.63 | 1.91 ± 0.78 | 3.71 ± 1.57 | 28.19 ± 3.30 | 13 |
| 8 | 40/5/4 | 0.84 ± 0.80 | 2.27 ± 1.10 | 1.87 ± 1.89 | 23.81 ± 5.02 | 8 |
| 9 | 36/6/4 | 1.42 ± 1.0 | 1.95 ± 1.19 | 29.73 ± 2.80 | 5 |
| 9½ | 30/5/3 | 0.43 ± 0.38 | 1.65 ± 1.40 | 0.67 ± 0.46 | 24.00 ± 2.21 | 3 |
| 12 | 30/5/4 | 0.75 ± 0.46 | 1.82 ± 1.79 | 29.83 ± 5.74 | 4 |
| Early antral 0.1-0.2 mm diam | Unmated | 33/5/3 | 1.35 ± 0.63 | 1.61 ± 0.40 | 2.32 ± 1.57 | 23.88 ± 5.06 | 10 |
| 9 | 34/4/4 | 1.00 ± 0.92 | 1.51 ± 0.64 | 1.95 ± 2.03 | 23.01 ± 3.88 | 9 |
| 12 | 30/4/3 | 1.17 ± 0.31 | 1.96 ± 0.85 | 2.47 ± 1.37 | 25.46 ± 1.64 | 10 |

* Not all cells had nexuses, therefore the values in this column are calculated on a per nexus basis, rather than a per cell basis.

† All standard deviations are calculated on a per follicle basis in order to demonstrate follicular variance.

‡ Refers to follicles that have actually ruptured.

### Table II

| Follicle size | Hours postcoitus | Cells/follicles/rabbits | Avg cytoplasmic area | Interiorized nexuses/cell | Interiorized nexuses/100 μm² cytoplasm |
|---------------|------------------|-------------------------|----------------------|--------------------------|--------------------------------------|
| Mature 1.0-1.2 mm diam | Unmated | 200/12/8 | 45.57 ± 12.88* | 1.05 ± 0.32 | 2.30 |
| 8 | 253/10/4 | 33.37 ± 19.00 | 0.72 ± 0.12 | 1.26 |
| 9 | 204/12/6 | 41.92 ± 16.17 | 0.73 ± 0.23 | 1.75 |
| 9½ | 207/8/3 | 34.74 ± 9.91 | 0.61 ± 0.34 | 1.76 |
| 12 | 255/16/7 | 50.61 ± 20.95 | 0.50 ± 0.26 | 0.99 |
| Early antral 0.1-0.2 mm diam | Unmated | 287/14/7 | 30.40 ± 12.29 | 0.06 ± 0.08 | 0.20 |
| 9 | 225/9/6 | 26.24 ± 8.18 | 0.23 ± 0.12 | 0.88 |
| 12 | 245/12/7 | 31.38 ± 2.38 | 0.12 ± 0.15 | 0.38 |

* All standard deviations are calculated on a per follicle basis in order to demonstrate follicular variance.

‡ Refers to follicles that have actually ruptured.

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nexuses. In examining this hypothesis, Bjersing and Cajander (4) reported (by empirical observation) a decrease in the surface nexuses and a "significant" increase in the interiorized nexuses at the time of ovulation. However, the present data do not confirm either our initial hypothesis or its subsequent support by other investigators. Although there may be a slight reduction in the number of surface nexuses at the time of ovulation, there is not an increase in the number of interiorized nexuses. Therefore, the functional value of the interiorization phenomenon remains undetermined.

Although there was no increase in interiorized nexuses, there were more granulosa cells without any surface nexuses at the time of follicular rupture. It was not possible to determine whether this decline in surface nexuses is the result of a dissociation of established junctions, or whether it is the result of an increase in mitotic activity in the granulosa. The latter explanation is reasonable, if the rate of cell division is greater than the rate of formation of new surface nexuses during ovulation.

We conclude that the specific functions of the surface nexuses in the granulosa cell layer are basically identical to the functions of such junctions in other tissues, i.e., metabolic and electrical coupling, and some degree of intercellular cohesion. The reason why these surface nexuses are interiorized into the granulosa cells remains undetermined, but appears unrelated to the disruption of cell-to-cell cohesion. Elucidation of this question may be derived from the collective information that accumulates in the future regarding the function of nexuses in other tissues.

SUMMARY

Electron microscopy was used in a semi-quantitative study to determine changes in the abundance and size of surface nexuses and changes in the abundance of interiorized nexuses in growing and mature ovarian follicles during the ovulatory process. Mature follicles contain larger granulosa cells than follicles in the early stage of antral formation. Also, the granulosa cells of mature follicles have a slightly greater number of surface nexuses (without a change in nexus length), and more interiorized nexuses, compared to immature follicles. As a mature follicle approaches rupture, there is an appreciable decrease in the number of surface nexuses per granulosa cell. There is also a slight reduction in the number of interiorized nexuses at this time. It is concluded that this decrease in both surface nexuses and interiorized nexuses may be a consequence of ovulatory changes during which the rate of granulosa cell division is greater than the rate of formation of new nexuses. Additionally, the disruption of cell-to-cell cohesion during the ovulatory process appears to be independent of the interiorization of surface nexuses.

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REFERENCES

1. ALBERTINI, D. F., and E. ANDERSON. 1974. The appearance and structure of intercellular connections during the ontogeny of the rabbit ovarian follicle with particular reference to gap junctions. J. Cell Biol. 63:234-250.
2. ALBERTINI, D. F., and E. ANDERSON. 1975. Structural modifications of lutein gap junctions during pregnancy in the rat and the mouse. Anat. Rec. 181:171-194.
3. ALBERTINI, D. F., D. W. FAWCETT, and P. J. OLDS. 1975. Morphological variations in gap junctions of ovarian granulosa cells. Tissue Cell. 7:389-405.
4. BJERSING, L., and S. CAJANDER. 1974. Ovulation and the mechanism of follicular rupture. IV. Ultrastructure of membrana granulosa of rabbit Graafian follicles prior to induced ovulation. Cell Tissue Res. 153:1-14.
5. ESPETT, L. L., and R. H. STUTTS. 1972. Exchange of cytoplasm between cells of the membrana granulosa in rabbit ovarian follicles. Biol. Reprod. 6:168-175.
6. LOEWENSTEIN, W. R. 1976. Permeable junctions. Cold Spring Harbor Symp. Quant. Biol. 15:49-63.
7. MERK, F. B., C. R. BOTTICELLI, and J. T. ALBRIGHT. 1972. An intercellular response to estrogen by granulosa cells in the rat ovary; an electron microscopic study. Endocrinology. 90:992-1007.
8. MERK, F. B., J. T. ALBRIGHT, and C. R. BOTTICELLI. 1973. The fine structure of granulosa cell nexuses in rat ovarian follicles. Anat. Rec. 175:107-126.