HORMONAL MODULATION OF OVARIAN INTERSTITIAL
CELLS WITH PARTICULAR REFERENCE TO GAP JUNCTIONS

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ABSTRACT
Thin-section and freeze-fracture studies on the rat ovarian interstitial cells reveal reductions in the size and the number of gap junctions after pituitary ablation. Small gap junctions, however, persist as long as 90 days after hypophysectomy, even though regressive cytoplasmic changes are completed 75 d earlier. Administration of exogenous human chorionic gonadotrophin (HCG) results in the restoration of the normal interstitial cell morphology which is accompanied by amplification of junctional membrane. Within 24 h of hormone application, gap junction growth is characterized by the appearance of formation plaques. These studies suggest that the effect of hormone on interstitial cell gap junctions is to modulate the junctional surface area.

KEY WORDS gap junctions • interstitial cells • hormonal modulation • rat ovary

Freeze-fracture studies of the mammalian ovary have indicated that cellular populations within several ovarian compartments exhibit membrane specializations implicated in cell-to-cell communication. These specializations termed gap junctions have been identified between granulosa cells (2, 25), corpus luteum cells (3), theca interna cells (1), and ovarian interstitial cells (23). In addition, heterocellular gap junctions, i.e., gap junctions between two distinct cell types, have been observed between cumulus cells and the oocyte (4). In the case of the cumulus-oocyte and granulosa cell contacts, demonstration of ionic coupling and movement of tracers (17, 19) suggests a possible role for the gap junction in metabolic cooperation during follicular development.

In view of the reciprocal relationship between ovarian compartments and the pituitary involving both trophic and steroid hormones, it is of interest that the formation, maintenance, and/or turnover of gap junctions in a number of tissues including the mammalian ovary may be profoundly affected by hormones, including both steroid and trophic peptides (reviewed in reference 22). The role of luteinizing hormone (LH) on the organization of interstitial cell gap junctions is of special interest since the structural integrity of ovarian interstitial tissue is believed to be dependent on LH (8, 18, 32). However, in contrast to the other steroidogenic compartments of the mammalian ovary including the Graafian follicle destined to ovulate and the corpus luteum, interstitial cells do not degenerate after a functional period; rather, they exhibit cycles of glandular activity followed by regression to a less-differentiated state in which they are capable of further response to LH (27).

The present studies were designed to examine whether the integrity of gap junctional contacts between interstitial cells is also sensitive to levels of LH. In these investigations, the interstitial tissue response of adult rats has been examined
MATERIALS AND METHODS

30 rats of the CD strain, hypophysectomized at 90 d of age, were obtained from the Charles River Breeding Laboratories (North Wilmington, Mass.). Animals were caged in pairs and were provided with 5% glucose water ad lib. in addition to standard laboratory diet. Body weights of all animals were taken 1 d after arrival in the laboratory.

Groups of three untreated hypophysectomized animals were sacrificed at intervals of 14, 30, 60, and 90 days after pituitary ablation. Additional groups of hypophysectomized animals, consisting of four animals each, received the following daily intraperitoneal injections (beginning 90 d posthypophysectomy): group 1 received 20 IU of HCG; group 2, 0.5 mg of estradiol (E2) dissolved on 0.5 ml of sesame oil; group 3, 0.5 mg of progesterone (P) dissolved in 0.5 ml of sesame oil; group 4, 0.5 ml of sesame oil only. (HCG rather than LH was used in these studies, since both hormones share the same receptor but HCG is more stable [31].) Two animals from each group receiving injections were sacrificed 24 h after the first injection, and the remaining animals were sacrificed 96 h after the start of the hormonal regimen (four injections total). Interstitial tissue from intact rats was obtained from six 90- and six 180-d-old rats to monitor ovarian morphology at the time of hypophysectomy. These animals were also used to monitor normal body and ovarian weights.

Rats were weighed and then sacrificed by ether anesthesia. The sella turcica of each hypophysectomized animal was inspected with a dissecting microscope to verify that pituitary ablation was complete. Ovaries and uteri were excised, weighed, and the ovaries were sliced in half and immersed in fixative, at room temperature, containing 2% paraformaldehyde, 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After 5 min in fixative, interstitial tissue was dissected out in fixative, and the tissue fragments from each group were divided in half for microtomy and freeze-fracture analysis.

Interstitial tissue is easily recognized in ovarian slices by its foamy appearance due to accumulations of cytoplasmic lipid. After hypophysectomy, interstitial tissue appears to be reduced in volume and is much more dense, but is relatively easily dissected away from corpora lutea and small follicles. Before tissue samples were subjected to freeze-fracture, all samples processed for thin-section studies were examined in semithin sections after staining for light microscopy with 0.1% toluidine blue in 1% sodium borate to confirm the isolation of interstitial tissue.

For thin-section studies, tissue was kept in fixative for 1–2 h and then washed several times in 0.1 M cacodylate buffer followed by postfixation in 1% OsO4 in 0.1 M cacodylate. Some of the tissue was en bloc stained in 1% aqueous uranyl acetate and subsequently washed several times in distilled water over 24 h. Tissues were then dehydrated in a graded ethanol series and embedded in a mixture of Epon-Araldite. Thin sections were mounted on Formvar-coated 150-mesh copper grids and examined with a Philips 200 electron microscope after staining with uranyl acetate and lead.

Tissue processed for freeze-fracture was fixed for 20-30 min, washed several times in 0.1 M cacodylate buffer, and infiltrated with 25% glycerol in cacodylate buffer. Tissue fragments measuring 1 mm² or less were mounted on paper disks, frozen in the liquid phase of Freon 22, cooled with liquid nitrogen, and subsequently stored in liquid nitrogen. Samples were then fractured at −115°C and shadowed with platinum and carbon in a Balzers apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.). Replicas were cleaned in a solution containing 1% NaOH dissolved in full-strength Chlorox. Micrographs are oriented so that the direction of shadowing is from bottom to top.

Measurements of junctional surface area and nuclear pore frequency were derived from freeze-fracture replicas, using a compensating polar planimeter (model no. 620015, Keuffel and Esser Co., Morristown, N. J.). Replicas of P-face views of the plasma membrane were used in the calculation of gap junction and formation plaque surface areas to avoid the uncertainty in determining the boundaries of formation plaques of E-face views of the plasmalemma. Junctional surface area is expressed as gap junction or formation plaque area (μm²) per 100 μm² of P-face membrane. From each ovary preparation, at least 10 junctional regions were randomly selected from available micrographs and measured. Nuclear pores were counted on a total of 15 nuclear fragments from each of the following interstitial cell preparations: control, 90 d posthypophysectomy, and 96 h after the onset of hormonal stimulation. Only those nuclear fragments measuring 10 μm² or larger were used in the calculations. Pore frequency is expressed as the number of nuclear pores per μm² of nuclear envelope. Nuclear surface area was estimated and used to calculate the total number of nuclear pores per nucleus. Estimates were made from thin-sectioned cells revealing a large cross section of the nucleus with...
nucleoli present. Two measurements were made at right angles to each other and averaged. This average diameter was used to calculate surface area, making the assumption that the nucleus was spherical. At least 25 nuclei per experimental treatment were used to estimate nuclear diameter.

RESULTS

Support of Experimental Data

The efficacy of hypophysectomy was indicated by comparison of body weights in control and experimental animals and by the lack of detectable pituitary fragments in the cranial floor at autopsy. Analysis of ovarian weights (Fig. 1) and the uterine atrophy in experimental animals also indicated the inhibitory effect of pituitary ablation on ovarian function. Increases in body weight of untreated hypophysectomized and hypophysectomized rats receiving exogenous hormone never exceeded 10% of arrival body weights. Analysis of variance indicated significant ovarian weight gain ($P < 0.05$) in rats receiving exogenous HCG and E$_2$ but not P injections. In addition, light and electron microscope observations were in agreement with earlier reports of dramatic cellular changes occurring in response to hypophysectomy and replacement therapy (6, 7, 18, 32). As shown in Fig. 2, the rat interstitial gland consists of clusters of polygonal cells separated by scattered fibroblast-like cells and bundles of collagen. Nuclei are oval with sparse heterochromatin and prominent nucleoli while the cytoplasm contains relatively large accumulations of lipid. In response to hypophysectomy, interstitial cells exhibited marked regressive changes which were reversed by administration of HCG (compare Figs. 3 and 4). The most obvious regressive changes include extensive condensation of chromatin and reduction in both nuclear and cytoplasmic volume. While HCG reversed many of the regressive changes, not restored were extensive accumulations of cytoplasmic lipid droplets.

Gap Junctions in Control Interstitial Tissue

Before summarizing the response of interstitial cell gap junctions to experimental treatments, a brief description of the appearance of these membrane specializations in untreated interstitial tissue is in order. When interstitial tissue from adult animals was examined in thin sections, profiles of gap junctions were frequently encountered at low magnification (Fig. 5). Freeze-fracture revealed that these gap junctions appear as aggregates of 9-nm intramembrane particles organized into plaques which average 1.7 μm in Diam and range in size from 0.5 to 2.1 μm. Occasionally, regions of interstitial cell membranes revealed small aggregates of clustered 10-nm particles which were associated only at the periphery of these relatively large gap junctions (Fig. 5 b). These regions of membrane are termed formation plaques and are thought to represent areas of formation and/or enlargement of gap junctions (12, 21). Quantitative measurements of freeze-fracture replicas indicated that ~1.25 μm$^2/100$ μm$^2$ of the P face of control interstitial cell membrane is composed of gap junction plaques while 0.03 μm$^2/100$ μm$^2$ is devoted to formation plaques. In addition, annular gap junctions were occasionally observed within the cytoplasm of untreated interstitial cells. The quantitative measurements of junctional surface area in both control and experimental animals are summarized in Table I.

Ultrastructural Comparison of Hypophysectomized and Treated Rats

The frequency of detection and the apparent
size of gap junction profiles in thin-sectioned interstitial cells from hypophysectomized animals, when compared to control tissue, appeared to be substantially reduced. However, small gap junctions were detected in all interstitial tissue preparations fixed as long as 90 d after hypophysectomy, even though the regressive cytoplasmic changes had been completed 75 d earlier.

Although gap junctions persisted for as long as 90 d after withdrawal of gonadotrophins, quantitative freeze-fracture analysis of interstitial cell membranes indicated that structural modifications of junctional membrane resulted from pituitary ablation and replacement therapy with exogenous HCG. Fig. 6 a and b show the appearance of junctional surfaces between interstitial cells from untreated hypophysectomized animals. Measurements of junctional profiles from hypophysectomized animals revealed a remarkable uniformity in size, averaging 0.2 μm but ranging from 0.12 to 0.23 μm in Diam. Compared to control tissue the gap junctional surface area measured ~0.11 μm²/100 μm², representing an 11.3-fold reduction in area. Furthermore, no evidence of formation plaques or annular gap junctions was encountered in interstitial cells from hypophysectomized animals.

Substantial amplification of the junctional surface area resulted from four daily injections of 20 IU of HCG (Fig. 7 a and b). In addition to the
Figure 5 Thin section of control interstitial tissue showing morphology typical of steroidogenic cells. A relatively large gap junction is shown at the arrow. × 12,200. Inset a: Higher magnification of the junctional profile seen at the arrow. × 36,000. Inset b: Freeze-cleaved appearance of an interstitial cell gap junction from the same fixation. Note the aggregates of 10-nm particles (at arrowheads) in areas thought to be formation plaques. × 48,000. Inset c: Annular gap junction from control interstitial cell. × 36,000.
TABLE I
Effects of Experimental Treatments* on Gap Junction and Formation Plaque Surface Area

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Junctional surface area (μm²/100 μm²)</th>
<th>Macular gap junctions</th>
<th>Formation plaques</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.25</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>HX</td>
<td>0.11</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>HX + HCG (24 h)</td>
<td>0.18</td>
<td>2.12</td>
<td></td>
</tr>
<tr>
<td>HX + HCG (96 h)</td>
<td>1.09</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>HX + E₂</td>
<td>0.13</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>HX + P</td>
<td>0.10</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

* Values obtained represent at least 40 measurements from each treatment.

Increased size of junctional membrane, regions of aggregation of smaller junctional plaques about a larger gap junction are apparent. Quantitative assessment of freeze-fracture replicas reveals surface areas of gap junctions and formation plaques averaging 1.09 and 0.26 μm²/100 μm², respectively, representing a 12.3-fold increase in junctional membrane when compared to preparations from hypophysectomized animals.

Indications of junctional growth, however, were most prominent in interstitial cell membranes examined 24 h after a single injection of 20 IU of HCG (Figs. 8 and 9). In thin sections, these junctional sites appear as regions of close apposition between plasma membranes of adjacent cells. The width of the extracellular space ranges from 2 to 10 nm at the junctional sites to 15 to 20 nm at nonjunctional regions. Subjacent to the junctional membranes, a fine network of microfilaments is apparent. Freeze-fracture reveals gap junctions in all of the phases of construction as suggested by Decker (12). Although the area encompassed by "macular" junctions (0.18 μm²/100 μm²) is only slightly larger than the junctional surface of interstitial cells from hypophysectomized animals, the total junctional surface including macular junctions and formation plaques measures 2.3 μm²/100 μm². This represents a 21-fold increase in junctional membrane as compared to preparations from hypophysectomized animals.

Further effects of pituitary ablation and replacement therapy on interstitial cells are seen in Figs. 10-13. In addition to the previously reported cellular changes (6, 18, 32), freeze-fracture revealed an altered distribution and a reduction in the number of nuclear pores in preparations from hypophysectomized animals when compared to HCG-restored cells. Interstitial cells from hypophysectomized animals were found to have a mean of 4.76 ± 0.48 pores/μm² while HCG-restored cells were found to have 8.7 ± 0.62. The difference in nuclear pore frequency was significant between interstitial cells from hypophysectomized animals and treated interstitial cells (P < 0.01) while no significant difference in pore frequency was observed between control (8.3 ± 0.33) and HCG-treated cells. The nuclei of interstitial cell preparations from hypophysectomized animals had an estimated surface area of 70.4 μm² whereas the surface area of HCG-treated cells was 100.6 μm². Using these estimates, the total number of nuclear pores was calculated to be 329 and 870, respectively. These data suggest that hypophysectomy also results in a 2.6-fold reduction in nuclear pore frequency. Furthermore, certain nuclear structures termed perichromatin granules, which are positioned at the interface between heterochromatin and euchromatin, were reduced by approximately half in interstitial cells from hypophysectomized animals.

DISCUSSION
The results of the present study indicate that regressive changes in the gap junctions of interstitial cells are detected following gonadotrophin withdrawal. Ovarian interstitial tissue, which is a target tissue for the peptide hormone LH, loses the unique ultrastructural organization associated with steroidogenesis after hypophysectomy (6). The loss of the differentiated interstitial cell morphology is the result of quantitative reductions in the volume and number of organelles as well as qualitative changes in morphology (9). Similarly, the frequency of detection and the size of gap junctions are substantially reduced after pituitary ablation. It is noteworthy that although hypophysectomy leads to a reduction in gap junctional contacts, these membrane specializations persist as long as 90 d in the absence of gonadotrophin stimulation, even though the regressive cytoplasmic changes had been completed 75 d earlier.

Restoration of the structural characteristics of differentiated interstitial cells results from administration of exogenous LH (8) or HCG. Replace-
FIGURES 6 and 7 Freeze-fracture appearance of interstitial cell membranes from hypophysectomized rats. × 130,000.

**Figure 6** (a) P-face view of interstitial cell gap junction from a rat hypophysectomized 30 days earlier. (b) E-face view of a gap junction 90 d after pituitary ablation. The junctions rarely exceed 0.2 μm.

**Figure 7** (a) Gap junction from HCG-restored cell. Amplification of junctional area has resulted. (b) E- and P faces of a gap junction from an HCG-treated cell. Arrows indicate E-face views of forming junctional plaques which appear during the enlargement of a gap junction.
Figures 8 and 9  Interstitial cell membranes from 90-d hypophysectomized rat, 24 h after a single injection of 20 IU of HCG.

Figure 8  Thin section of probable formation plaque, showing close apposition of membranes with gap junctions (at arrowheads). Note attachment of microfilamentous material subjacent to plaque membrane. × 90,000.

Figure 9  P-face view of freeze-cleaved membrane, showing the developmental stages as summarized by Decker (12) including: particle clustering (A), small aggregates (B), growing junctions (C), and macular junctions (D). × 90,000.

Growth therapy also leads to the amplification of gap junction contacts in discrete steps as a result of the appearance of peripherally associated intermembrane particles about existing junctions and by the apparent aggregation of smaller gap junction plaques. This growth phenomenon was first described by Johnson et al. (21) in Novikoff hepatoma cells which were dissociated and reaggregated, and has since been observed in other systems (2, 12, 14, 30).

The present studies suggest that the action of LH on the dynamics of interstitial cell gap junctions may be a modulatory one, i.e. qualitative and quantitative alterations in interstitial cell gap junctions may result from fluctuations in levels of LH, yet these specializations of the plasma membrane are maintained in the absence of hormonal stimulation. Rather than acting on any single organelle, LH may produce a general stimulation of cellular metabolism, a conclusion suggested by the study of Enders and Lyons (16) on the effects of prolactin on the ultrastructure of the rat corpus luteum. Similarly, the amplification of gap junctions in a number of tissues can be correlated with altered or elevated biochemical changes which result from the administration of exogenous hormones or other stimulatory agents. Gap junction growth has been reported to result during the ACTH stimulation of adrenocortical Y-1 tumor cells (13), thyroxine-induced differentiation of ependymoglial cells in Rana pipiens embryos (12), and vitamin A-induced mucous metaplasia in chick shank epidermis in organ culture (15). In each case, gap junction growth was induced in
tissues which exhibit small gap junctions before stimulation. Recent studies on the effects of estrogen on granulosa, and of HCG on theca cell gap junctions in hypophysectomized rats, also indicate that the effects of these hormones on the target tissue gap junctions is to modulate or augment a process that has already been started (R. C. Burghardt and E. Anderson, unpublished observations).

The variability of the distribution and frequency of nuclear pores and perichromatin granules in rat interstitial tissue during the response to hypophysectomy and replacement therapy is also consistent with the stimulatory effects of gonadotrophin on RNA and protein synthesis (28, 29). While nuclear pore frequency is not an absolute indicator of nuclear RNA synthesis activity, an increase in nuclear surface and also in pore frequency has been correlated with the activation of nuclear transcription in lymphocytes (24, 35). Similarly, the response of perichromatin granules may be of significance since these nuclear ribonucleoprotein particles have been suggested to contain RNA species of the messenger type, based on their perichromatin location, their similarity to Balbiani granules, drug-induced alterations, and evidence that they are able to migrate into or from the cytoplasm (reviewed in reference 5).

Administration of exogenous estrogen and progesterone was without effect on either interstitial cell morphology or junctional membrane. Although the exact nature of rat interstitial cell secretion is not known, there is evidence that rabbit interstitial cells secrete 20α-hydroxyprog-4-ene-3-one (10, 20). The effect of progesterone on interstitial cell gap junctions is of interest since it has been shown by Merck et al. (26) that estrogens which are produced by granulosa cells also exert growth-promoting effects on granulosa gap junctions. However, it does not appear that either estrogen or progesterone is capable of exerting any comparable morphogenetic effects on interstitial cell gap junctions.

No direct evidence has been provided which relates gap junctions to any specific function (22), and the significance of cell-to-cell communication in the interstitial cell compartment of the mammalian ovary likewise remains obscure. Definition of the structural and behavioral characteristics of gap junctions within functional units within the ovary, however, may provide a direction from which to approach the question of the role of cell-to-cell communication in the physiology of the ovary. Recent studies by Sheridan et al. (33) have demonstrated a direct correlation between electronephysiological measurements of mean junctional conductance and the mean area of gap junction contacts calculated from freeze-fracture replicas of cultured Novikoff hepatoma cells. It is reasonable to expect that alterations in the junctional membrane of interstitial cells resulting from fluctuations in the levels of LH might be related to quantitative changes in the transfer of electrical signals and/or cellular metabolites.

The maintenance of junctional contacts during periods of cytoplasmic regression may, therefore, be of significance since it has been reported that interstitial gland cells normally exhibit recurring cycles of glandular differentiation followed by periods of dedifferentiation and glandular inactivity (11, 27). It is also noteworthy that the HCG binding activity in the ovaries of adult hypophysectomized rats remains almost constant (34), indicating that the HCG receptors of interstitial gland cells may represent very stable receptors or are largely independent of pituitary hormones. It appears likely that the prolonged maintenance of both HCG receptors and gap junction contacts

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**Figures 10–13** Nuclei of interstitial cells from hypophysectomized rats.

**Figure 10** Altered distribution and reduction in number of nuclear pores 90 d after hypophysectomy. × 18,000.

**Figure 11** Nuclear envelope morphology after HCG administration. Pore frequency and distribution is identical to that of control nuclei.

**Figure 12** Thin-sectioned nucleus of interstitial cell 90 d after hypophysectomy. Perichromatin granules (arrowheads) are reduced in number compared to HCG-restored cells.

**Figure 13** Nucleolar amplification and heterochromatin reduction resulting from HCG administration. Perichromatin granules are numerous at the boundary of heterochromatin and euchromatin.
may facilitate the return to the differentiated functional state in response to elevated circulating levels of luteinizing hormone.

The present studies provide the baseline morphological data regarding the appearance of interstitial cell gap junctions after long-term hypophysectomy followed by replacement therapy. Further studies are being conducted to establish the fate of macular and annular gap junctions after withdrawal of gonadotrophin support as well as the effects of HCG hyperstimulation on gap junction growth and turnover.

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