Abstract. After ovulation of an oocyte, granulosa cells of the ovarian follicle differentiate into luteal cells and become a major factory dedicated to the synthesis of the steroid hormone progesterone. We recently established granulosa cell lines by cotransfection of granulosa cells with SV-40 and Ha-ras oncogene. In these cells progesterone secretion can be induced by cAMP as in normal rat granulosa cells. The induction of progesterone secretion is observed only after ~24 h and closely follows the delayed but quantitatively dramatic induction of the mitochondrial cytochrome P450scc which catalyzes the first step in steroid hormone biosynthesis. The mitochondrial P450 system electron transport proteins, adrenodoxin and adrenodoxin reductase, are also induced but adrenodoxin shows a faster induction. Immunofluorescence studies show that the three enzymes are induced in all cells and incorporated into all mitochondria uniformly. Electron microscopic examination using immunogold technique further confirms this and reveals that adrenodoxin is predominantly located on the matrix side of the inner mitochondrial membrane. Thus, adrenodoxin, which is a small highly charged protein, shows a distribution similar to P450scc which is an integral membrane protein. The uniformity of the response of the cells provides further evidence for the homogeneity of the cell line and makes this new granulosa cell line a highly promising system for the study of the molecular mechanisms involved in changes in gene expression during the process of granulosa cell differentiation.

In mammals, the processes of ovarian follicle maturation, ovulation, corpus luteum formation, and finally its dissolution are repeated at each estrus and menstrual cycle (24). These processes are initiated with a preovulatory increase in gonadotropic hormones. The gonadotropin-induced follicle maturation is accompanied by a sharp increase in blood progesterone level to prepare the uterus for possible ovum implantation (24). This increase results from two concomitant processes: induction of steroidogenic enzymes in the granulosa cells of the maturing follicle that differentiate into highly steroidogenic luteal cells and proliferation of these cells to form the corpus luteum after ovulation (14, 16, 18, 21–23, 32–34, 39, 42).

The induction of steroidogenesis in granulosa cells is apparently initiated by the gonadotropic hormones acting directly on these cells. The first step in the biosynthesis of progesterone is catalyzed in mitochondria by cytochrome P450scc which converts cholesterol to pregnenolone by cleaving its side chain (hence the designation “scc”) (14, 16, 25, 28). This enzyme and its ancillary electron transport proteins, adrenodoxin and adrenodoxin reductase, can be induced by gonadotropins in granulosa cells in primary culture (5, 9–12, 41). The increase in the levels of these enzymes reflects enhanced transcription of their genes (5, 11, 37, 44, 45). Gonadotropins bind to cell surface receptors and activate intracellular signaling systems including adenylate cyclase (1, 2, 4, 47). Their inductive effects can be duplicated by stimulation with cAMP suggesting that this may be an intracellular messenger of the gonadotropins (8, 32, 36).

The effect of gonadotropins on the granulosa cells is dependent on the stage of maturation of the follicle from which they derive (10, 12, 46). Thus, studies of gonadotropin action in these cells is technically difficult because of the heterogeneity of response of granulosa cells from a whole ovary or even a single follicle (12, 46), and the small number of cells that can be obtained from follicles. In contrast, the recently established granulosa cell lines easily provide a homogeneous population of cells (3, 38). Since the induction of the steroidogenic enzymes is one of the most important facets of granulosa cell differentiation, the present studies were undertaken to examine the cAMP inducibility, and intracellular distribution of a key steroidogenic enzyme system in these newly established granulosa cell lines.
**Materials and Methods**

The granulosa cell lines were established by cotransfection of granulosa cells (from preovulatory follicles of pregnant mare serum gonadotropin treated immature rats) with SV-40 DNA and Ha-ras oncogene (PO-GRS line) (38). The culture conditions, and protein and steroid assays were as previously described (3, 38). For studies on enzyme induction, cells were cultured in 35- or 100-mm petri dishes (Nunc, Belgium), at a density of \(~40,000\) cells/cm\(^2\) in DME and Ham's F12 (1:1) with 5% fetal calf serum (Biolab, Israel). At this density 50% confluence was reached in \(~24\) h. The medium was then replaced with fresh medium with or without the stimulant tested.

For Western blot analysis cells were collected in Eppendorf tubes (Brinkmann Instruments Co., Westbury, NY), lysed by freezing and thawing and kept frozen at a concentration of \(~2-3\) mg protein/ml. Aliquots of this suspension were electrophoresed on polyacrylamide gels, and Western blots were prepared and reacted with antibodies and \(^{125}\)I-protein A as described (17, 18).

Immunofluorescence was performed essentially as described (10). The cells were observed in a Zeiss photomicroscope III equipped with a vertical fluorescence illuminator.

For electron microscopic immunocytochemistry the cultured cells were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 h. The cells were then scraped with a rubber policeman and pelleted by centrifugation for 30 min in an Eppendorf centrifuge (Brinkmann Instruments Co.). The pellet was cut into small squares and infiltrated with 2.3 M sucrose in PBS, sectioned using ultracut E (Reichert Jung, Vienna, Austria) with a FC4D cryo-attachment and labeled as described (15, 40). The second antibody used was goat anti-rabbit IgG coupled to 10-nm gold particles (Janssen Pharmaceutica, Beerse, Belgium). Electron micrographs were taken either on a Phillips EM 410 or EM 300 at 80 kV.

**Results**

**Correlation of Progesterone Synthesis and Induction of Cytochrome P450scc**

In the PO-GRS1 cell line, the induction of progesterone synthesis requires nearly 24 h of treatment with 8-Br-cAMP or forskolin (Fig. 1). This delay could reflect induction of steroidogenic enzymes or the synthesis of another component necessary for steroid biosynthesis. Western blot analysis showed that in unstimulated cells the enzymes of the cholesterol side chain cleavage system are present at undetectable levels (Fig. 1). After stimulation with 8-Br-cAMP, the concentrations of these enzymes show a large increase. The time course of progesterone accumulation in the medium closely follows the induction of the P450scc but not that of adrenodoxin (Fig. 1).

The induction of P450scc shows a small but detectable increase at 24 h and reaches a peak after 48 h after the addition of medium with 8-Br-cAMP. In contrast, the induction of adrenodoxin is faster; it is observed in 6 h, and reaches a peak in 24 h (Fig. 1). The peak levels of the enzymes are similar to those in gonadotropin-stimulated normal granulosa cells. It should be emphasized that the time courses noted here were consistently observed in several experiments; thus the earlier induction of adrenodoxin appears to be a consistent phenomenon in these cells.

Since in Western blots we quantitate the enzyme levels using purified enzyme standards, the concentrations are shown in molar amounts in Fig. 1, rather than in relative units of densitometric intensity. But the purified standards are from bovine tissue and not from rat. Therefore these estimates should not be interpreted as the true concentrations of the enzymes in the cells.

**Intracellular Distribution of Newly Synthesized Cytochrome P450scc System Enzymes**

To examine the intracellular distribution of the enzymes in
PO-GRSI cells, we grew the cells on coverslips, fixed them at different times after forskolin or 8-Br-cAMP treatment, and followed the localization of the enzymes with fluorescently labeled antibodies (Fig. 2). In the absence of stimulation only a very faint fluorescence could be detected (Fig. 3). After treatment immunofluorescence observed for all three of these enzymes increased after a time course of induction similar to that revealed by the Western blot studies (Fig. 1). At maximal induction the intensity of fluorescence was nearly uniform in all cells (Fig. 2). There was a moderate difference in the intensity of staining in individual cells cultured in the presence of 5% serum. However, cells cultured in serum free medium showed a more uniform staining.

Throughout the time course of induction of the enzymes, the immunofluorescence was associated with mitochondria (Figs. 3 and 4). There was no detectable staining of nuclei and other vesicular organelles that could be seen by phase-contrast microscopy. Patches of fluorescence could be observed over some nuclei but at a higher focal plane this could be identified distinctly as mitochondria over the nuclei. Within each cell the fluorescence intensity was uniform in all mitochondria including those within cell processes (Figs. 3 and 4).

Intramitochondrial Localization of Adrenodoxin in Normal and Transformed Granulosa Cells

Recent immunogold studies elucidated the intramitochondrial localization of cytochrome P450scc (7, 9). Hence, in this study we examined the localization of adrenodoxin by

Figure 2. Phase-contrast (a) and immunofluorescent (b) microscopic photographs of PO-GRSI cells reacted with anti-adrenodoxin antibody. PO-GRSI cells were incubated for 48 h with 0.1 mM forskolin and reacted with antibodies to adrenodoxin. The numbers indicate identical cells visualized in phase contrast and fluorescent microscopy. The nuclei (Nu) are unlabeled in all cells. Bar, 30 μm.
immunogold staining of ultrathin cryosections of both normal granulosa and the PO-GRSI cells. The electron microscopy of these sections showed that the induction of the enzymes was associated with an enlargement and rounding of the mitochondria in both normal and transformed cells (cf. Fig. 5, a and b, and c and d).

In primary nonstimulated granulosa cells, adrenodoxin was localized almost exclusively in mitochondria (Fig. 5). Similar densities of gold particles were found in individual mitochondria of different cells (Table I). The gold particles were very often associated with mitochondrial cristae. However, some particles seemed to be localized at a distance >20 nm from membrane cristae (Fig. 5). Labeling in the cytoplasm was very low and occasionally seemed to be associated with polyribosomes (Fig. 5). Other cytoplasmic organelles and nuclei did not show specific labeling.

In the absence of stimulation, PO-GRSI cells showed an extremely low level of label within mitochondria (Figs. 5 and 6). After stimulation with forskolin the density of gold particles increased significantly. Most of the gold particles were localized over or on the matrix side of the inner mitochondrial membrane forming the cristae (Figs. 5 d and 6 b).

**Figure 3.** Appearance of adrenodoxin in the mitochondria of PO-GRSI cell line after stimulation with forskolin. Cells were cultured for 48 h in the absence (a) or presence (b) of 0.1 mM forskolin and reacted with antibodies to adrenodoxin for fluorescence microscopy. In a the arrowheads mark the faint fluorescence of mitochondria in nonstimulated cells. In b the arrowheads mark some regions where individual mitochondria can be distinctly visualized. Bar, 10 μm.
Discussion

During ovarian follicle maturation the granulosa cells differentiate into highly steroidogenic luteal cells. This process requires a dramatic induction of the cytochrome P450scc which catalyzes the first step in steroid hormone biosynthesis (16, 18, 21, 22, 28, 32–34, 42). In this study we examined the inducibility of the enzymes of the P450scc system, and the intramitochondrial localization of adrenodoxin in the newly established granulosa cell line and compared these with the response of normal granulosa cells.

Induction of Steroidogenesis and Cytochrome P450scc System Enzymes by cAMP

In the granulosa cell lines the induction of progesterone synthesis requires nearly 24 h. The studies here reveal that this delay in steroidogenesis reflects the slow induction of cytochrome P450scc (Fig. 1). The time course of induction of cytochrome P450scc and its final induced level in these cells are similar to that in normal granulosa cells in primary culture (our data and reference 41).

In a maturing follicle in vivo, the hormonal induction of P450scc is first observed in cells in the periphery of the follicle and then progressively becomes visible towards the center of the follicle (12, 46). Granulosa cells in primary culture show varying degrees of induction of P450scc, probably reflecting the variability observed in the follicles (10). In contrast, our immunofluorescence studies reveal that the cAMP-induced enzymes are observed nearly uniformly in all the cells of PO-GRS1 line. Thus, these cells show a uniform response as if they represent a specific stage in granulosa cell differentiation. This uniformity of response also provides further evidence for the homogeneity of the cell line.

The cell line described here was derived from preovulatory follicles (38). A previous cell line from preantral follicles shows a much lower steroidogenic response (3). Moreover, we obtained additional cell lines with gradations of steroidogenic activity (38). This raises the possibility that the protocol of transformation used results in entrapment of the cells at a particular level of responsiveness to steroidogenic stimulation as occurs in vivo (see paragraph above). Understanding the basis of this differential response may elucidate one of the major puzzles in ovarian follicle development, and these cell lines may be useful in this endeavor.

In the corpus luteum of several species, the increase in P450scc concentration is accompanied by an increase in the levels of its electron transport proteins, adrenodoxin reduc-
tase and adrenodoxin (18, 33, 42). In cultured granulosa cells these enzymes and their mRNAs can be induced by gonadotropins and by cAMP analogues suggesting that cAMP may be a second mediator of the inductive effects of gonadotropic hormones (5, 9-13, 32, 36, 39, 41, 43). Similarly, in the granulosa cell line the de novo synthesis of all three enzymes of the P450scc system shows a dramatic increase after stimulation with cAMP. However, the time course of induction for adrenodoxin observed in the cell line is much faster than that of P450scc revealing a significant difference in the regulation of the genes of these two proteins.

Most cAMP-regulated genes that have been characterized to date are very rapidly induced within an hour after stimulation (35). In contrast, the enzymes studied here can be in-

Figure 5. Localization of adrenodoxin in cryoultrathin sections of normal and transformed granulosa cells by immunogold labeling. (a and b) Primary granulosa cells from preovulatory follicles cultured for 48 h in the absence (a) or presence (b) of 0.1 mM forskolin. Highly sparse labeling is observed over particulate material in the cytoplasm (probably polyribosomes, circled). (c and d) PO-GRSI cells cultured for 48 h in the absence (a) or presence (b) of 0.1 mM forskolin. The arrowheads mark gold particles in close proximity to mitochondrial (M) cristae (wide empty arrows). Bar, 0.3 μm.
Table 1. Incorporation of Adrenodoxin into Mitochondria of Granulosa Cells

| Gold particles/μm² of section | Total mitochondria* | Individual mitochondria† |
|-------------------------------|---------------------|--------------------------|
| cAMP                          |                     |                          |
| Granulosa cells               |                     |                          |
| −                             | 34.8 ± 1.5          | 32.1 ± 3.2               |
| +                             | 92.2 ± 6.6          | 96.4 ± 6.9               |
| PO-GRS1 line                  |                     |                          |
| −                             | 7.2 ± 0.4           | 6.9 ± 0.9                |
| +                             | 76.2 ± 5.1          | 71.7 ± 5.6               |

Analysis was performed on electron micrographs taken at a magnification of 29,000 and enlarged to 100,000. Only mitochondria showing at least 0.15 μm² were selected for analysis to avoid errors in measurement of mitochondrial area in tangential sections. Areas were measured using a Namonics Digitizer connected to an Apple II computer. Background showed about one to two particles/μm² often associated with particulate material in the cells.

* The values are the mean ± SEM of 2 measurements over a total 20 mitochondria.
† The values are the mean ± SEM calculated per each mitochondria.

Produced only after prolonged stimulation for many hours. Hence, the mechanism of cAMP induction of these enzymes may be different from that of rapidly induced genes. The 5'-flanking region of the P450scc gene does not appear to have a consensus sequence similar to the well-characterized cAMP regulated elements (31).

The response of the cell line to forskolin indicates that the cells contain a highly functional adenylate cyclase system. However, one deficiency of these new cell lines is their apparent lack of gonadotropin receptors (38). The gene encoding the LH/hCG receptors have been recently cloned (26, 27). This may enable us to transfect the cDNA into the cell line, in an attempt to express these receptors in the cells and reconstitute the gonadotropin-stimulated steroidogenic response. Thus, this deficiency of the cells may prove to be an advantage in studying the functions of this hormone receptor in this granulosa cell line.

Intracellular and Intramitochondrial Localization of the Enzymes

Throughout the full time course of induction of the enzymes studied, the immunofluorescent and immunogold label associated with them was observed within the mitochondria. The very low background in immunogold staining of adrenodoxin outside of the mitochondria shows that it does not accumulate in the cytoplasm. The enzyme is incorporated into all mitochondria within a cell uniformly. There does not ap-

Figure 6. Intramitochondrial localization of adrenodoxin in PO-GRS1 cells. The cells were cultured for 48 h in the absence (a) or presence (b) of 0.1 mM forskolin. The arrowheads mark gold particles in close proximity to or over mitochondrial cristae (wide empty arrows). Note that the vast majority of the gold particles are associated with the cristae in both nonstimulated and stimulated cells. Bar, 0.1 μm.
pear to be any selective translocation of the enzyme into a sub-population of mitochondria (Table I). These results contradict previous observations of differential distribution of proteins within mitochondria in a single adrenal cortex cell (29, 30). One possible reason for this may be technical limitations of previous horseradish peroxidase staining method (29, 30).

Previous evidence indicated that the mitochondrial P450 system enzymes are located in the membranes of the cristae (6, 29, 30). Recent immunogold studies refined the localization of the mitochondrial cytochrome P450s in the matrix side of the inner mitochondrial membrane (7, 9). In contrast to these P450s that behave as integral membrane proteins and require detergents for solubilization, their electron donor, adrenodoxin, is a small easily solubilized protein that associates with the P450 and its reductase mainly by ionic interactions (19, 20, 25). The immunogold labeling results shown here establish that despite its very different biochemical behavior and structure, adrenodoxin is also localized on the matrix side of the inner mitochondrial membrane (Figs. 5 and 6). Thus, adrenodoxin is clearly a peripheral membrane protein that probably associates with the membrane components by ionic interactions, and it does not appear to float in the matrix as pictured in some models of mitochondrial P450 systems. Although the vast majority of the gold particles were associated with the mitochondrial cristae, some gold particles were also found far away from the mitochondrial inner membrane. This phenomenon can arise from the technical limitations of the method which does not always clearly resolve the cristae especially at tangential sections. In addition, because of the indirect labeling methods, the size of the first and second antibody linked to the gold particle should also be considered. Nonetheless, even if the gold particles in the matrix reflect a true localization, these would represent only a small portion of the total adrenodoxin molecules in the mitochondria.

Conclusion

The newly established granulosa cell line, PO-GRS1, shows a behavior similar to normal granulosa cells from preovulatory follicles in the patterns of induction of steroidogenesis and steroidogenic enzymes by cAMP, and the levels and intracellular distribution of steroidogenic enzymes after induction. Yet, unlike primary granulosa cells in culture, in the newly established granulosa cell line, PO-GRS1, the uninduced levels of the enzymes remain extremely low but uniformly and dramatically increase in all cells after induction. As opposed to primary cultures of mixed cells, the uniformity of the response and the technical convenience of the new cell line make it a highly promising system for the study of the molecular mechanisms involved in the induction of steroidogenic enzymes. The apparent maintenance of some differentiated responses of normal granulosa cells in the newly developed cell line may also make it useful in the study of other processes related to steroidogenesis and granulosa cell differentiation. The specificity of the requirement for cotransfection with Ha-nas oncogene in establishing these cell lines may hold some clues for our understanding of these processes.

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