Immunoelectron microscopic localization of three key steroidogenic enzymes (cytochrome P450$_{scc}$, 3β-hydroxysteroid dehydrogenase and cytochrome P450$_{c17}$) in rat adrenal cortex and gonads

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Abstract

The biosynthesis of steroid hormones in endocrine steroid-secreting glands results from a series of successive steps involving both cytochrome P450 enzymes, which are mixed-function oxidases, and steroid dehydrogenases. So far, the subcellular distribution of steroidogenic enzymes has been mostly studied following subcellular fractionation, performed in placenta and adrenal cortex. In order to determine *in situ* the intracellular distribution of some steroidogenic enzymes, we have investigated the ultrastructural localization of the three key enzymes: P450 side chain cleavage (scc) which converts cholesterol to pregnenolone; 3β-hydroxysteroid dehydrogenase (3β-HSD) which catalyzes the conversion of 3β-hydroxy-5-ene steroids to 3-oxo-4-ene steroids (progesterone and androstenedione); and P450$_{c17}$ which is responsible for the transformation of C$_{21}$ into C$_{19}$ steroids (dehydroepiandrosterone and androstenedione). Immunogold labeling was used to localize the enzymes in rat adrenal cortex and gonads. The tissues were fixed in 1% glutaraldehyde and 3% paraformaldehyde and included in LR gold resin. In the adrenal cortex, both P450$_{scc}$ and 3β-HSD immunoreactivities were detected in the reticular, fascicular and glomerular zones. P450$_{scc}$ was exclusively found in large

mitochondria. In contrast, 3β-HSD antigenic sites were mostly observed in the endoplasmic reticulum (ER) with some gold particles overlying crista and outer membranes of the mitochondria. P450$_{c17}$ could not be detected in adrenocortical cells. In the testis, the three enzymes were only found in Leydig cells. Immunolabeling for P450$_{scc}$ and 3β-HSD was restricted to mitochondria, while P450$_{c17}$ immunoreactivity was exclusively observed in ER. In the ovary, P450$_{scc}$ and 3β-HSD immunoreactivities were found in granulosa, theca interna and corpus luteum cells. The subcellular localization of the two enzymes was very similar to that observed in adrenocortical cells. P450$_{c17}$ could also be detected in theca interna cells of large developing and mature follicles. As observed in Leydig cells, P450$_{c17}$ immunolabeling could only be found in the ER. These results indicate that in different endocrine steroid-secreting cells P450$_{scc}$, 3β-HSD and P450$_{c17}$ have the same association with cytoplasmic organelles (with the exception of 3β-HSD in Leydig cells), suggesting similar intracellular pathways for biosynthesis of steroid hormones.

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Introduction

The biosynthesis of steroid hormones in the adrenal cortex, testis, ovary and placenta results from a series of successive steps involving both cytochrome P450 enzymes, which are mixed-function oxidases, and steroid dehydrogenases (for review see Miller 1988). The first step is the conversion of cholesterol into pregnenolone by the cholesterol side-chain cleavage P450 (P450$_{scc}$) enzyme. Pregnenolone can be either directly hydroxylated at the 17α-position or dehydrogenated at the 3β-position by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) to yield progesterone. The hydroxylation of pregnenolone and progesterone on the 17α-position and the scission of the

C17,20-carbon bond of the 17α-hydroxylated steroids are catalyzed by the steroid 17α-hydroxylase/17,20-lyase P450 (P450$_{c17}$). This enzyme is a key branch point in the pathways of biosynthesis of steroid hormones, such as glucocorticoids and sex steroids. All these enzymes are distributed throughout different cellular compartments.

So far, most of the data on the subcellular localization of steroidogenic enzymes have been obtained following subcellular fractionation studies performed in placenta and adrenal cortex (Katagiri *et al.* 1976, Kominami *et al.* 1980, Pudney *et al.* 1985, Shinzawa *et al.* 1988, Luu-The *et al.* 1989, 1990, 1991, Cherradi *et al.* 1993, Sauer *et al.* 1994, Thomas *et al.* 1999). The ultrastructural localization of steroidogenic enzymes has, so far, not been extensively
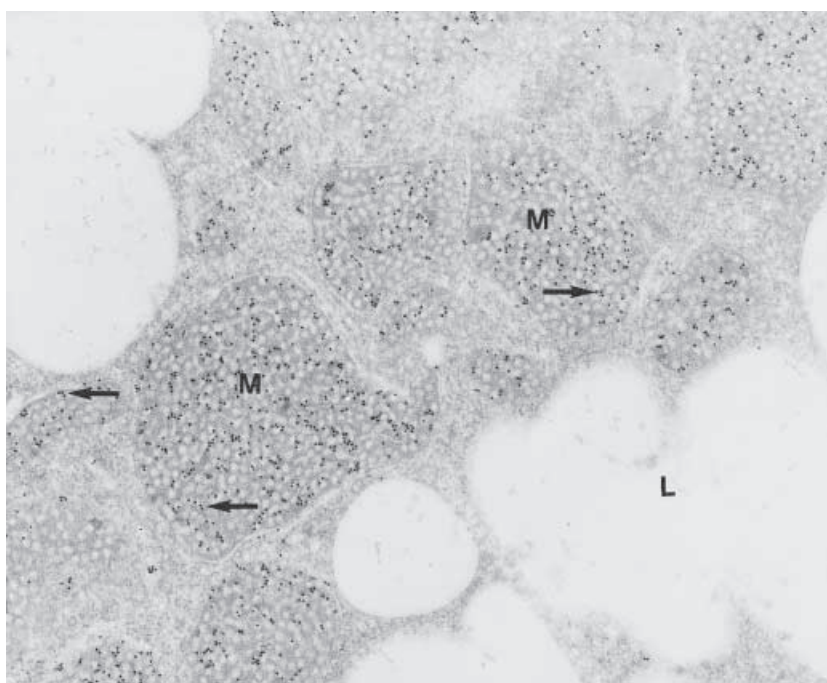
Figure 1 Localization of $P450_{scc}$ in fascicular zone of the adrenal cortex. Colloidal gold particles are located in mitochondria (M), overlying vesicular structures (→). Note the absence of labeling over the endoplasmic reticulum and lipid inclusions (L). Magnification × 64 000.

investigated and the few reports on the subject are restricted to the adrenal cortex (Ishimura *et al.* 1988, Shinzawa *et al.* 1988, Whitnall *et al.* 1993, Cherradi *et al.* 1997, Ishimura & Fujita 1997).

From the previous biochemical and morphological studies it appears that $P450_{scc}$ is almost exclusively associated with mitochondria and that 3β-HSD is predominant in microsomal fractions (endoplasmic reticulum) and has also been found in mitochondrial preparations (Katagiri *et al.* 1976, Luu-The *et al.* 1989, 1990, 1991, Cherradi *et al.* 1994, 1997, Ishimura & Fujita 1997, Thomas *et al.* 1999). $P450_{c17}$ has been found to be associated only with microsomal fractions (Kominami *et al.* 1980, Miller 1988, Ishimura & Fujita 1997).

In order to clarify the subcellular distribution of these three enzymes in different steroid-secreting cell types, we felt it was of interest to study the comparative *in situ* localization of the enzymes in adult rat adrenal cortex and gonads. The studies were performed at the electron microscopic level using specific antibodies for each enzyme.

Materials and Methods

Animals

Four adult male (225–250 g) and female (175–200 g) Sprague-Dawley rats were housed under constant temperature (21±1 °C) and light (lights on from 0600 to 2000 h) regimens. Purina Chow (Ralston-Purina, St Louis, MO, USA) and tap water were available *ad libitum*. The rats were all perfused between 0900 and 1000 h for histological procedures, as described below. The females were at random stages of the estrous cycle.

Histological procedures

The animals were perfused transcardially with 200 ml 1% gluturaldehyde and 3% (w/v) paraformaldehyde in 0·1 M phosphate buffer (pH 7·4). The different tissues, namely testes, ovaries and adrenals were excised and post-fixed in the same fixative for 24 h at 4 °C.

Preparation of the tissue blocks in LR gold resin (London Resin Co., Reading, Berks, UK) was performed as previously described (Thorpe 1999). Briefly, following fixation, tissue fragments were rinsed with phosphate buffer and dehydrated in graded series of up to 100% ethanol. The tissue fragments were then infiltrated with LR gold resin and placed in gelatin capsules for polymerization under UV lamps at 4 °C for 24 h.

Immunocytochemistry

Semithin sections were first performed for selection of the areas of interest in each gland. Ultrathin sections were then cut with a diamond knife and collected on nickel grids.
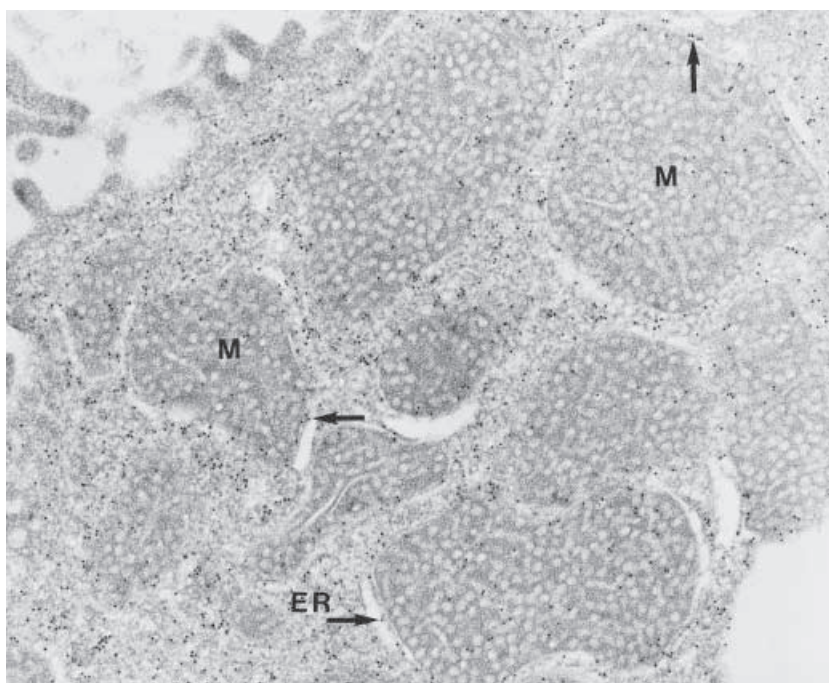
Figure 2 Localization of 3β-HSD in the adrenal cortex (fascicular zone). The colloidal gold particles are mostly associated with the endoplasmic reticulum (ER). In the mitochondria (M), the gold particles are overlying vesicular cristae membranes as well as the outer membranes (arrows). Magnification × 64 000.

They were immunostained using protein A–gold complex (10 nm; British Biocell Int., Cardiff, UK), as described (Roth *et al.* 1978). The antisera to human P450$_{scc}$ (supplied by Dr Miller; Black *et al.* 1993), human type 1 3β-HSD (Luu-The *et al.* 1989, 1990, Pelletier *et al.* 1992) and human P450$_{c17}$ (Tremblay *et al.* 1994) were all used at dilutions ranging from 1:500 to 1:1000. Control experiments were performed by substituting non-immunized rabbit serum (1:500) or the antiserum (1:500) absorbed with an excess of their respective antigen (10^{-6} M). Following immunostaining procedures, the sections were counterstained with 1% uranyl acetate and lead citrate.

Results

In all the tissues examined, the fixation with aldehydes and embedding in LR gold did not always provide a clear distinction between the smooth and rough endoplasmic reticulum (ER). Since the steroid-secreting cells contain large amounts of smooth ER, especially in close proximity of lipid inclusions and mitochondria, we consider that in general the labeling occurring in ER was mostly associated with smooth ER.

Adrenal cortex

In the adrenal cortex, immunolabeling for P450$_{scc}$ and 3β-HSD was observed in secretory cells of reticular, fascicular and glomerular zones. The adrenocortical cells are characterized by the presence of large amounts of smooth ER, numerous lipid inclusions and large mitochondria with tubular or vesicular cristae. P450$_{scc}$ immunogold labeling was restricted to mitochondria, with the majority of colloidal gold particles overlying vesicular structures (Fig. 1). Neither ER nor lipid inclusions were labeled. In contrast, 3β-HSD immunolabeling was mostly observed over ER (Fig. 2; Table 1). Labeling also occurred in mitochondria, with gold particles being located over the vesicular crista membranes of mitochondria as well as over the outer membranes. No staining was obtained with the antibodies to P450$_{c17}$.

Testis

In the testis, the three enzymes were immunolocalized exclusively in Leydig cells. The cytoplasm of these cells is characterized by abundant smooth ER, numerous mitochondria and a few lipid inclusions. As observed in the adrenal cortex, P450$_{scc}$ immunoreactivity was exclusively observed in mitochondria, although the intensity of labeling was less than that observed in the adrenocortical cells (Fig. 3). Colloidal gold particles were observed over the limiting and crista membranes of the mitochondria. In contrast to what has been observed in the adrenal cortex, 3β-HSD immunostaining was exclusively detected in mitochondria, with no specific labeling occuring in the

Table 1 Subcellular distribution of 3β-HSD (percent of gold particles). Data were calculated from the analysis of 820-1310 gold particles per cell type

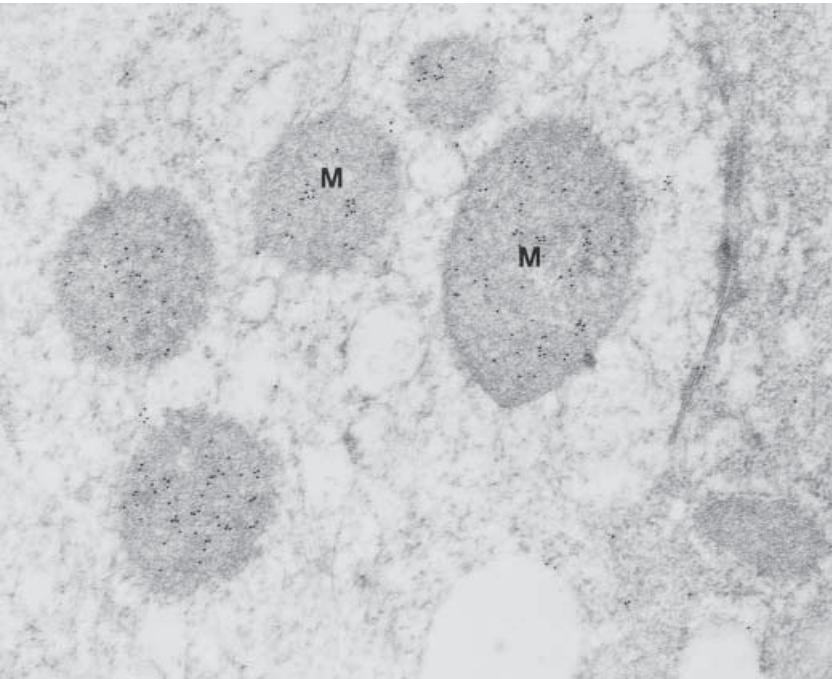| | **Cell types** | | | | |
| | | | Ovarian cells | | |
Subcellular compartments	Adrenocortical cells	Leydig cells	Granulosa cells	Thecal cells	Luteal cells
Mitochondria	25·5	100	25·8	30·6	26·1
Endoplasmic reticulum	74·5	0	74·2	69·4	73·9

Figure 3 Localization of $P450_{scc}$ in a Leydig cell of the testis. The labeling is seen in mitochondria (M). Very few particles, which can be associated to background, are observed over the endoplasmic reticulum. Magnification × 64 000.

ER (Fig. 4; Table 1). As shown in Fig. 5, $P450_{c17}$ immunoreactivity was only observed in ER. The very few gold particles which were occasionally seen overlying mitochondria could possibly be attributed to background.

Ovary

In the ovary, the ultrastructural characteristics of the steroid secreting cells are similar to those observed in the Leydig cells with abundant smooth ER and lipid inclusions varying in number and size. The lipid inclusions appeared larger and more numerous in luteal and thecal cells than in granulosa cells. $P450_{scc}$ could be immunolocalized in granulosa cells and theca interna cells in large antral and preovulatory follicles (Fig. 6). In small developing follicles, the labeling was weak and inconsistant in both granulosa and thecal cells. Interstilial and corpus luteum cells were also immunolabeled (Fig. 7). As observed in the Leydig and adrenocortical cells, in all the positive cells, $P450_{scc}$–associated gold particles were only observed over mitochondria, mostly in association with crista membranes.

3β–HSD immunoreactivity was detected in thecal and granulosa cells of growing and preovulatory follicles. Primary follicles remained unstained. Interstitial and luteal cells were also strongly labeled. (Figs 8 and 9; Table 1). As observed in adrenocortical cells, in all the reactive cells,
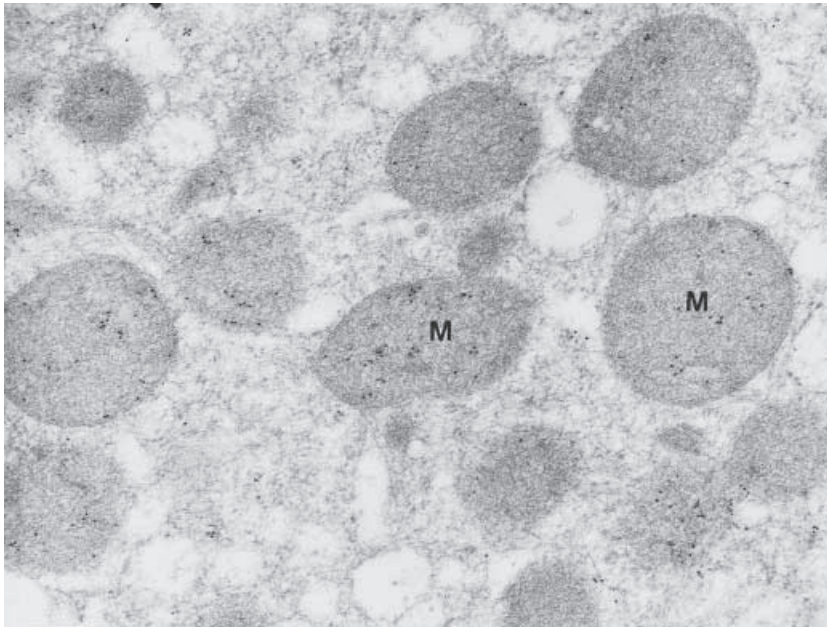
Figure 4 Localization of 3β-HSD in a Leydig cell of the testis. Immunolabeling is restricted to mitochondria (M). Magnification × 64 000.
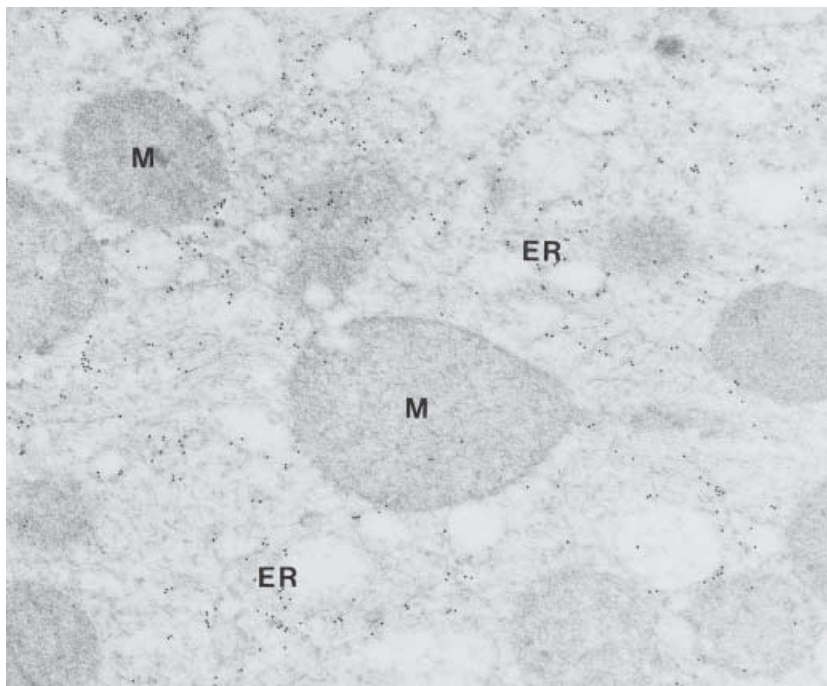

Figure 5 Localization of P450$_{c17}$ in a Leydig cell of the testis. Labeling is exclusively detected in the endoplasmic reticulum (ER). Very few particles, which correspond to background level, are present over mitochondria (M). Magnification × 64 000.

most of the immunoreactivity had an extra–mitochondrial localization, most of the gold particles being associated with the ER. In the mitochondria, the labeling was associated with the crista membranes.

P450$_{c17}$ was detected in the theca interna cells of large antral and preovulatory follicles (Fig. 10) and interstitial cells while granulosa cells remained unlabeled. Few luteal and interstitial cells were observed to exhibit
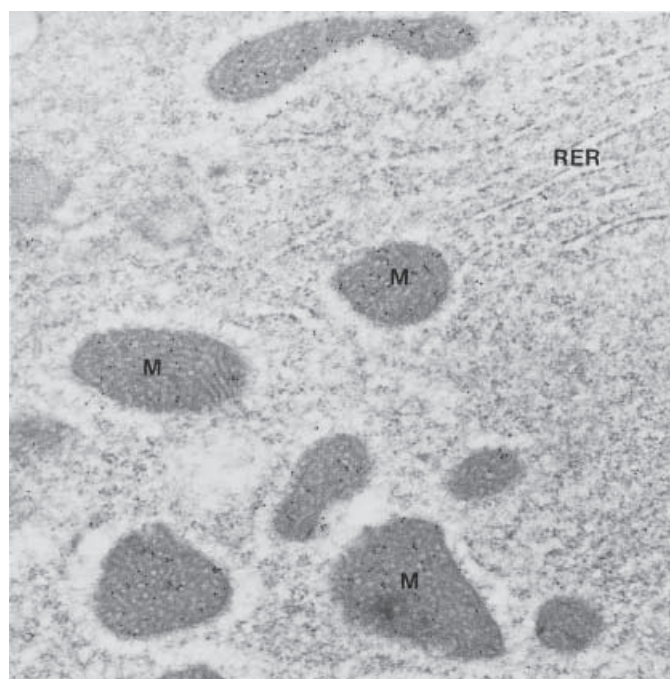
Figure 6 Localization of P450$_{scc}$ in the ovary: theca interna cell. Colloidal gold particles are almost exclusively associated with the tubular-vesicular cristae membranes of mitochondria (M). Very few particles, corresponding to the background, are present outside mitochondria. RER: rough endoplasmic reticulum. Magnification × 64 000.

immunolabeling. The subcellular localization of the enzyme was identical to that observed in the Leydig cells of the testis with labeling exclusively associated with ER.

When non-immunized rabbit serum or antisera immunoabsorbed with their respective antigens were used, no association of gold particles with any organelle in the reactive cell types could be observed. Only a few dispersed gold particles could be detected throughout the sections (Fig. 11).

Discussion

The present data obtained at the ultrastructural level provides new information about the *in situ* localization of three key steroidogenic enzymes. Although due to the fixation and embedding methods used it was not always possible to clearly identify the smooth ER, the abundance of smooth ER in the steroid-secreting cells suggest that most of the extra-mitochondrial immunolabeling was in fact associated with smooth ER. Moreover, the rough ER was not seen to be consistently labeled in any of the P450$_{scc}$-, 3β-HSD- or P450$_{c17}$-positive cells.

P450$_{scc}$ immunoreactivity was detected in the three layers of the adrenal cortex, the Leydig cells in the testis as well as in thecal, granulosa, interstitial and luteal cells in the ovary. These results are in good agreement with previous localization studies performed in several species including the rat (Miller 1988, Sasano *et al.* 1989, LeGoascogne *et al.* 1991, Ishimura & Fujita 1997, Sanders & Stouffer 1997). In all the reactive cell types, immunolabeling was restricted to mitochondria, with gold particles overlying vesicular structures. These results are in agreement with a previous immunoelectron microscopic study indicating that P450$_{scc}$ immunoactivity was associated with the matrix side of the inner mitochondrial membranes in rat adrenocortical cells as well in granulosa, theca interna and interstitial cells in the ovary (Farkash *et al.* 1986). They are also in agreement with previous subcellular fractionation studies indicating that in rat and bovine adrenal glands P450$_{scc}$ activity was associated with mitochondrial fractions (Hanukoglu *et al.* 1981, Kramer *et al.* 1984, Cherradi *et al.* 1994). Submitochondrial fractionation studies combined with immunolabeling have shown that immunoreactive P450$_{scc}$ was associated with inner mitochondrial membranes (Cherradi *et al.* 1997). These biomedical and morphological studies thus suggest that in steroid-secreting cells cholesterol is transferred from the outer to the inner membranes (crista membranes facing matrix) where P450$_{scc}$ is present to initiate the steroid synthesis.
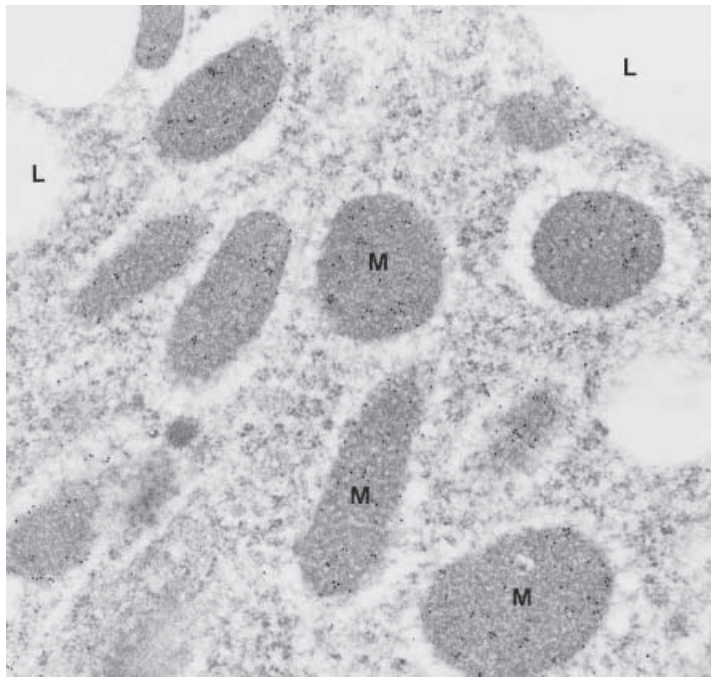
Figure 7 Localization of P450$_{scc}$ in the ovary: corpus luteum cell. Immunolabeling is observed only over cristae membranes of mitochondria (M). Neither the endoplasmic reticulum nor the lipid inclusions (L) are labeled. Magnification × 64 000.

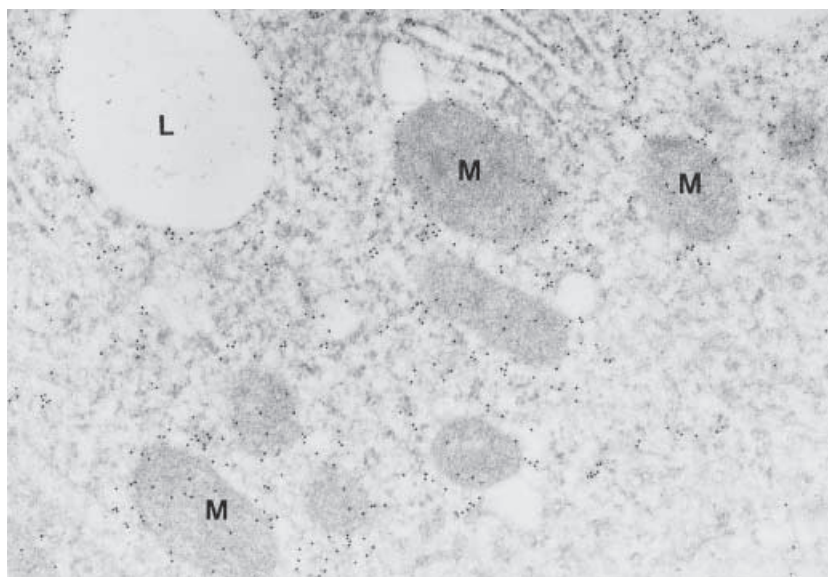**Figure 8** Localization of 3β-HSD in the ovary: granulosa cell. Most of the colloidal gold particles are associated with the endoplasmic reticulum, with a few particles overlying mitochondria (M). L: lipid inclusion. Magnification × 64 000.

With antibodies which have been extensively used to localize 3β-HSD in tissues of several species including rat (Dupont *et al.* 1990*a*,*b*, Pelletier *et al.* 1992), we have shown that, in steroid-secreting cells of the adrenal cortex, 3β–HSD is mostly observed in association with membranes of the ER, with consistent labeling of mitochondria. In bovine and rat adrenal cortex, 3β-HSD has been shown to be associated with both the mitochondrial and
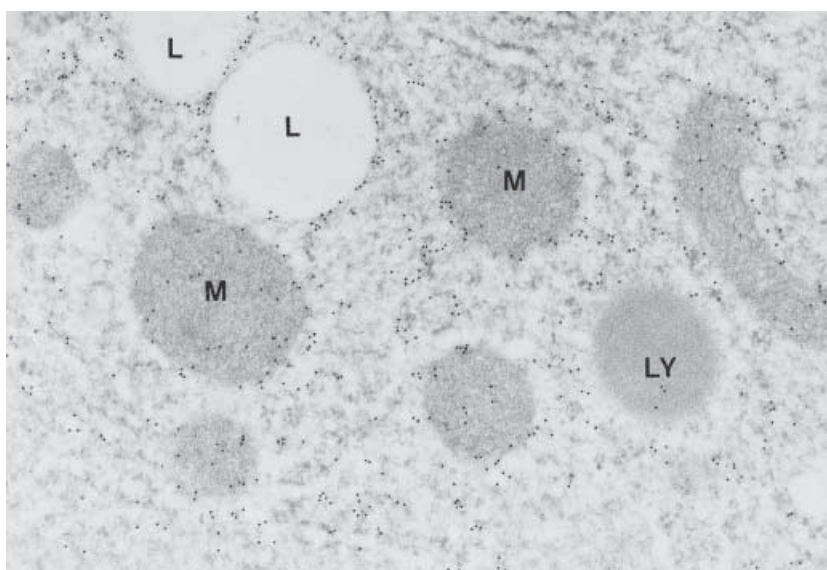
Figure 9 Localization of 3β-HSD in the ovary: corpus luteum cell. Gold particles are detected over the endoplasmic reticulum with a high density in membranes surrounding the lipid inclusions (L). Immunolabeling can also be observed inside the mitochondria (M) and their outer membranes. LY: lysosome. Magnification × 64 000.
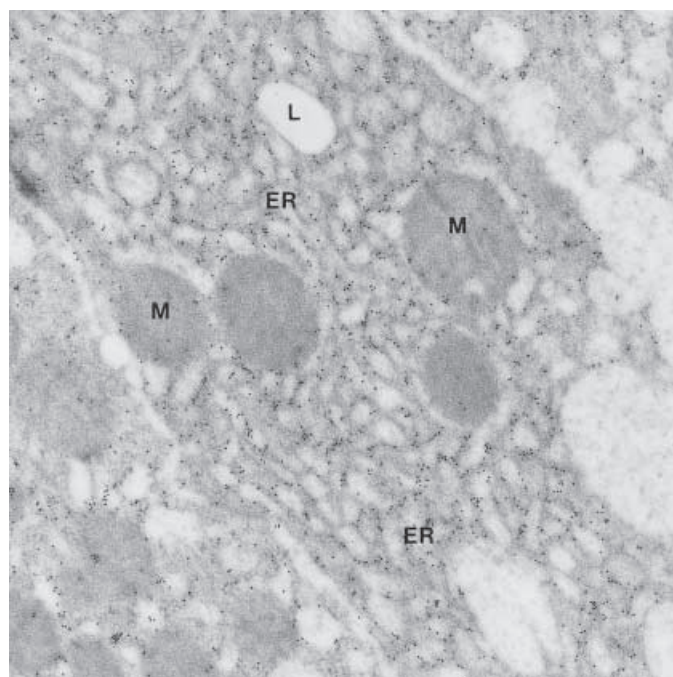

Figure 10 Localization of P450$_{c17}$ in a theca interna cell of the ovary. Strong immunolabeling of endoplasmic reticulum (ER) can be observed. Neither mitochondria (M) nor lipid inclusions (L) appear to be significantly labeled. Magnification × 64 000.

microsomal fractions (Cherradi *et al.* 1993, 1994, Sauer *et al.* 1994). By immunoelectron microscopy, 3β–HSD immunoreaction was found in the ER in bovine adrenal cortex (Ishimura *et al.* 1988, Ishimura & Fujita 1997). Recently, Cherradi *et al.* (1997) using immunoelectron microscopy have reported that 3β–HSD immunoreactivity
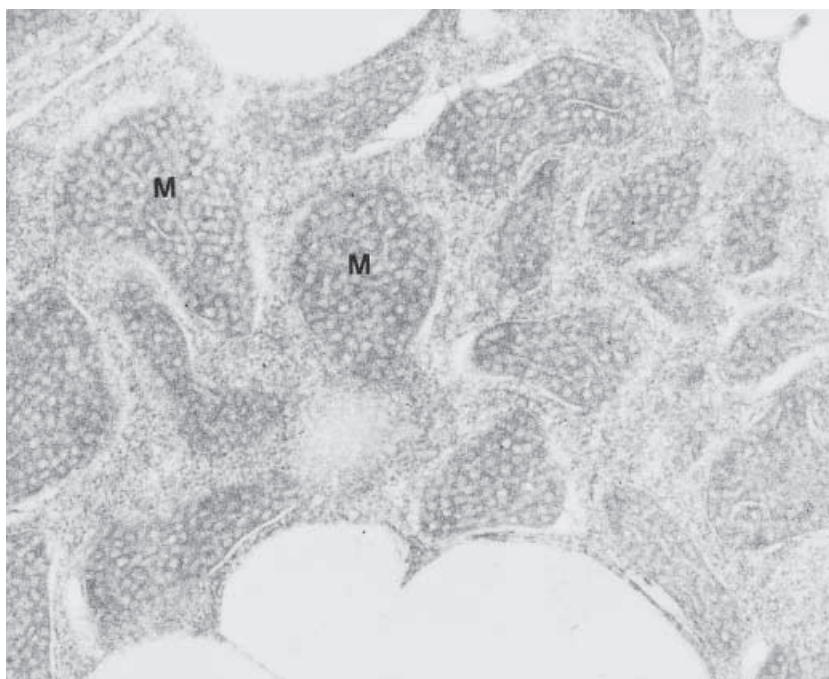
Figure 11 Control adrenal cortex (fascicular zone) section. Immunoabsorption of the antiserum to 3β-HSD with the antigen has completely prevented any staining (compare with Fig. 2). Very few dispersed gold particles can be detected. M: mitochondria. Magnification × 64 000.

could be detected in both ER and mitochondria, the highest density being observed in the ER, as shown in the present study. In mitochondria, we observed that 3β-HSD labeling was mostly associated with crista membranes and was also found over the outer membranes. Cherradi *et al.* (1997) using immunogold-labeling also observed that 60% of 3β-HSD immunoreactivity was associated with the crista membranes, while the rest of antigenic sites were distributed between the outer membranes, the mitochondrial matrix and the intermembrane spaces.

In the ovary, we confirm previous studies obtained in the rat which indicated that 3β-HSD was expressed in all ovarian steroid-secreting cells including granulosa cells in growing follicles (Dupont *et al.* 1990*b*). At the ultrastructural level, we clearly establish that the subcellular localization of 3β-HSD is identical in all the positive ovarian cells. As in adrenocortical cells, 3β-HSD was mainly found in the ER with consistent labeling of mitochondrial crista membranes. This is the first report on the *in situ* ultrastructural localization of 3β-HSD in ovarian tissue. The present data strongly suggest that the steroid metabolism involving 3β-HSD follows an intracellular pathway similar to that observed in adrenocortical cells.

In the testis, immunoreactive material was only observed in Leydig cells, in agreement with previous results obtained at the light microscopic level (Dupont *et al.* 1990*a*,*b*, Pelletier *et al.* 1992). Contrary to what has

been observed in the adrenal cortex and ovary, 3β–HSD immunoreactivity was confined to the mitochondria. Using immunoblot analysis, Cherradi *et al.* (1997) had previously shown the presence of immunoreactive 3β-HSD in MH-10 mouse Leydig cell mitochondrial fractions. Such a localization suggests that the type of 3β-HSD expressed in the rat testis is different, although immunologically related, from that found in the adrenal cortex and ovary. The exact reason for such a differential localization is unclear.

No P450$_{c17}$ immunoreactivity could be detected in the adrenal cortex, as previously shown by light microscope immunostaining studies (LeGoascogne *et al.* 1991) This is in complete agreement with the biochemical data indicating the absence of 17α-hydroxylase/17,20 lyase activity in the rat adrenal cortex (Miller 1988). In the testis, P450$_{c17}$ immunoreactivity was only detected in the Leydig cells, confirming previous light microscope studies performed in the rat and guinea pig (LeGoascogne *et al.* 1991, Suzuki *et al.* 1992). Strong immunolabeling was only observed in the ER. In the ovary, the immunostaining was mostly restricted to theca interna cells, with a few interstitial and luteal cells being weakly labeled. At the light microscopic level, immunoreactive P450$_{c17}$ was only found in theca interna of rat ovaries (LeGoascogne *et al.* 1991). In the bovine ovary, both theca interna and interstitial cells were shown to express P450$_{c17}$ mRNA and the

immunoreactive protein (Suzuki *et al.* 1992). In the human ovary, immunoreactive $P450_{c17}$ was confined to theca interna cells and a few luteinized theca cells in corpora lutea (Sasano *et al.* 1989). As observed in the Leydig cells, gold particles were only detected over ER. Up until now, there had been no report on the subcellular localization of $P450_{c17}$ in testis and ovary. The exclusive detection of immunoreactive material in the ER is in agreement with previous biochemical studies indicating that 17α-hydroxylase and 17,20-lyase activities were associated with bovine and guinea pig adrenocortical microsomes (Inano *et al.* 1969, Kominami *et al.* 1982, Shinzawa *et al.* 1988). By immunoelectron microscopy involving the use of the direct peroxidase–labeled antibody technique, Shinzawa *et al.* (1988) reported that staining for $P450_{c17}$ occurred on smooth-surface ER in the guinea pig adrenal cortex. In their studies, due to the diffusion of the reaction product, it was not possible to identify clearly all the labeled structures. From the present data obtained in the rat gonads, it appears that the subcellular distribution of the $P450_{c17}$ is identical in all steroidogenic endocrine cells, thus extending previous results obtained by subcellular fractionation and immunoelectron microscopy in the adrenal cortex.

In summary, it clearly appears that in steroid-secreting cells of endocrine glands three key enzymes involved in steroid biosynthesis are associated with the same organelles. The only exception is the exclusive localization of 3β-HSD to mitochondria in Leydig cells. The present data indicate that, in general, steroid metabolism follows similar intracellular pathways in different steroid-secreting cell types.

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