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Binding and Internalization *In Vivo* of [^{125}I]hCG in Leydig Cells of the Rat

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The present study was performed to demonstrate the binding, mode of uptake, pathway and fate of iodinated human chorionic gonadotropin ([^{125}I]hCG) by Leydig cells *in vivo* using electron microscope radioautography. Following a single injection of [^{125}I]hCG into the interstitial space of the testis, the animals were fixed by perfusion with glutaraldehyde at 20 minutes, 1, 3, 6 and 24 hours. The electron microscope radioautographs demonstrated a prominent and qualitatively similar binding of the labeled hCG on the microvillar processes of the Leydig cells at 20 minutes, 1, 3, and 6 hours. The specificity of the [^{125}I]hCG binding was determined by injecting a 100-fold excess of unlabeled hormone concurrently with the labeled hormone. Under these conditions, the surface, including the microvillar processes of Leydig cells, was virtually unlabeled, indicating that the binding was specific and receptor-mediated. In animals injected with labeled hCG and sacrificed 20 minutes later, silver grains were also seen overlying the limiting membrane of large, uncoated surface invaginations and large subsurface vacuoles with an electron-lucent content referred to as endosomes. A radioautographic reaction was also seen within multivesicular bodies with a pale stained matrix. At 1 hour, silver grains appeared over dense multivesicular bodies and occasionally over secondary lysosomes, in addition to the structures mentioned above, while at 3 and 6 hours, an increasing number of secondary lysosomes became labeled. At 24 hours, binding of [^{125}I]hCG to the microvillar processes of Leydig cells persisted but was diminished, although a few endosomes, multivesicular bodies and secondary lysosomes still showed a radioautographic reaction. No membranous tubules that were seen in close proximity to, or in continuity with, endosomes and multivesicular bodies were observed to be labeled at any time interval. Likewise, an attempt to correlate silver grains with small coated or uncoated pits, the stacks of saccules of the Golgi apparatus and other Golgi-related elements including GERL, proved unsuccessful, since these structures were mostly unlabeled. These *in vivo* experiments thus demonstrate the specific binding of [^{125}I]hCG to the plasma membrane of Leydig cells predominantly on their microvillar processes, and the subsequent internalization of the labeled hCG to secondary lysosomes. In addition, binding and internalization of hCG persisted for long periods of time.

Key words: Leydig cells, receptor-mediated endocytosis, [^{125}I]hCG, secondary lysosomes.

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Receptors to the human chorionic gonadotropin (hCG) or luteinizing hormone (LH) have been postulated from light microscope (Mancini et al, 1967; de Kretser et al, 1969, 1971; Castro et al, 1972; Dal Lago et al, 1977) and biochemical studies on interstitial tissue fractions or isolated Leydig cells (Catt et al, 1980; Chan et al, 1981; Payne et al, 1980, 1982;

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Rebois and Fischman, 1984) to be present on the surface of Leydig cells. Using a strain of Leydig tumor cells (MA-10), Ascoli and collaborators have shown by biochemical techniques that hCG, which shares similar biologic properties with LH and binds to the same receptors (Amsterdam and Lindner, 1984), is transferred after binding and internalization to lysosomes (Ascoli and Puett, 1978a, 1978b; Ascoli, 1982a, 1982b; Lloyd and Ascoli, 1983) where the hormone-receptor complex appears to be degraded (Ascoli, 1984). Studies on dispersed Leydig cells in culture using iodinated hCG and electron microscope radioautography have also stated that hCG could be found in lysosomes following binding of the ligand to the Leydig cell surface (Amsterdam et al, 1981; Amsterdam and Lindner, 1984).

Recent studies on endocytosis (fluid phase, adsorptive, receptor-mediated) have shown that the initial uptake, subsequent pathway and fate of different ligands vary depending on the cell type and the ligand. While in some cells the initial uptake of a given ligand has been shown to be mediated via coated pits (Goldstein et al, 1979; 1985; Brown et al, 1983; Willingham and Pastan, 1984; Pastan and Willingham, 1985), no such involvement has been documented in others (Bergeron et al, 1985). Following uptake, the intercellular pathway may also vary. In some instances, the ligand (α_2-macroglobulin, low density lipoprotein, epidermal growth factor, etc.) is found after internalization in lysosomes where it is presumably degraded (Goldstein et al, 1979; Steinman et al, 1983; Pastan and Willingham, 1983; Willingham and Pastan, 1984). Other ligands such as transferrin, after entering a prelysosomal compartment referred to as endosomes (Helenius et al, 1983), do not dissociate from their receptor but rather are recycled to the cell surface along with their receptors (Dautry-Varsat et al, 1983). In yet other cases, a route to the Golgi region has been suggested for a wide variety of ligands (Posner et al, 1982; 1984), including localization within Golgi saccules and/or GERL (reviews, Farquhar, 1983; 1985; Willingham and Pastan, 1984).

In a recent study on the endocytotic activity of Leydig cells in the rat testis, it has been shown that these cells are actively involved in fluid phase and adsorptive endocytosis (Hermo et al, 1985). Both fluid phase and adsorptive tracers reached lysosomes via a pathway involving large uncoated surface invaginations, endosomes and multivesicular bodies. However, in addition to the lysosomal pathway, the adsorptive tracers were also seen in spherical, often C-shaped, intermediate vesicles (200 to 300 nm) in the Golgi region, although such labeled vesicles were never in continuity with the Golgi saccules, GERL or other Golgi elements, nor was the tracer ever observed within these structures (Hermo et al, 1985).

In light of our earlier work using non-specific tracers, as well as recent findings on the endocytotic activity of other cell types, we have undertaken to examine *in vivo* the mode of uptake, pathway and fate of [^{125}I]hCG using electron microscope radioautography.

Materials and Methods

Highly purified hCG (CR-121; 13,450 IU/mg) for iodination was a gift from the Center for Population Research of the NICHDD of the NIH. [^{125}I]hCG was prepared and characterized as described by Ireland and Richards (1978). The specific activity of the labeled hCG was approximately 40 μCi/μg. The unlabeled hCG (1000 IU/mg) was obtained from Ayerst Laboratories (Montreal, Quebec). A dose of 0.05 ng of hCG in 100 μl saline was injected into the interstitial space of each animal.

Under anesthesia, the left and the right testis of adult Sherman rats (350 to 450 g) were exposed through an abdominal incision. Following a single injection of [^{125}I]hCG into the interstitial space of each testis, the animals (three per time interval) were fixed by perfusion at 20 minutes and 1, 3, 6 and 24 hours later. To determine whether or not the binding of [^{125}I]hCG was specific, a 100-fold excess of unlabeled hormone was injected concurrently with the labeled hormone into the testicular interstitial space of three animals. Twenty minutes later, the animals were fixed by perfusion. Fixation was carried out by perfusion through the abdominal aorta with 2.5% glutaraldehyde buffered in sodium cacodylate (0.1 M) containing 0.05% $CaCl_2$ at *p*H 7.2. Two minutes prior to fixation by perfusion, the interstitial space was cleared with lactated Ringer's solution to remove unbound hormone.

After fixation, each testis was removed from the scrotum, cut into small mm^3 pieces, placed in fixative for an additional hour at 4 C and then left overnight in 0.1 M sodium cacodylate buffer at 4 C. Testicular tissue from four rats injected with physiologic saline solution served as controls. Samples of testicular tissue were collected from regions close to or at a short distance from the site of injection. For a given experiment, the distribution of [^{125}I]hCG on the surface or within Leydig cells was identical in these various samples, since the ligand diffuses freely in the open lymphatic interstitial space in which rat testicular Leydig cells are suspended.

For cytochemical studies, testicular tissue from two rats was fixed with 2% glutaraldehyde buffered in sodium cacodylate, washed in sodium cacodylate buffer containing 5% sucrose, and then sectioned (75 μm) with a Sorvall TC-2 tissue chopper. To demonstrate acid phosphatase activity, the tissue sections were incubated for 60 minutes at 37 C in a medium containing cytidine monophosphate as substrate at *p*H 5 (Novikoff, 1963) with cerium as a cap-

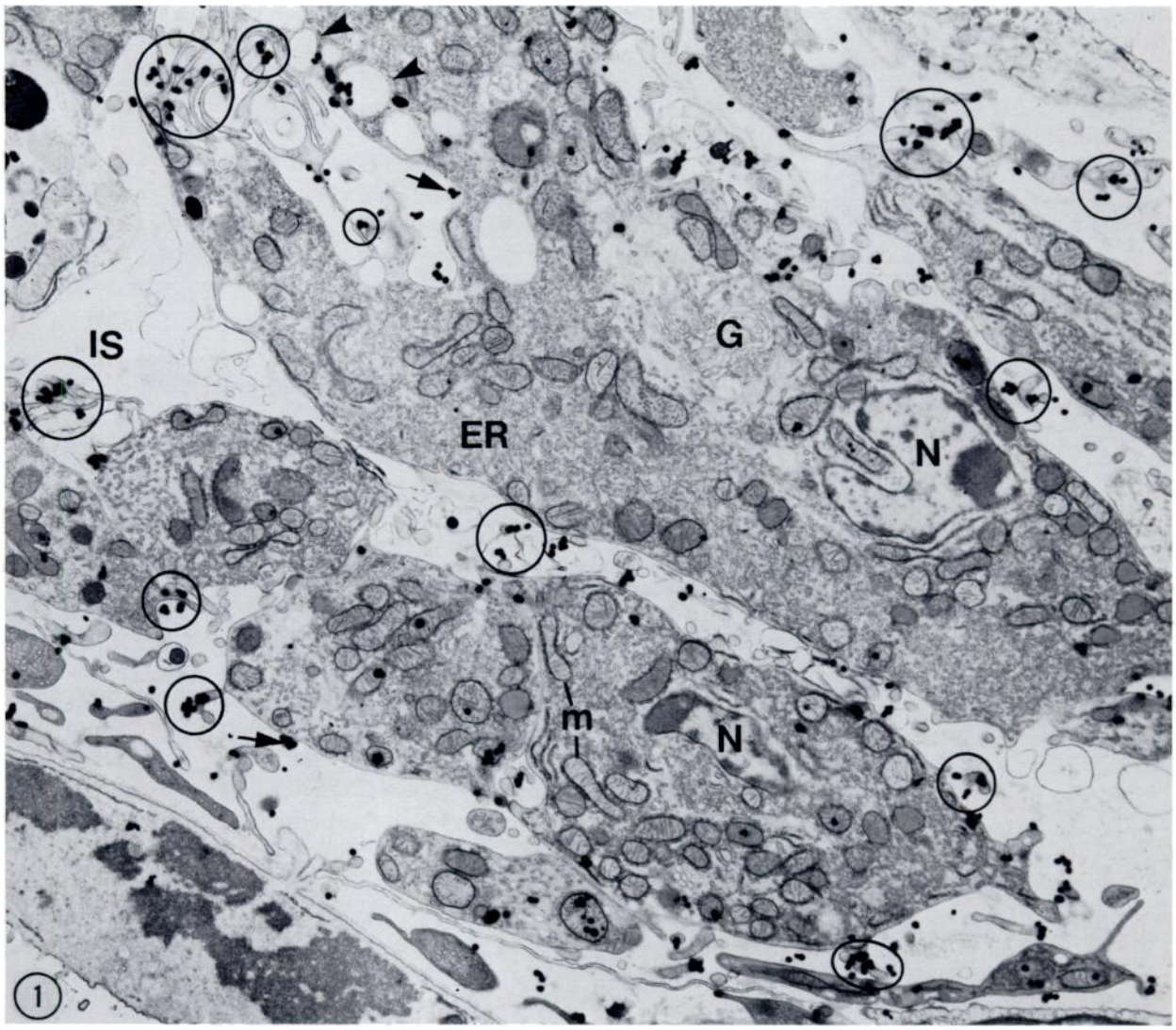

Fig. 1. Low power electron micrograph of the interstitial space (IS) showing several Leydig cells 20 minutes after an injection of [125I]hCG. The label is seen to be localized predominantly over their microvillar processes (circled). The odd cluster of silver grains is also seen on its surface (arrows). A few endosomes are also labeled (arrowheads). G: Golgi apparatus, N: nucleus, ER: smooth endoplasmic reticulum, m: mitochondria (× 7,920).

ture agent according to the method of Robinson and Karnovsky (1983). Incubation of tissue in the absence of substrate served as controls.

After an overnight wash in buffer, all testicular tissue was postfixed in potassium ferrocyanide-reduced osmium tetroxide (Karnovsky, 1971) for 1 hour at 4 C, dehydrated in alcohol and embedded in Epon. Semi-thin sections (0.5 μm) were cut for light microscope observation and stained with iron hematoxylin for radioautography. After radioautography, thin sections of selected areas of interstitial space were stained with uranyl acetate (5 minutes) and lead citrate (2 minutes) and examined with the Philips 400T electron microscope. All radioautographs were prepared according to the technique of Kopriwa (1973) and exposed in dry air at 4 C for 4 days for light microscopy and 30 days for electron microscopy. The radioautographs were developed for 7 minutes in Agfa-Gevaert solution physical developer preceded by gold latensification, a procedure that yields small, compact silver grains (Kopriwa, 1975).

Results

Leydig cells are large elongated cells with an indented nucleus that bathe in the lymphatic fluid of the interstitial space (Fig. 1). Several short or long and occasionally branching microvillar processes are

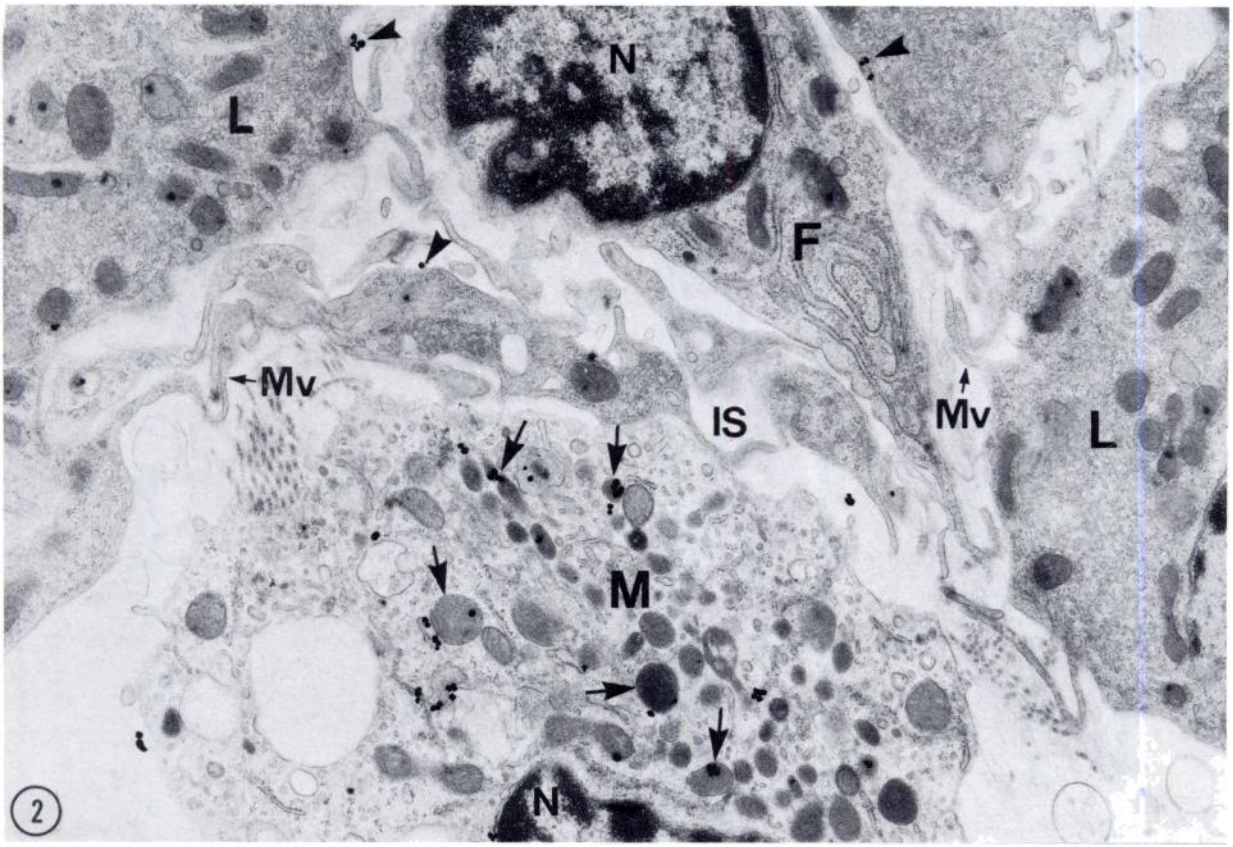

Fig. 2. Low power electron micrograph of the interstitial space (IS) showing portions of Leydig cells (L), a fibrocyte (F) and macrophage (M), 20 minutes after a coinjection of [^{125}I]hCG and a 100-fold excess of unlabeled hormone. Except for the odd silver grain (arrowheads), the microvillar processes (Mv) and surface of Leydig cells are now virtually devoid of label. Note that several lysosomal elements (arrows) of the macrophage (M) are labeled, indicating that [^{125}I]hCG was present in that area of interstitial space. No surface labeling was ever observed in the case of macrophages or fibrocytes. N: nucleus (× 12,000).

distributed along their surface (Figs. 1, 3, 9 and 11). The latter often appear as fold- or flap-like extensions of the cell surface. A large spheroidal Golgi apparatus, an extensive network of smooth endoplasmic reticulum and numerous mitochondria and peroxisomes (Fig. 1) are present in the cytoplasm.

Various types of vesicular profiles distinct from Golgi elements and smooth endoplasmic reticulum were present in the cytoplasm. First, large uncoated invaginations of the cell surface were often seen adjacent to microvilli (Figs. 4 and 5). Second, large uncoated spherical or slightly irregular vacuoles (usually about 500 nm or larger) with an electron-lucent content were found subjacent to the cell surface. Such elements, occasionally seen adjacent to or continuous with membranous tubules (Figs. 3 and 4), were acid phosphatase-negative and will be referred to as endosomes. Third, vesicular elements (200 to 300 nm diameter) showing a clear content were also evident. Such vesicles, usually spherical or C-shaped in appearance, will be referred to as the intermediate vesicles. While a few of these vesicles were observed adjacent to the cell surface (Fig. 12), they were also prominent in the Golgi region (Figs. 6 and 10). Lastly, small coated and uncoated pits (diameter of about 100 nm) were also observed on the cell surface (Figs. 4, 6, and 11 inset), as well as occasional subsurface coated and uncoated vesicles of similar size.

Several multivesicular bodies showing a pale, moderate or densely stained matrix were present next to the cell surface or deeper in the cytoplasm (Figs. 6, 7, 10 and 13 inset). Frequently, multivesicular bodies were accompanied by small membranous tubules with which they were at times directly continuous (Figs. 8 and 13 inset). Such membranous tubules were also found next to the cell surface (Fig. 6). A few pale multivesicular bodies showed a small plaque of fuzzy material along their unit membrane (Figs. 10

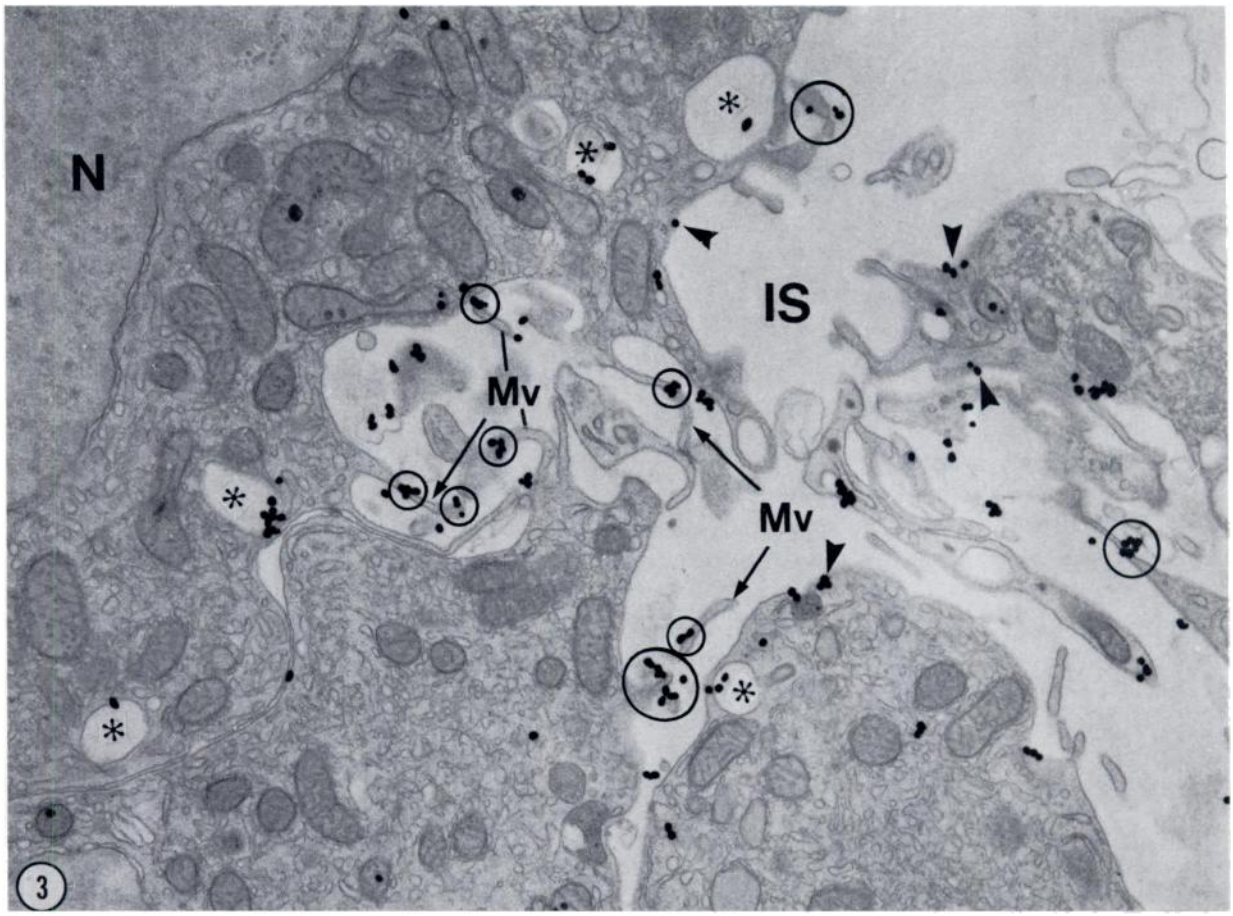

Fig. 3. Portions of Leydig cells 20 minutes after injection of [125I]hCG. Silver grains are seen predominantly in association with the microvillar processes (Mv) of Leydig cells (circled), although a few are distributed along its surface (arrowheads). Label is also found over several endosomes (asterisks). In such structures, the silver grains are commonly present in close association with their limiting membrane. N: nucleus, IS: interstitial space (× 10,800).

inset and 12 inset). Several moderately dense to dense membrane-bound bodies, with a spherical or elongated form and homogeneous content, were scattered throughout the cytoplasm (Figs. 6, 7, 10, and 13). Such bodies were acid phosphatase-positive (Fig. 8), indicating that they were lysosomal in nature and will be referred to as secondary lysosomes.

Receptor Mediated Endocytosis of [125I]hCG

Seen at low magnification, [125I]hCG was localized predominantly over the microvillar processes of Leydig cells at the earliest time interval after injection (20 minutes) (Figs. 1 and 3). Much less frequently, the label was also distributed in a random manner along the Leydig cell surface (Figs. 1 and 3). To determine whether the binding of [125I]hCG was specific, a 100-fold excess of unlabeled hCG was injected along with the labeled hormone. Under these conditions, the microvillar processes and other surface areas of the Leydig cells were virtually unlabeled (Fig. 2). However, macrophages of the interstitial space, while not showing surface labeling, clearly incorporated the label, since silver grains were observed overlying their numerous lysosomal elements (Fig. 2).

At 20 minutes, the large uncoated invaginations of the cell surface frequently adjacent to microvillar processes were labeled as well as the endosomes (Figs. 3 and 4). At higher magnification and in appropriately cut sections, the silver grains were frequently seen in both of these structures to be closely related to their unit membrane and not freely distributed in their content (Fig. 5). Several multivesicular bodies with a pale (Fig. 6) or moderately stained matrix (Fig. 7) showed a radioautographic reaction at this early interval, while secondary lysosomes did not (Figs. 6 and 7). An attempt to correlate labeling with

small coated and uncoated pits and vesicles (100 nm diameter) proved unsuccessful, since silver grains were not usually found overlying or in close proximity to these structures (Figs. 4 and 6). Similarly, there was no association of silver grains with membranous tubules found adjacent to or continuous with endosomes (Fig. 4), multivesicular bodies (Figs. 6 and 7) or close to the cell surface (Fig. 6).

At 1 hour after injection of [^{125}I]hCG, silver grains were still found to be present over numerous microvillar processes of the Leydig cells as well as in occasional patches along their surfaces (Fig. 9). In addition to the labeling of large uncoated surface invaginations, endosomes and pale multivesicular bodies (Fig. 10 inset), silver grains were now present over multivesicular bodies with a dense staining matrix and occasionally over secondary lysosomes (Fig. 10).

At 3 and 6 hours after injection, there was still a prominent labeling of the microvillar processes in addition to occasional, sporadic grains over the Leydig cell surface (Fig. 11). A qualitative assessment of [^{125}I]hCG binding at these different intervals indicated that the number and distribution of silver grains overlying Leydig cell microvillar processes appeared to be similar to that observed at 20 minutes or 1 hour (compare Figs. 1, 9 and 11). At 3 and 6 hours, the label also was present within large uncoated surface invaginations and endosomes (Fig. 12) as well as pale and dense multivesicular bodies (Figs. 12 inset and 13 inset), indicating that internalization was still continuing. However, more labeled secondary lysosomes (2 to 3 times more) were observed in a given Leydig cell at these late time intervals (Fig. 13). At 24 hours after injection, labeling of the microvillar processes and the cell surface persisted but was considerably diminished, and silver grains could be seen over only a few endosomes, multivesicular bodies and secondary lysosomes.

Small coated and uncoated pits and vesicles (100 nm diameter) of the cell surface were still predominantly unlabeled at 3, 6, and 24 hours (Fig. 11 inset). No labeling of the membranous tubules connected to or associated with endosomes and multivesicular bodies was observed at these late time intervals (Fig.

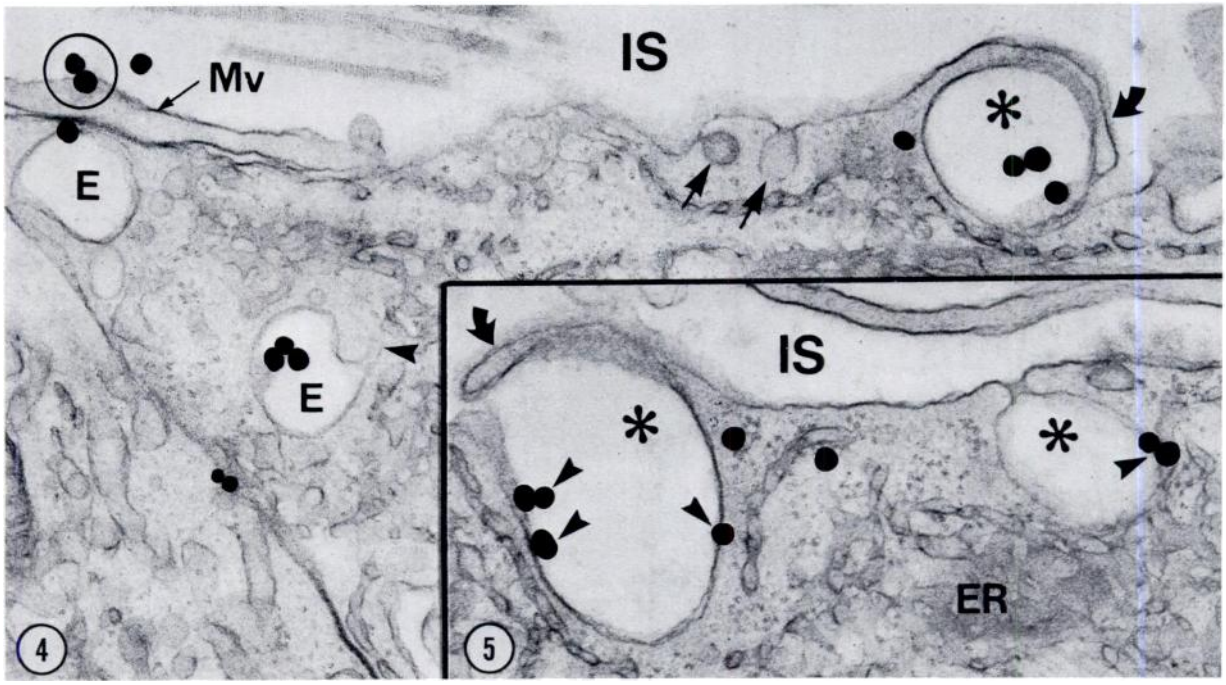

Fig. 4. High power electron micrograph of a portion of a Leydig cell 20 minutes after injection of [^{125}I]hCG. Silver grains are found over a microvillar process (Mv, circled) and in a large uncoated surface invagination (asterisk) that appears to be formed by partial involvement of a microvillus (curved arrow). Several endosomes (E) are also labeled. No labeling is observed in the case of the small pits of the cell surface (arrows) or the tubule connected to an endosome (arrowhead). IS: interstitial space (× 44,200).

Fig. 5. High power electron micrograph of a portion of a Leydig cell 20 minutes after injection of [^{125}I]hCG. Silver grains are found over the limiting membrane (arrowheads) of large uncoated surface invaginations (asterisks). The invagination on the left appears to be forming by partial involvement of a microvillar process (curved arrow). ER: smooth endoplasmic reticulum, IS: interstitial space (× 47,600).

13 inset). At no point in time were any of the spherical, often C-shaped, intermediate vesicles (200 to 300 nm) seen adjacent to the cell surface (Fig. 12) or in the Golgi region (Fig. 10) ever found to be labeled. The Golgi stacks of saccules and associated small vesicles, including GERL, were also consistently found to be unlabeled.

Discussion

Specific Binding Sites for [125I]hCG

Receptors for the gonadotropic hormones LH and hCG have been shown to exist on the surface of Leydig cells with the aid of biochemical techniques (Catt et al, 1980; Chan et al, 1981; Payne et al, 1980; 1982; Ascoli, 1982a; 1982b; 1984; Rebois and Fishman, 1984), light microscope studies using radioautography or immunohistochemistry (Mancini et al, 1967; de Kretser et al, 1969; 1971; Catt et al, 1971; Castro et al, 1972), immunofluorescence (Hsueh et al, 1976) and ferritin-labeled hCG or iodinated hCG in conjunction with electron microscope radioautography (Amsterdam and Lindner, 1984). These various studies, however, have been performed on either interstitial tissue fractions, isolated Leydig cells or different strains of Leydig tumor cells. The need to verify these studies using an *in vivo* approach is apparent and is the subject of the present study.

The *in vivo* radioautographic method has been used extensively for recognizing receptors for various hormones in a wide variety of cell types (review, Bergeron et al, 1985), including the localization of FSH binding sites in the testis (Orth and Christensen, 1977). This specific binding assay, based on the application of the law of mass action, states that an injection of labeled together with an excess of unlabeled hormone will produce a competition for binding on the saturable specific receptor sites. Specific binding can then be determined by the difference noted between the bound labeled hormone in experimental and control animals.

Using an *in vivo* approach and electron microscope radioautography, the present study shows that Leydig cells have specific binding sites for hCG. Twenty

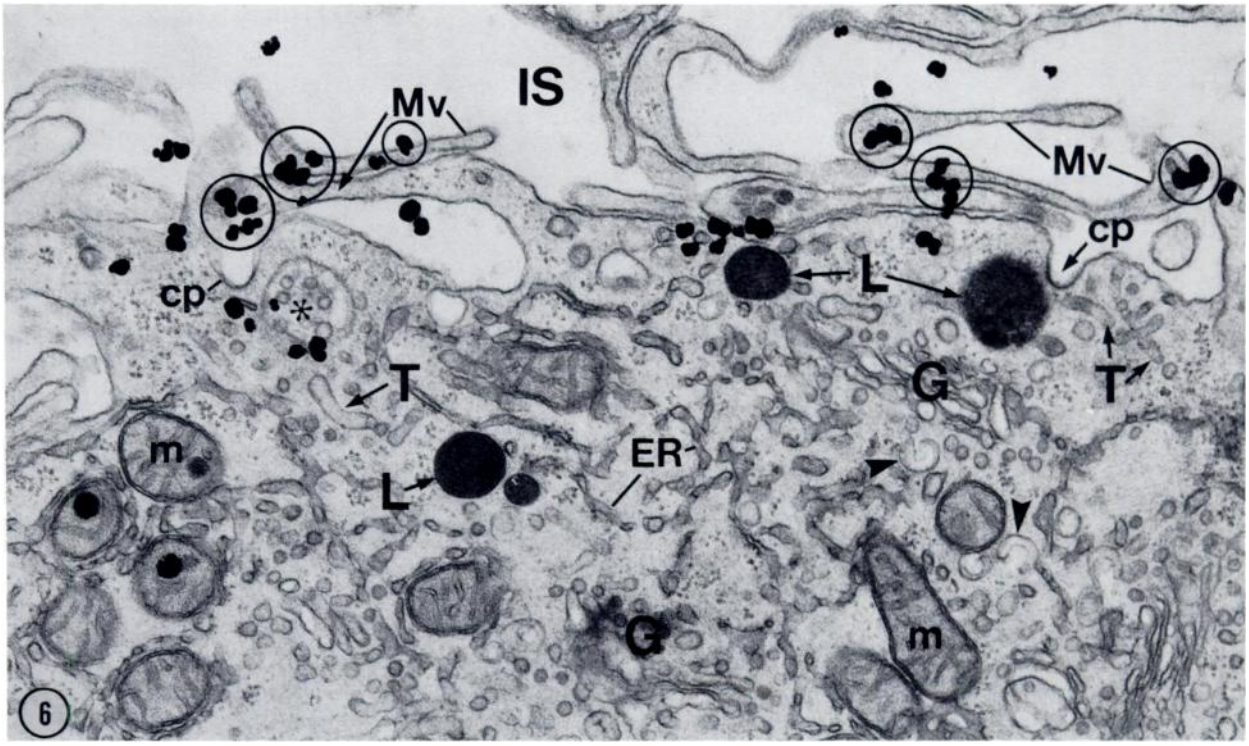

Fig. 6. Portion of a Leydig cell 20 minutes after injection of [125I]hCG. Silver grains are seen over microvillar process (Mv) of the Leydig cell (encircled) and in association with the limiting membrane of a pale multivesicular body (asterisk). Coated pits (cp) of the cell surface are unlabeled as are membranous tubules (T) found next to the multivesicular body or close to the cell surface. Dense membrane-bound bodies identified as secondary lysosomes (L) are also unlabeled at this time interval. The stacks of saccules of the Golgi apparatus (G) and related C-shaped intermediate vesicles (arrowheads) show no radioautographic reaction. ER: smooth endoplasmic reticulum, IS: interstitial space, m: mitochondria (× 31,000).

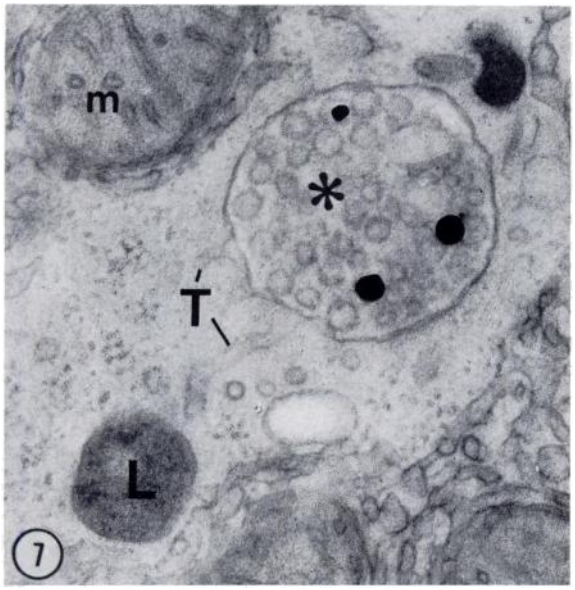

Fig. 7. Labeled multivesicular body (asterisk) showing a moderately dense staining matrix 20 minutes after an injection of [^{125}I]hCG. Membranous tubules (T) connected or adjacent to the multivesicular body are unlabeled as is a dense membrane-bound secondary lysosome (L). m: mitochondrion (× 56,000).

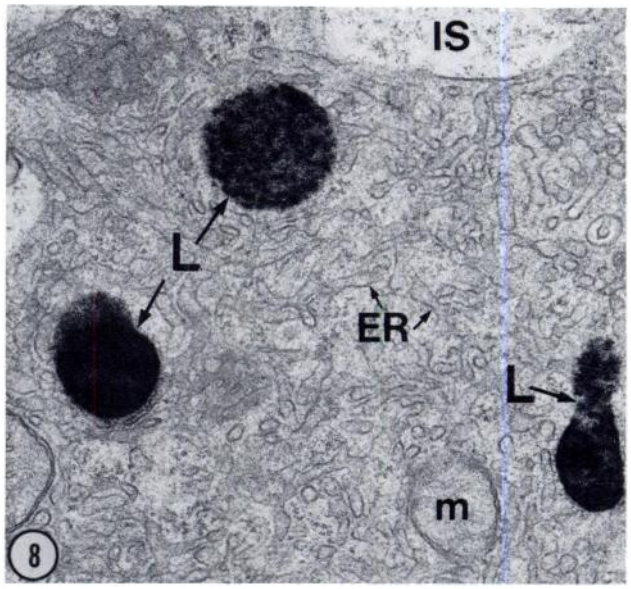

Fig. 8. Acid phosphatase reaction product over dense membrane-bound bodies in Leydig cell identifying them as lysosomal elements (L); such elements are referred to as secondary lysosomes. m: mitochondrion, IS: interstitial space, ER: smooth endoplasmic reticulum (× 42,900).

minutes after a single injection of the ligand, silver grains were located on the surface of Leydig cells, predominantly over their microvillar processes. Control experiments in which [^{125}I]hCG was coinjected with a 100-fold excess of unlabeled hormone revealed that under these conditions no significant binding was observed on the Leydig cell surface or its microvilli. Such results indicate that hCG binds to specific binding sites on Leydig cells and that these are located mainly on their microvillar processes. A radioautographic reaction was noted over the numerous dense lysosomal elements of interstitial macrophages at the 20-minute interval in both experimental and control animals, but never on the macrophage cell surface. These results indicate that [^{125}I]hCG was present in the area of the interstitial space examined in control animals, and also that specific binding sites for hCG do not exist on the surface of macrophages. Presumably, hCG was internalized by fluid phase endocytosis or phagocytosis, which are well known functions of macrophages.

Internalization of [^{125}I]hCG

At 20 minutes after injection of [^{125}I]hCG, the label was seen over the Leydig cell surface, predominantly over the microvillar processes. A similar distribution had been noted on Leydig cells maintained in culture (Amsterdam et al, 1981; Amsterdam and Lindner, 1984). However, while these investigators noted an accumulation of cytoskeletal elements beneath aggregates of receptor-bound hormone, no evidence for such an association was noted in the present *in vivo* work. At the earliest interval (20 minutes), large uncoated invaginations of the cell surface were found to be labeled. In such instances, silver grains were frequently found in close association with the limiting membrane, suggesting a close relationship between hCG and its receptor. After binding to their receptor, various ligands, especially in cultured cells, including low density lipoprotein, α_2-macroglobulin, epidermal growth factor, transferrin, asialoglycoproteins and certain viruses have been shown to concentrate in coated pits prior to their entry (Pastan and Willingham, 1981; 1983; Goldenthal et al, 1984; Willingham and Pastan, 1984; Goldstein et al, 1985). In Leydig cells, small coated or uncoated pits were rarely observed to become labeled with hCG. This is similar to the situation described *in vivo* for insulin, prolactin and other peptide hormones where attempts to document the participation of coated pits in internalization have not proven successful (Bergeron et al, 1979; 1980; 1983; 1985). In contrast, the present study suggests (Figs. 4 and 5) that following binding

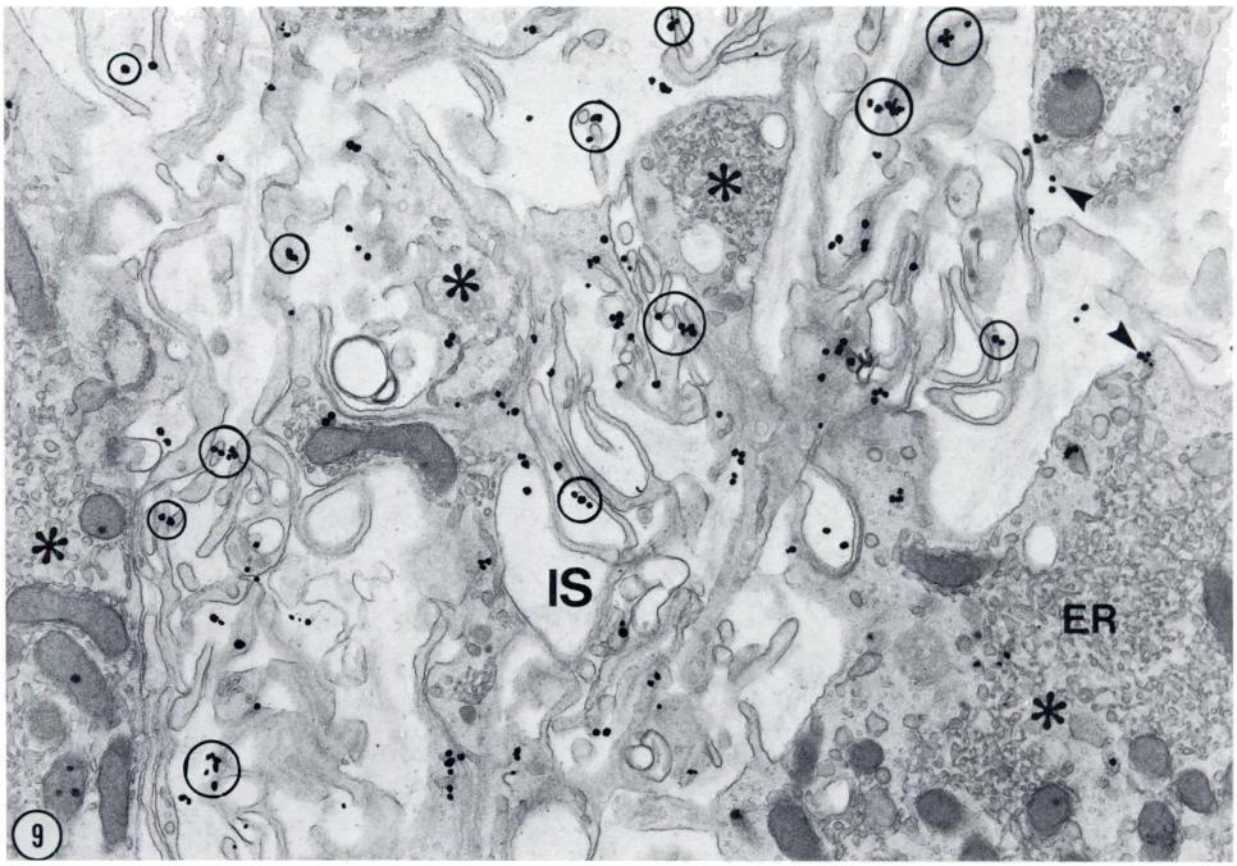

Fig. 9. Low power electron micrograph of portions of Leydig cells (asterisks) 1 hour after injection of [125I]hCG. Note the distribution and quantity of silver grains, predominantly overlying microvillar processes of Leydig cells (circled), are similar to that observed at 20 minutes. The odd silver grain is also seen over its surface (arrowheads). IS: interstitial space, ER: smooth endoplasmic reticulum (×15,400).

to the microvillar processes, the internalization of hCG may occur by the folding over of the microvillus and the fusion of its membrane with the adjacent Leydig cell surface, resulting in the internalization of the bound hCG and eventual formation of a large subsurface endocytic vacuole referred to as an endosome. While this remains to be proven, it can be stated from the present work that small coated or uncoated pits as well as small subsurface coated and uncoated vesicles (100 nm diameter) do not appear to be involved in the internalization of hCG in Leydig cells, since silver grains were rarely seen overlying or next to these structures.

Pathway of [125I]hCG to Secondary Lysosomes

At 20 minutes, large endocytic vacuoles located beneath the cell surface were labeled. Such structures had a clear content, were acid phosphatase-negative and showed a close association or continuity with short membranous tubules. These features suggest that they represent the prelysosomal compartment referred to as endosomes (Helenius et al, 1983). A close relationship of silver grains with the limiting membrane of these structures was noted. A similar distribution of silver grains over endosomes was observed by Lai et al (1986) for epidermal growth factor in human term placental cells, suggesting that the ligand and its receptor were still bound to one another. At 20 minutes, spherical, pale multivesicular bodies, some bearing a plaque of fuzzy material, also showed a radioautographic reaction, while at 1 hour, dense multivesicular bodies became labeled. Silver grains were mainly found overlying the matrix or limiting membrane of these structures, and no relationship of silver grains was observed with membranous tubules seen next to or in continuity with them.

The possibility exists that some of these structures correspond to CURL (compartment for uncoupling of receptor and ligand), a term proposed by Geuze et

al (1983a) in which ligands dissociate from their receptors. However, whether or not such tubules are involved in recycling the receptor for hCG back to the cell surface remains to be determined. Indeed, there is even some debate as to whether or not the receptor does (Habberfield et al, 1986) or does not (Ascoli, 1984) recycle back to the cell surface.

It has been suggested from biochemical studies on Leydig tumor cells (Ascoli and Puett, 1978; Ascoli 1982a, 1982b; 1984) and on dispersed Leydig cells in culture (Amsterdam et al, 1981; Amsterdam and Lindner, 1984) that internalized hCG is eventually found within lysosomal elements where presumably it is degraded. The present work illustrates the presence of silver grains at the 1-hour interval over dense membrane-bound elements that were found to be acid phosphatase-positive, indicating that they are lysosomal elements. These results agree with the findings of Habberfield et al (1986) who showed that radioactivity associated with highly degraded hCG was released from Leydig cells 1 hour after internalization of hCG. While such structures (secondary lysosomes) were occasionally labeled at 1 hour, an increasing number were found to be labeled in a given Leydig cell at the later time intervals (3 and 6 hours).

Following binding to its receptor, hCG was internalized by large uncoated surface invaginations and subsequently found in endosomes, pale and dense multivesicular bodies and secondary lysosomes. Transformation of endosomes into secondary lysosomes has been suggested for a wide variety of cell types (De Bruyn et al, 1983; Hornick et al, 1984; Morales et al, 1984; 1985; Hermo and Morales, 1984) and also appears to be the case in Leydig cells (Hermo et al, 1985). In the present work, small vesicles that could serve to carry the ligand from one compartment to the next, referred to as shuttle vesicles (Helenius et al, 1983; vesicle shuttle model), were not observed in association with these structures.

Pathway [^{125}I]hCG to the Golgi Region

In an earlier work, Hermo et al (1985) noted that after an injection of an adsorptive tracer, cationic

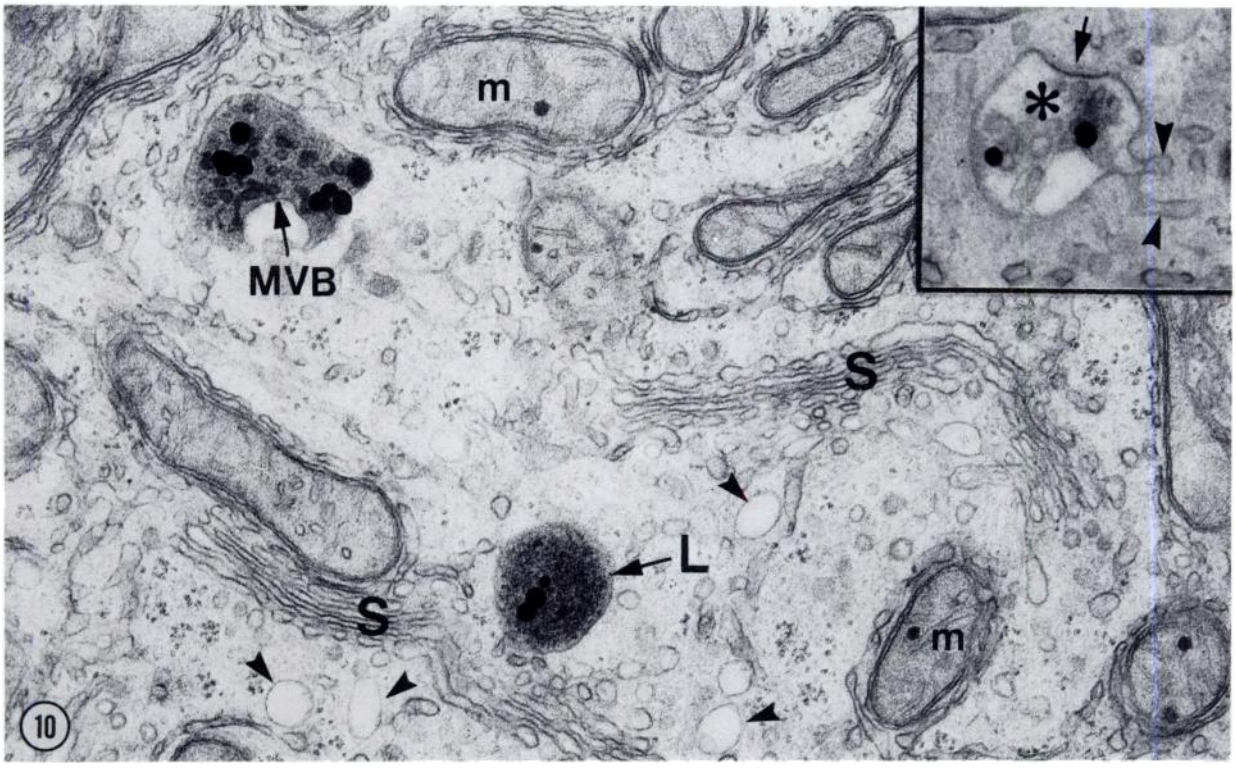

Fig. 10. One hour after injection of [^{125}I]hCG, multivesicular bodies (MVB) with a densely stained matrix become labeled as do an occasional secondary lysosome (L). Note that the stacks of saccules (S) of the Golgi apparatus show no radioautographic reaction. Intermediate vesicles (arrowheads) in the Golgi region are also repeatedly unlabeled. m: motochondria (× 41,040). **Inset**: Labeled pale multivesicular body (asterisk) 1 hour after injection of [^{125}I]hCG. Membranous tubules continuous with or adjacent to the multivesicular body are unlabeled. Note the plaque of fuzzy material and associated thickened unit membrane in the indentation of this body (arrow). (× 40,800).

ferritin, into the interstitial space, intermediate vesicles named because of their size (200 to 300 nm) were found to be labeled at early time intervals near the cell surface and at later time intervals in the Golgi region. In the present study, silver grains were not seen overlying such vesicles located at the cell surface or in the Golgi region, nor was a radioautographic reaction ever observed over the stacks of saccules of the Golgi apparatus or other Golgi elements including GERL. This is similar to observations in some cell types (Morales et al, 1984; 1985; Gonnella and Neutra, 1984), but differs from others where different ligands, including hormones, have been noted in the vicinity of the Golgi apparatus (Bergeron et al, 1978; 1979; 1983; Posner et al, 1980; 1982; 1984), in the reticular portion of the Golgi referred to as GERL (Geuze et al, 1983b, Pastan and Willingham, 1983; Willingham and Pastan, 1984) or within the Golgi saccules themselves (Farquhar, 1983; 1985).

Prolonged Binding of [125I]hCG to the Leydig Cell Surface

The binding of [125I]hCG to the Leydig cell surface persisted at 1, 3 and 6 hours. Although not quantitated, it could readily be observed at these late time intervals that a considerable radioautographic reaction remained over the microvillar processes of Leydig cells, the functional significance of which is not known. Our findings on the prolonged binding of hCG differ from those of Habberfield et al (1986), who worked with cultured Leydig cells and who suggest a rapid internalization of hCG and its receptor. However, a slow internalization of hCG has been reported for cultured (Amsterdam et al, 1981) or isolated (Hsueh et al, 1976) Leydig cells as well as for granulosa and luteal cells (Chan et al, 1977; Conn et al, 1978; Amsterdam et al, 1979; Ahmed et al, 1981). Our present observations on the prolonged binding of hCG correlate well with the known half life of the

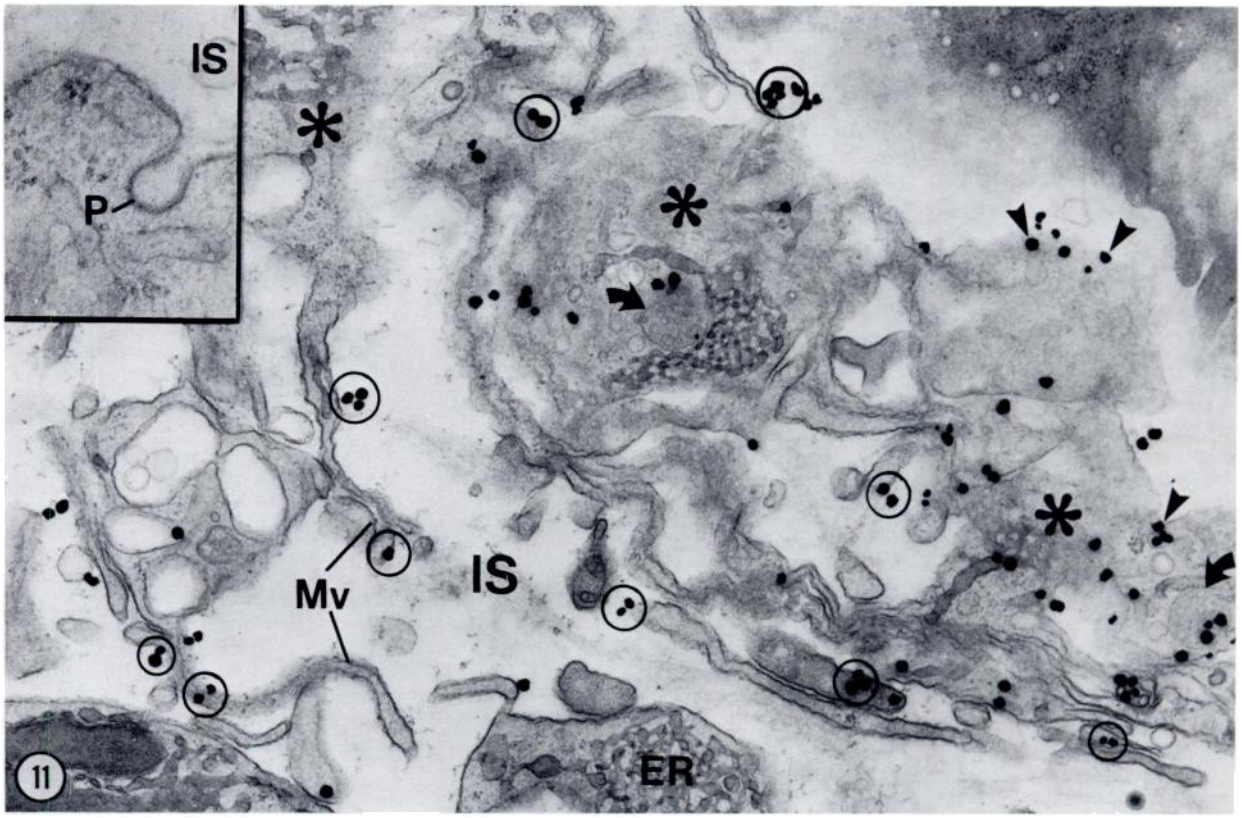

Fig. 11. Portions of several Leydig cells (asterisks) 6 hours after injection of [125I]hCG. Note prominent labeling of microvillar processes (Mv) of Leydig cells (circled) at this time interval that is still qualitatively similar to that observed at 20 minutes and 1 hour. A few silver grains are also seen over or next to small pits of cell surface (arrowheads), but such findings are rare. Silver grains are also associated with dense multivesicular bodies (curved arrows). ER: smooth endoplasmic reticulum, IS: interstitial space (× 25,740). **Inset**: Three hours after injection [125I]hCG (and at all other time intervals), small coated or uncoated pits (p) of the cell surface are predominantly unlabeled. IS: interstitial space (× 54,400).

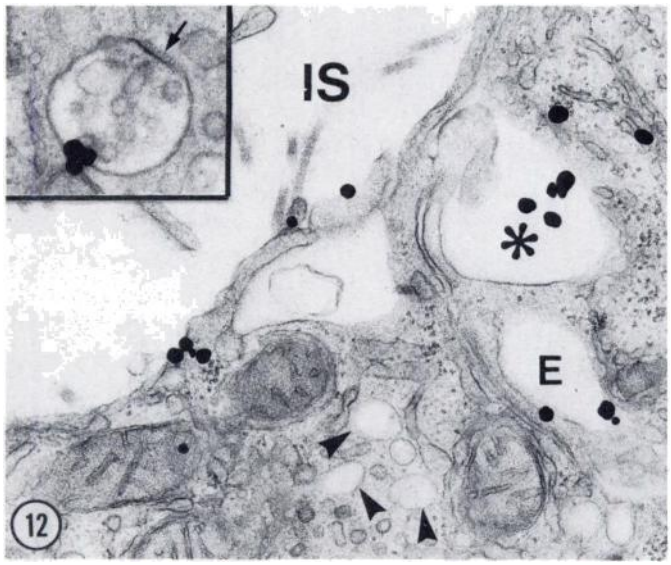

Fig. 12. Six hours after injection of [^{125}I]hCG, a large uncoated surface invagination (asterisk) and endosome (E) of a Leydig cell are still labeled. No labeling, as observed at earlier time intervals, is found over the intermediate vesicles situated near the cell surface (arrowheads). IS: interstitial space (× 33,440). **Inset:** Labeled pale multivesicular body of a Leydig cell 6 hours after injection of [^{125}I]hCG. Note silver grains related to its limiting membrane and the plaque of fuzzy material (arrow). (× 37,700).

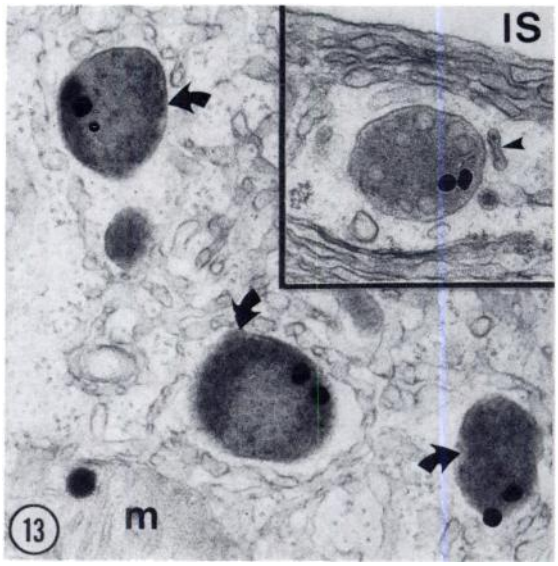

Fig. 13. Three hours after injection of [^{125}I]hCG, several secondary lysosomes of a Leydig cell are labeled (arrows). m: mitochondrion (× 39,600). **Inset:** Labeled dense multivesicular body 6 hours after injection of [^{125}I]hCG. Membranous tubule adjacent to the body is unlabeled (arrowhead). IS: interstitial space (× 39,000).

hCG hormone receptor complex, which has been shown *in vitro* to be about 24 hours at 24 C (Amsterdam and Lindner, 1984), and with our finding that hCG is not taken up by coated pits, structures that have been shown to be involved in a very rapid internalization of receptor-bound proteins (Goldstein et al, 1979; Pastan and Willingham, 1983; Willingham and Pastan, 1984). While numerous silver grains were still present on the microvillar processes of Leydig cells at the late time intervals, the large surface invaginations, or endosomes, and the pale and dense multivesicular bodies and secondary lysosomes continued to be labeled, indicating that internalization was still occurring. Similar observations were noted at 24 hours, although binding to the microvillar processes was diminished and fewer labeled endosomes, multivesicular bodies and secondary lysosomes were observed than at the 1-, 3- and 6-hour intervals.

While hCG is internalized, it is well known that its internalization does not appear to be involved in the expression of its biologic action. In Leydig cells, this action is mediated by the receptor-mediated activation of adenylate cyclase and the formation of cAMP (Mendelson et al, 1975), resulting in increased testosterone production within 5 to 10 minutes (Amsterdam et al, 1981). Indeed, the endocytotic uptake has been thought to be important in the termination of hormone action (Segaloff and Ascoli, 1981) and in the long term regulation of the number of receptors on the cell surface (Freeman and Ascoli, 1981).

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