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Immunocytochemical Demonstration of a Lipid Droplet-Specific Capsule in Cultured Leydig Cells of the Golden Hamsters

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Abstract In this report, we provide direct evidence for the presence of a lipid droplet-associated capsule in hamster steroidogenic Leydig cells by using a monoclonal antibody A2. Leydig cells are characterized by containing many lipid droplets and having 3β-hydroxysteroid dehydrogenase activity. Immunofluorescence staining with this antibody demonstrated a rim or capsule surrounding the lipid droplets in Leydig cells, a pattern not seen with anti-vimentin antibody. Immunogold labelling confirmed ultrastructurally that antibody binding was distributed on the lipid droplet surface. In order to investigate the possible function of the capsule, we examined the morphological changes induced in the capsule following stimulation with LH or dibutyryl cAMP; the fluorescent intensity of the capsule was seen to gradually decrease, accompanied by a decrease in number and size of lipid droplets, and the response to both reagents was time- and concentration-dependent. We thus conclude that hormonal stimulation resulting in the detachment of certain capsular proteins from the surface of lipid droplets is mediated via the cAMP signaling pathway and may allow cholesterol ester hydrolytic enzyme direct access to its substrate in the lipid droplet.

Key words: capsule, lipid droplet, Leydig cell, monoclonal antibody, immunocytochemistry

Steroidogenic lipid droplets in Y-1 mouse adrenal tumor cells are surrounded by a complete electron-dense capsule with a thickness of 5 nm [Almahbobi et al., 1992]. Recently, Blanchette-Mackie et al. [1994, 1995] reported that perilipin A (56 kD), a hormone-regulated phosphoprotein, is detected on the surface layer of lipid droplets in both adrenal Y-1 cells and 3T3-L1 adipocytes. Concomitant with the activation of lipolytic activity in these cells, perilipin A is hyperphosphorylated by protein kinase A, suggesting that these proteins may have a role in lipid metabolism [Egan et al., 1990; Greenberg et al., 1991, 1993; Servetnick et al., 1995]. Another perilipin, perilipin C (42 kD), is also found in adrenal Y-1 cells [Servetnick et al., 1995]. Another type of steroidogenic cell, the MA-10 Leydig cell, also expresses both perilipin A and C, which are detected in the lipid droplet fraction [Servetnick et al., 1995]. However, the exact role of the perilipins in lipolysis still remains to be determined.

We have produced a monoclonal antibody, A2, which specifically immunolabels the capsule of lipid droplets in rat adrenal cells [Wang and Fong, 1995], and were therefore interested to know whether this antibody could be a universal probe for labelling steroidogenic lipid droplets in other cell types, such as Leydig cells. During preliminary screening, we observed the positive immunoreactivity in the cytoplasm of the hamster Leydig cell but not in seminiferous tubule cells. Further studies showed labelling to be restricted to the surface of the lipid droplets [Wang and Fong, 1995]. These results suggest that antibody A2 may be a useful probe for studying the surface structure and function of the capsule of steroidogenic lipid droplets.

Steroid secretion in Leydig cells is stimulated by the binding of LH to specific high-affinity receptors on the cell membrane [Hsueh et al., 1976]; this activates adenylate cyclase and increases the intracellular concentration of cAMP, which then activates cAMP-dependent protein kinases [Podesta et al., 1978]. The subsequent

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phosphorylation of unidentified proteins enhances the mobilization of cholesterol from lipid droplets, and its transport to mitochondria, where side-chain cleavage of cholesterol occurs, resulting in increased testosterone secretion [Hall et al., 1979]. The action of LH on steroidogenesis in Leydig cells can be mimicked by cAMP analogue, such as dibutyryl cAMP [Hsueh, 1980].

In the present study, this antibody probe was used to directly demonstrate the presence of the lipid droplet capsule in Leydig cells by electron microscopy and to study the effect of LH and dibutyryl cAMP stimulation on the capsule morphology.

MATERIALS AND METHODS

Animals

Adult male Syrian hamsters (*Mesocricetus auratus*), aged 8 to 12 weeks, were used in this study, and were housed under standard laboratory conditions of light (14L:10D) and temperature (22°C), with free access to food and water.

Isolation and Culture of Leydig Cells

Animals were anesthetized by intraperitoneal injection of 7% chloral hydrate and Leydig cells isolated as described by Eskeland et al. [1989], with a few modifications. The testes were aspetically removed, and all subsequent procedures performed under sterile conditions. They were decapsulated and incubated in an enzyme solution containing 0.5 mg/ml of Type II collagenase (Sigma, St. Louis, MO), 0.1% bovine serum albumin, 25 mM HEPES in Dulbecco's modified Eagle's medium (pH 7.2) for 20 min at 34°C in a gently shaking water bath. The cell suspension was diluted with 4 volumes of 0.1% bovine serum albumin in HEPES-Dulbecco's medium and then left at room temperature for 2 min to allow the seminiferous tubules to settle. The supernatant was filtered through four layers of gauze and collected, the tubules washed once, and the supernatant pooled with the previously collected supernatant. Leydig cells were pelleted by low-speed centrifugation of the supernatants, further purified by Percoll gradient centrifugation [Eskeland et al., 1989], then plated on coverslips in medium 199 supplemented with 5% fetal calf serum, 100 IU/ml of penicillin and streptomycin and cultured in a humidified atmosphere of 95% air: 5% CO_2 at 37°C. Equine luteinizing hormone (Sigma, St. Louis, MO) was added to 2-day cultures at final concentrations of 36 IU/ml (low concentration) or 180 IU/ml (high concentration). Dibutyryl cAMP was added at final concentrations of 0.1 or 1 mM.

Enzyme Histochemistry

Staining for 3β-hydroxysteroid dehydrogenase was carried out by the method of Klinefelter et al. (1987). Cells on cover-slips were air dried, then incubated in substrate solution containing nitro blue tetrazolium, etiocholan-3β-o1-17-one and β-nicotinamide adenine dinucleotide in Dulbecco's phosphate-buffered saline for 90 min at 37°C. After a rinse in water, cells were fixed in 10% formalin. Positive staining was seen as blue-purpose formazan granules.

Antibodies

The monoclonal antibody A2 was produced as described previously [Wang and Fong, 1995]. Mouse monoclonal anti-vimentin, FITC-conjugated goat anti-mouse IgG (whole molecule), FITC-conjugated goat anti-mouse IgM μ chain, rhodamine-conjugated goat anti-mouse IgG γ chain, as well as 10 nm gold-conjugated goat anti-mouse IgM were all purchased from Sigma. Alkaline phosphatase conjugated goat-anti-mouse IgG was obtained from Promega (Madison, WI).

Immunofluorescence

Cells were briefly rinsed with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and then fixed in 0.15% glutaraldehyde in lysis buffer (60 mM PIPES, 25 mM HEPES, pH 6.9, containing 0.15% Triton X-100, 10 mM EGTA, and 2 mM $MgCl_2$,) for 5 min. Nonspecific binding was blocked by treatment with $NaBH_4$ (1 mg/ml) for 30 min. After a PBS wash, cells were incubated overnight at 4°C with first antibody, washed with PBS and reacted with FITC-conjugated goat anti-mouse IgG whole molecule. The cells on cover-slips were then washed and mounted on slides in PBS, pH 8.0, containing 2% n-propyl gallate and 50% glycerol, which were then sealed in place with nail polish.

Immunoelectron Microscopy

Leydig cells were directly cultured in plastic culture dishes. After a PBS rinse, cells were fixed in 0.15% glutaraldehyde in lysis buffer and

nonspecific staining blocked by $NaBH_4$ treatment. They were then incubated overnight at 4°C with antibody A2 in PBS containing 10% bovine serum albumin, washed with PBS, then reacted for 2 h at 37°C with 10 nm gold-conjugated goat anti-mouse IgM diluted in PBS containing 0.1% BSA, 0.5 M NaCl. They were washed in PBS and postfixed in 2% glutaraldehyde for 1 h, followed by 0.5% OsO_4 for 3 min. After dehydration in a series of alcohols, cells were embedded in Epon, and the Epon blocks separated from the culture dishes, trimmed into small blocks, and sectioned. Thin sections were collected on grids and double stained with uranyl acetate and lead citrate.

Western Blotting

Isolated Leydig cells were washed in PBS and hypotonically lysed in a lysis medium containing 1 mM EDTA, 1 mM bezamidine, 0.1 mM PMSF in 10 mM Tris-HCl, pH 7.4 [Servetnick et al., 1995]. The lysed cells were disrupted by 10 strokes in glass homogenizer and the homogenates centrifuged at 27,000*g* for 30 min. The floating lipid droplets were collected and dissolved in electrophoresis sample buffer at 95°C for 3 min. The protocols for gel electrophoresis and immunoblotting were as described by Fritz et al. [1989] and Towbin et al. [1979]. Each lane was loaded with 100 μg of protein. After electrotransfer, the protein sheet was blocked in 5% nonfat milk in Tris-buffered saline (50 mM Tris, pH 8.2, containing 150 mM NaCl and 0.1% Tween-20) and incubated overnight at 4°C with first antibody. After washing in Tris-buffered saline, membrane sheets were incubated 2 h at 37°C with alkaline phosphatase-conjugated goat anti-mouse IgG (1:7,500) and the reactive band visualized by substrate development with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

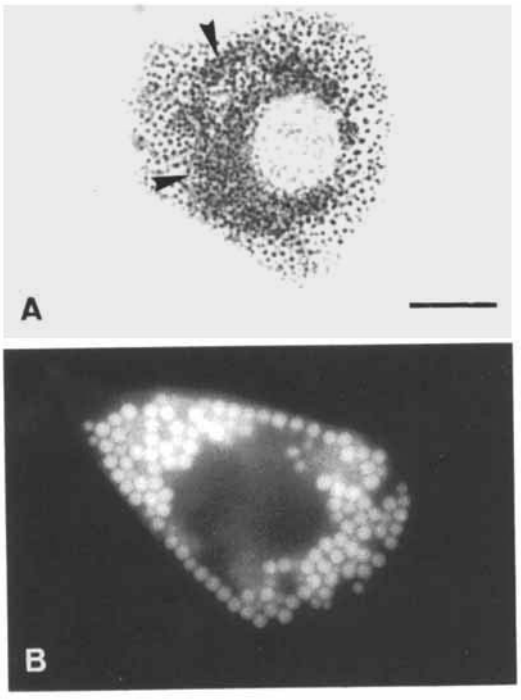

Fig. 1. Identification of cultured hamster Leydig cells. **A:** Histochemical demonstration of 3β-hydroxysteroid dehydrogenase enzyme activity in Leydig cells. Particles of bluish reaction products (arrowheads) are observed in the cytoplasm. **B:** Nile-red fluorescent stain for lipid droplets. Leydig cells contain many lipid droplets in the cytoplasm. N, nucleus. Bar = 10 μm.

RESULTS

Leydig cells were identified by the presence in the cytoplasm of bluish products of the 3β-hydroxysteroid dehydrogenase reaction (Fig. 1A), whereas contaminating Sertoli and spermatogenic cells were negative. The Leydig cells also contained numerous cytoplasmic Nile-red positive lipid droplets (Fig. 1B), most of which were uniform in size. Antibody A2 detected a 230 kD polypeptide from the lipid droplet fraction of Leydig cells (Fig. 2). Double-labelling with this

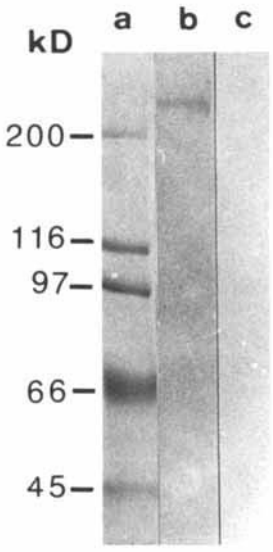

Fig. 2. Immunoblot characterization of antibody A2. **a:** Molecular weight markers. **b:** Immunoblot of lipid droplets fraction from Leydig cells with antibody A2. A 230 kD polypeptide is immunoreactive to this antibody. **c:** Control. Omission of primary antibody shows a clean background.

antibody and anti-vimentin antibody revealed different distribution patterns for the respective antigens. Each lipid droplet was surrounded by an A2-positive capsule (Fig. 3A), while vimentin filaments were seen as networks of interconnecting filaments between the lipid droplets, rather than closely investing the droplet (Fig. 3B). When extracted with Triton X-100, lipid droplets were enlarged and released from the cells in some case, however, a thin layer of fluorescent capsule

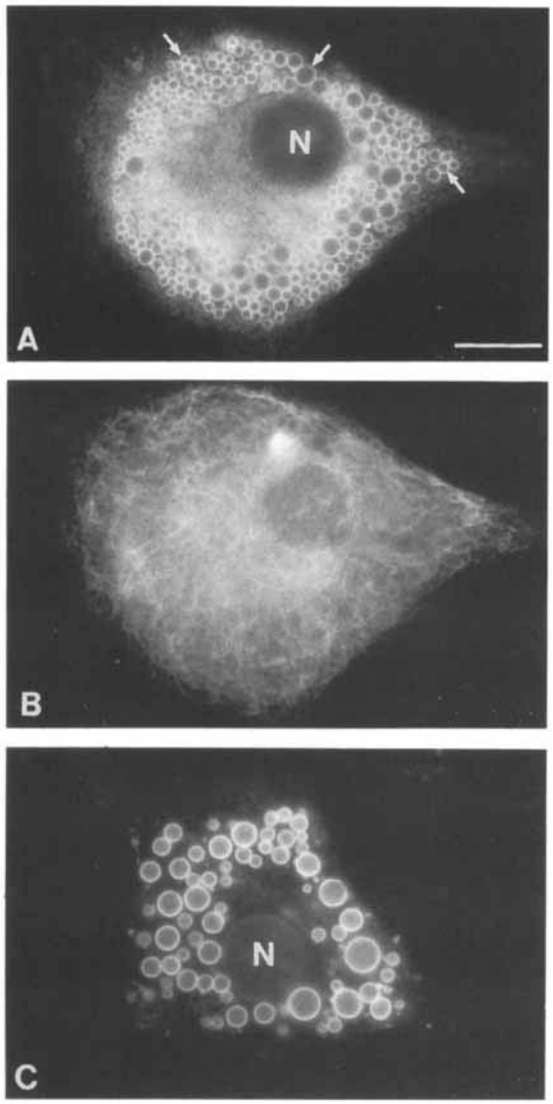

Fig. 3. Double-label immunofluorescence staining with anti-vimentin antibody and antibody A2 of Leydig cells. **A:** All lipid droplets are surrounded by A2-positive capsules (arrows). **B:** Vimentin intermediate filaments are seen as interconnecting networks, and no capsular structure is observed around the lipid droplets. **C:** Cells extracted with 0.5% Triton X-100. A2-positive capsule are still associated with enlarged lipid droplets. N, nucleus. Bar = 10 μm.

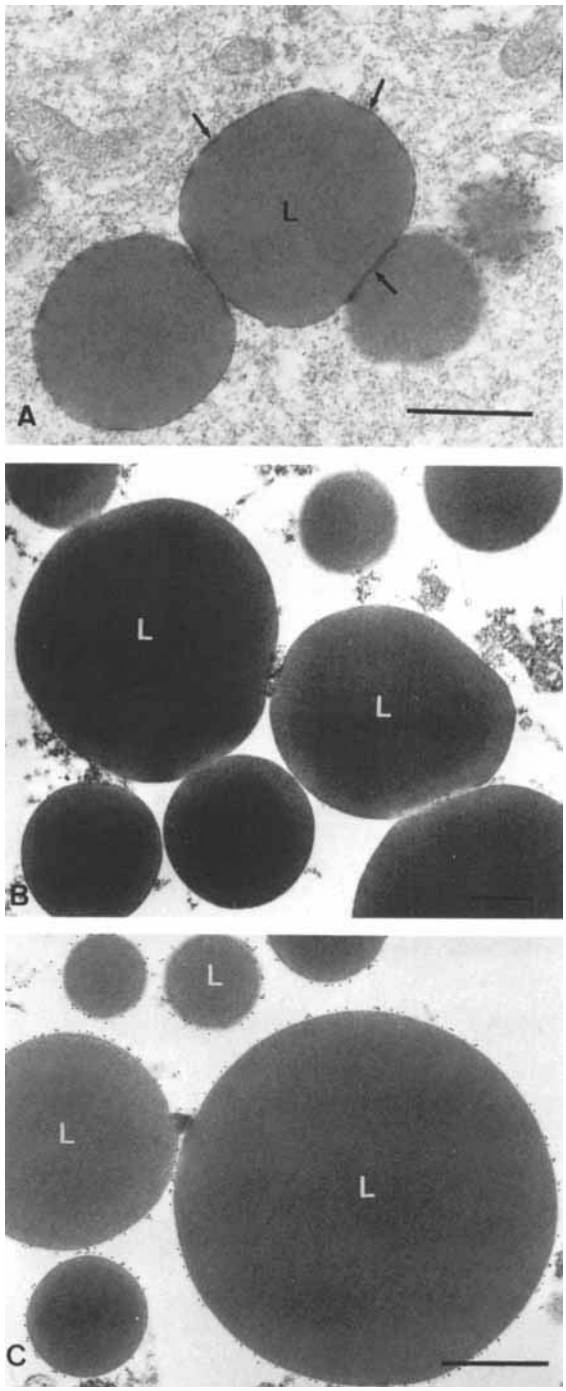

Fig. 4. Electron micrographs of lipid droplets and immunogold localization of antibody A2 in Leydig cells. **A:** An electron-dense layer surrounding the lipid droplet (arrows) is present in intact Leydig cells. Specimen prepared by conventional fixing and staining. **B:** Primary antibody blank control. The background and the lipid droplet surface are devoid of gold particles. **C:** Incubation with antibody A2 followed by 10 nm gold-conjugated secondary antibody. The periphery of the lipid droplets is clearly decorated by 10 nm gold particles showing the binding of antibody A2. L, lipid droplets. Bars = 1 μm.

remained associated with lipid droplets (Fig. 3C).

Ultrastructural studies on conventionally fixed and stained thin sections of cultured Leydig cells revealed an electron-dense substance investing each lipid droplet (Fig. 4A). Use of the immunogold labelling technique demonstrated that the use of A2 resulted in a heavy decoration of gold particles on the surface of lipid droplets (Fig. 4C), while other organelles were not labelled. In primary antibody blank control, no gold-labelling of lipid droplets was seen (Fig. 4B). A2-reactive polypeptides therefore seem to be unequivocally localized on the surface of lipid droplets.

Before activation by LH or dibutyryl cAMP, all the lipid droplets in Leydig cells were surrounded by a bright, continuous capsule, as shown by immunofluorescence staining with antibody A2 (Fig. 3A). After stimulation for 6 h with a low concentration of LH (36 IU/ml), the fluorescence intensity of the capsule was slightly decreased in 70% of the total Leydig cells (Fig. 5A and B), while when treatment was extended to 24 h, the number of lipid droplets decreased, the capsule was lost, and diffuse staining of the cytoplasm became obvious in 80% of the Leydig cells (Fig. 5C and D). When a higher concentration of LH (180 IU/ml) was used, decapsulation of lipid droplets was detectable in 70% of the Leydig cells as early as 2 h after treatment (Fig. 5E and F).

Since LH acts via the cAMP pathway, we also tested the effect of dibutyryl cAMP. Treatment with 0.1 mM dibutyryl cAMP for 6 h resulted in a discontinuous pattern of capsular staining in 70% of the Leydig cells (Fig. 6A and B) and after a prolonged incubation of 24 h, significant loss of capsular staining was found in 80% of the Leydig cells (Fig. 6C and D). At 1 mM dibutyryl cAMP, the time required for complete detach-

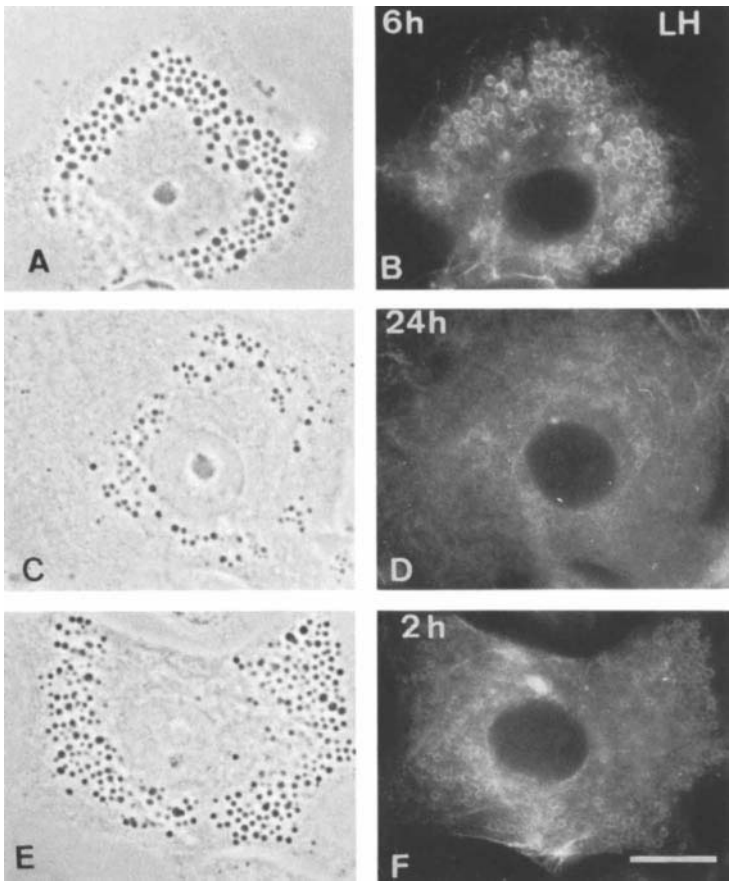

Fig. 5. Effect of LH on capsular staining of lipid droplets in Leydig cells. **A, C,** and **E** are the phase pair of **B, D,** and **F** (immunostained with antibody A2). A and B, 6 h after low concentration of LH stimulation. Most capsular staining has decreased. C and D, 24 h after low concentration of LH stimulation. The surface of many lipid droplets is not stained. E and F, 2 h after high concentration of LH stimulation. The fluorescent intensity of the capsule is reduced. Bar = 10 μm.

ment of the fluorescent capsule from the lipid droplets was decreased to 6 h (Fig. 6E and F). Thus the effect of both LH and dibutyryl cAMP treatment on the fluorescent capsule of lipid droplets is concentration- and time-dependent.

DISCUSSION

In the present study, we have confirmed the presence of the capsule on lipid droplets in another type of steroidogenic cell, the Leydig cell, in addition to adrenocortical cells, with monoclonal antibody A2 by immunofluorescence and immunoelectron microscopy. Moreover, the capsule of lipid droplets, although very stable to detergent extraction, undergoes progressive morphological change at different times following hormonal stimulation. Initially, capsular staining becomes discontinuous and punctate pattern; subsequently, the intensity of fluorescent staining of the capsule decreases, then, after a longer period of hormone treatment, the lipid droplets become smaller and the capsule staining is difficult to detect, concomitant with significant increase in diffuse cytosolic staining. We thus conclude that certain capsular proteins become detached in response to the lipolytic signal. Similar morphological changes of capsule were also observed in rat adrenocortical cells treated with ACTH [Wang and Fong, 1995]. These data suggest that the capsular protein may be involved in hormone-induced lipolysis of lipid droplets.

Since one mechanism of LH action involves the cAMP second messenger system with activation of adenylate cyclase [Cooke et al., 1992], we also examined morphological changes in the capsule induced by cAMP analogue. The results were similar to those induced by LH, confirming that detachment of capsular proteins after LH stimulation is mediated by an increased concen-

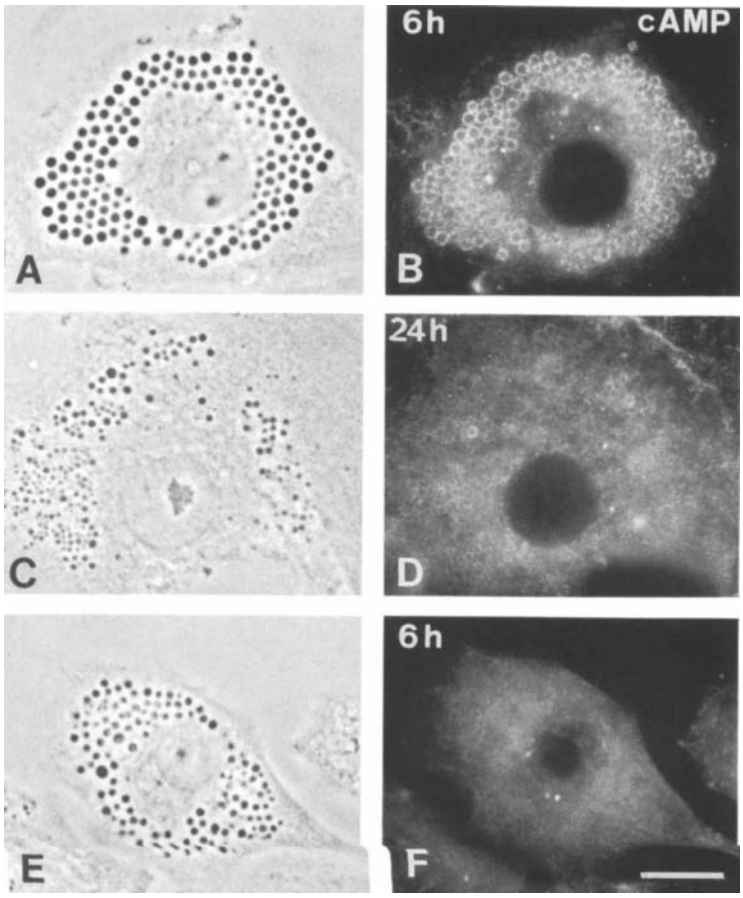

Fig. 6. Effect of dibutyryl cAMP on capsular staining of lipid droplets in Leydig cells. **A, C,** and **E** are the phase pair of **B, D,** and **F** (immunostained with antibody A2). A and B, 6 h after 0.1 mM dibutyryl cAMP treatment. Capsular staining becomes punctate or discontinuous. C and D, 24 h after 0.1 mM dibutyryl cAMP treatment. E and F, 6 h after 1 mM dibutyryl cAMP treatment. In D and F, no fluorescence is seen around the surface of the droplets. Bar = 10 μm.

tration of intracellular cAMP. It is clear that elevation of cAMP activates two types of protein kinase A in hormone-stimulated rat Leydig cells [Podesta et al., 1976]. Since both type I and type II are capable of activating steroidogenesis [Moger, 1991], it is possible that these kinases either directly or indirectly phosphorylate the capsular protein, resulting in its conformational change and thus the detachment. However, this hypothesis remains to be proved.

Lipolysis appears to proceed at a faster rate once the capsule is removed, as evidenced by the decrease in the number and size of lipid droplets in activated Leydig cells. In rat and bovine adrenal cortex, cholesterol ester hydrolase, which catalyzes the conversion of esterified cholesterol to cholesterol, is activated by cAMP in adrenal cells [Trzeciak and Boyd, 1973, 1974]. Whether the decapsulation provides direct exposure of activated cholesterol ester hydrolase to cholesterol ester contained in the lipid droplets, thus accelerating lipolysis, remains to be determined.

Testosterone production in Leydig cells is proportional to the concentration of LH or cAMP applied [Hall et al., 1979; Moyle and Ramachandran, 1972]. Our results indicating that the higher the concentration of LH or cAMP used, the shorter time required for decapsulation of lipid droplets, are in agreement with the physiological data. However, 2 h was needed for decapsulation of lipid droplets in response to high concentrations of LH (50 μg/ml) or dibutyryl cAMP (2 mM), whereas, at the same concentrations, testosterone production was found to be linearly increased from the onset of stimulation to 1 h [Hall et al., 1979]. This discrepancy in the latency period may be due to the presence of the plasma membrane pool for cholesterol mobilization [Freeman, 1987] which is available for the early increase of testosterone; it is possible that the later phase of testosterone production utilizes the cholesterol pool from decapsulated lipid droplets.

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