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Expression of caveolin-1 in rat Leydig cells

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ABSTRACT: Caveolin-1, the first member of caveolin family reported, is recognized as the structural component of caveola, a plasma membrane invagination or vesicles that are a subcompartment distinct from clathrin-coated pits. This protein is also known to be involved in cholesterol trafficking.

The aim of this study was to determine the expression of caveolin-1 in adult rat Leydig cells. Testis sections incubated with an antibody to caveolin-1 showed, by immunohistochemistry, a moderate number of Leydig cells with different degrees of immunoreaction and a strong reaction in endothelial cells and in the lamina propia of seminiferous tubules. Caveolin-1 was detected in the cell cytoplasm with a granular pattern and on the cell surface of Leydig cells cultured 24 h on uncoated, laminin-1 or type IV collagen coated coverslips. We also observed a milder reaction in 3 h cultures. Immunoreaction was also detected in Leydig cells with an antibody to tyrosine-phosphorylated caveolin-1. By double immunofluorescent technique, we observed co-localization of caveolin-1 and 3β-hydroxysteroid dehydrogenase. Western blot analysis revealed a band of about 22 kDa molecular weight that was recognized with both caveolin-1 and tyrosine-phosphocaveolin-1 antibodies. Caveolin-1 is one of a few proteins with a demonstrated ability to bind cholesterol *in vivo*. In this context, the presence of caveolin-1 in Leydig cells may be related to cholesterol traffic -a rate limiting step in steroid biosynthesis.

Introduction

Leydig cells are found in the testicular interstitial tissue intermingled with blood vessels, lymphatics, extracellular matrix proteins, connective tissue cells, and macrophages (Russel, 1998); they are present in clusters or groups of cells interconnected by gap junctions (Pérez-Armendariz *et al*., 1994).

Leydig cells in adult rats are surrounded by a discontinuous basement membrane mainly composed of laminin and type IV collagen distributed in a patchy pattern. These patches are found on the free surfaces of the Leydig cells, between two Leydig cell, and at Leydig cell-macrophage contact sites (Kuopio and Pelliniemi, 1989). Vernon *et al*. (1991) demonstrated the influence of extracellular matrix proteins on the shape, proliferation and expression of 3β-hydroxysteroid dehydrogenase in mouse Leydig cells. We previously reported the presence of laminin-1 and type IV collagen and the expression of integrin subunits, α_3, α_6, and β_1 in rat Leydig cell cultures (Denduchis *et al*., 1996). We also demonstrated that type IV collagen is able to modulate testosterone production in rat Leydig cells (Diaz *et al*., 2002).

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Leydig cells synthesize androgens from cholesterol in response to luteinizing hormone and to a large set of locally produced factors that act in a paracrine and/or autocrine way (Saez, 1994). Cholesterol utilized for testosterone biosynthesis is derived from: a) *de novo* synthesis, b) hydrolysis of cholesteryl esters in intracellular lipid droplets and/or c) cholesteryl esters in high density lipoprotein (HDL).

Caveolae are cell surface vesicular invaginations first discovered on the surface of endothelial cells (Palade, 1953). Caveolae, similarly to clathrin-coated pits, function as macromolecular vesicular transporters, while their unique lipid composition classifies them as plasma membrane lipid rafts, structures enriched in a variety of signaling molecules (Cohen *et al*., 2004). The caveolins proteins serve as the structural components of caveola, and caveolin-1 was the first member of caveolin family to be identified. It is an integral membrane protein of 22-24 kDa and the major component of caveolae (Glenney, 1992; Glenney and Soppet, 1992). This protein was identified as a major phosphorylated protein in v-Src transformed cells (Glenney and Zokas, 1989). In mammals, three caveolin genes have been identified that code for five isoforms of the protein: caveolin-1-α and 1–β, caveolin-2-α, 2-β, 2-γ and caveolin –3 whose molecular structure differ (Smart *et al*., 1999; Razani *et al*., 2002). Caveolin-1 and -2 form a hetero-oligomer, and their tissue distribution is similar with high expression in adipocytes, endothelial cells, pneumonocytes and fibroblasts (Scherer *et al*., 1997), while caveolin-3 is predominant in cardiomyocytes, skeletal and smooth muscle cells (Tang *et al*., 1996; Way and Parton, 1995; Razani *et al*., 2002). Caveolin-1 was shown to bind to the two primary components of lipid rafts, cholesterol and sphingolipids, both *in vitro* and *in vivo* (Fra *et al*., 1995). Caveolin-1 is an unusual protein in that it can be both an integral membrane protein and a soluble protein found in many cellular compartments (Liu *et al*., 2002). Caveolin-1 forms cytosolic complexes with chaperones, which seem to be involved in cholesterol trafficking from smooth endoplasmic reticulum to the plasma membrane (Smart *et al*., 1996; Uittenbogaard and Smart, 2000). It has been suggested that this protein participates in the process of cholesterol influx and efflux (Liu *et al*., 1999) and it is also involved in the integration of different signal transduction pathways playing a pathogenic role (Okamoto *et al*., 1998; Smart *et al*., 1999; Williams and Lisanti, 2004). In the latter context, Wei *et al*. (1999) have shown that caveolin-1 interacts with integrins and that the depletion of this protein by anti-sense methodology disrupts integrin-mediated adhesion and cell signaling. On the other hand, it has been proposed that integrins activate the extracellular signal-regulated kinase (ERK) cascade via caveolin-1, Src-family kinase Fyn and the adaptor protein Shc (Giancotti and Ruoslahti, 1999; Wary *et al*., 1998).

Considering that caveolin-1 can bind and sequester cholesterol within membrane microdomains and also that this lipid is required for steroidogenesis we evaluated for the first time the expression of caveolin-1 in rat Leydig cells.

Materials and Methods

Reagents and Antibodies

Culture medium 199, HAM's F-12, Dulbecco's modified Eagle Medium, human transferrin, insulin, collagenase, trypsin, soybean trypsin inhibitor, bovine serum albumin (BSA), vitamin E, anti-serum to α-smooth muscle actin and Percoll were obtained from Sigma Chemical Co. (St. Louis, MO). Rat transferrin was from Cappel Lab. (Cochranveille, PA). Laminin-1 and type IV collagen, isolated from an Engelbreth Holm Swarm sarcoma, were obtained from Gibco BRL, Life Technologies (Grand Island, NY). Mouse monoclonal antibodies against caveolin-1 (clone 2297) and phosphocaveolin Y14 (clone 56) were purchased from BD Transduction Labs (Bedford, MA). Polyclonal antibody against 3β-hydroxysteroid dehydrogenase was kindly provided by Dr. Hector E. Chemes (CEDIE-CONICET, Buenos Aires, Argentina). A fluorescein isothiocyanate (FITC)-conjugated horse anti-mouse IgG and the ABC detection system were obtained from Vector Laboratories (Burlingame, CA). Horseradish peroxidase-conjugated anti-mouse antibodies were purchased from Sigma Chemical Co. and a goat rhodamine-conjugated antibody to rabbit IgG from Chemicon (Temecula, CA). For Western blot, Immobilon-P transfer membranes were obtained from Millipore Corporation (Bedford, MA) and enhanced chemiluminiscence detection system from Amersham Pharmacia Biotech (Piscataway, NJ).

Rat Leydig cell cultures

Leydig cells were isolated from adult male Sprague-Dawley rats (60-70 days). The animals were treated according to protocols for animal use, following NIH guidelines for care and use of experimental animals.

Briefly, in each experiment 20 rats were killed by decapitation. Testes were removed and placed in ice-cold phosphate buffered saline (PBS), pH 7.4. Leydig cells were isolated as previously described (Dufau *et al*., 1974) and purified on a discontinuous Percoll gradient (21, 26, 40, 60%) (Lefèvre *et al*., 1983). Purified Leydig cells were resuspended in a medium consisting of 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle medium, supplemented with 20 mM Hepes, 100 IU/ml penicillin, 2.5 μg/ml amphotericin B, 1.2 mg/ml sodium bicarbonate, 10 μg/ml transferrin, 5 μg/ml vitamin E, and 0.1% BSA. Cells were incubated at 34ºC in a mixture of 5% CO_2/95% air for 90 min for Western blot analysis and for 3 and 24 h for immunocytochemistry. Cell viability was 95% as assessed by trypan blue exclusion. The 3 β-hydroxysteroid dehydrogenase activity revealed by a histochemical technique (Levy *et al*., 1959), was used to determine the purity of Leydig cell cultures, which ranged from 90 to 95%. Careful examination of the cultures by phase contrast microscope showed no Sertoli cells. This observation was further confirmed by the absence of rat transferring in Leydig cell-conditioned medium as demonstrated by a highly sensitive and specific radioimmunoassay (RIA) for this protein (Skinner and Griswold, 1982). Purified rat transferrin and a polyclonal rabbit antibody against rat transferrin were used in the RIA. The detection limit for this assay was 1,5±0.08 ng/tube (n=30 assays). The cross-reactivity of human transferrin in the rat transferrin assay was <0.003%. No myoid cell contamination was detected by immunofluorescence and immunoperoxidase techniques applied to Leydig cell cultures using a specific antiserum to α-smooth muscle actin diluted to 1/100-1/200.

Cells were cultured on glass coverslips uncoated or coated with 6 μg/cm^2 laminin-1 or 6 μg/cm^2 type IV collagen; cells were plated at a final cell density of 1x10^{5}cells/cm$''$ as previously described (Diaz *et al*., 2002).

Immunohistochemistry

Immunoperoxidase

Cells cultured on glass coverslips, uncoated or coated with laminin-1 or type IV collagen, were washed three times with PBS and fixed for 10 min at room temperature (RT) with 2-4% paraformaldehyde in PBS. Endogenous peroxidase was inactivated in 0.3% H_2O_2/methanol for 30 min. Cells rinsed with PBS and blocked with 1.5% normal horse serum in PBS for 20 min at RT were then incubated with the primary antibodies against caveolin-1 or tyrosine-phosphorylated caveolin-1 (1/20) at 37ºC in a humidified chamber for 60 min. Then, cells were processed following a standard ABC immunostaining procedure with an ABC kit. Immunoreaction product was visualized with diaminobenzidine/H_2O_2 solution, and cells were counterstained with hematoxylin.

Immunoperoxidase technique was also used on testis sections from adult normal Sprague-Dawley rats, fixed in Bouin's-solution and paraffin-embedded. Sections were deparaffinized and irradiated with microwaves in 0.1M sodium citrate buffer (pH6) for 15 min at 850W. After washing with PBS, the IP technique using an antiserum to caveolin-1 was performed as described above. As negative controls, Leydig cells and tissue sections were incubated with PBS instead of the primary antibody and observed by light microscopy.

Immunofluorescence (IF)

Leydig cells cultures were fixed as described above. Fixed cells were rinsed with PBS and permeabilized for 10 min with 0.1% Triton X-100 in PBS. After washing, cells were incubated with anti-caveolin-1 or anti-tyrosine-phosphorylated caveolin-1 (1/20) antibodies at 37°C in a humidified chamber for 60 min. After three washes with PBS, cells were incubated with conjugated anti-mouse IgG (1/100) at 37°C for 60 min.

A double immunofluorescent technique was applied to Leydig cell cultures using a rabbit antibody against 3β-hydroxysteroid dehydrogenase and a mouse antibody to caveolin-1.

Secondary antibodies were FICT-conjugated anti-mouse IgG and rhodamine-conjugated anti-rabbit IgG. Cells were washed, mounted in buffered glycerin, and observed in a Axiophot fluorescent microscope with epi-illumination (Carl Zeiss, Inc., Oberkochen, Germany).

Western blot analysis

Leydig cells were cultured for 90 min in 6-well plates uncoated or coated with 6 μg/cm^2 laminin-1 or 12 μg/cm^2 type IV collagen. Cells were washed twice with PBS and lysed with 100 μl of buffer pH 7.4, containing 150 mM NaCl, 50 mM Tris, 1% SDS, 2 mM Na_3VO_4, 50 mM NaF, 2mM EDTA, 1% sodium desoxycholate, 1% NP-40, 1 mM phenylmethysulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pestatin A and 10 μg/ml aprotinin. Cells were collected with a cell scraper and disrupted by ultrasonic irradiation for 2-5

sec; proteins were measured by Bradford assay Bio-Rad, (Hercules, CA, USA). Cellular extracts were boiled for 5 min with sample buffer (500 μM Tris-HCl pH 6.8, 10% SDS, 30% glycerol, 0.5% β-mercaptoethanol and 0.5% bromophenol blue). Samples (20 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12% acrylamide/bisacrylamide for the resolving gel and 5% acrylamide/bisacrylamide for the stacking gel) in Mini Protean Cell Bio Rad. After SDS-PAGE, gels were equilibrated in transfer buffer for 15 min and electrotransferred at 150 V for 60 min onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P) using a mini trans blot cell (Bio Rad). Membranes were stained with Ponceau S (Sigma) to confirm transfer and then blocked with Tris-buffered saline- 0.1% Tween 20 with 3% BSA and 3% non-fat dry milk for 90 min. Blots were probed overnight with a monoclonal antibody against tyrosine-phosphocaveolin-1 (1/250) in the blocking solution at 4ºC. Then, blots were washed and incubated with horseradish peroxidase-conjugated anti-mouse IgG diluted in blocking solution for 60 min at RT. Blots were reprobed with a monoclonal antibody against total caveolin-1 as a loading control. Prestained protein standards with a molecular weigt range of approximately 256-7 kDa were used. Protein immunoreactivity was revealed with an enhanced chemiluminescence detection system. Autoradiographic band intensities were quantified by densitometry and analyzed by the Scion Image program and expressed as arbitray units.

Statistics

Data were analyzed by one-way analysis of variance and the Student-Newman-Keuls multiple comparison test for unequal replicae using an Instat program. A value of $P<0,05$ was considered significant.

Results

Testis sections incubated with an antibody to caveolin-1 showed, by immuno-peroxidase technique, few Leydig cells with different degrees of immunoreaction and a strong reaction in endothelial cells and in the lamina propia of seminiferous tubules including a mild reaction in myoid cells (Fig. 1A). No reaction was observed in germ cells. Control was negative (Fig. 1B).

In vitro experiments, using the same technique, showed that caveolin-1 is localized in the cytoplasm of numerous Leydig cells cultured for 24 h on uncoated glass coverslips (Fig. 2A and B). In Leydig cells cultured on type IV collagen (Fig. 2C) or laminin-1 (Fig. 2D), caveolin-1 was seen as a brown reaction product mainly in the cell cytoplasm with a granular pattern and on the cell surface, respectively. Leydig cells cultured for 3 h on uncoated glass coverslips reacted with anti caveolin-1 showing a positive reaction in the cytoplasm and on the cell surface, milder compared to 24 h cultures. All controls were negative (Fig. 2E). Similar results were

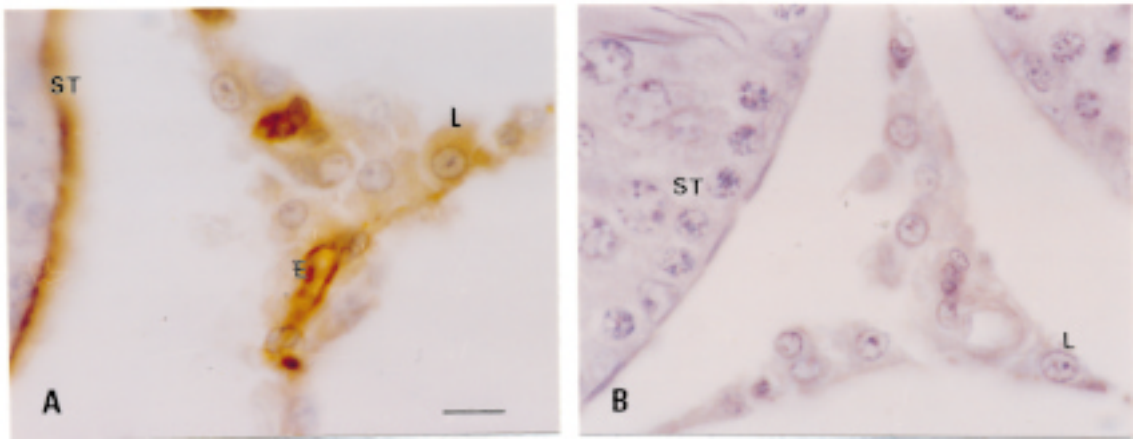

FIGURE 1. Expression of caveolin-1 on rat testis sections. Caveolin-1 was identified by immunoperoxidase technique using an anti-caveolin-1 antibody in the Leydig (L) cell cytoplasm, with different staining degrees (A). High expression of caveolin-1in endothelia (E) and in the lamina propia of seminiferous tubules (ST) is observed. No positive reaction is detected in controls (B). Scale bar represents 10 μm.

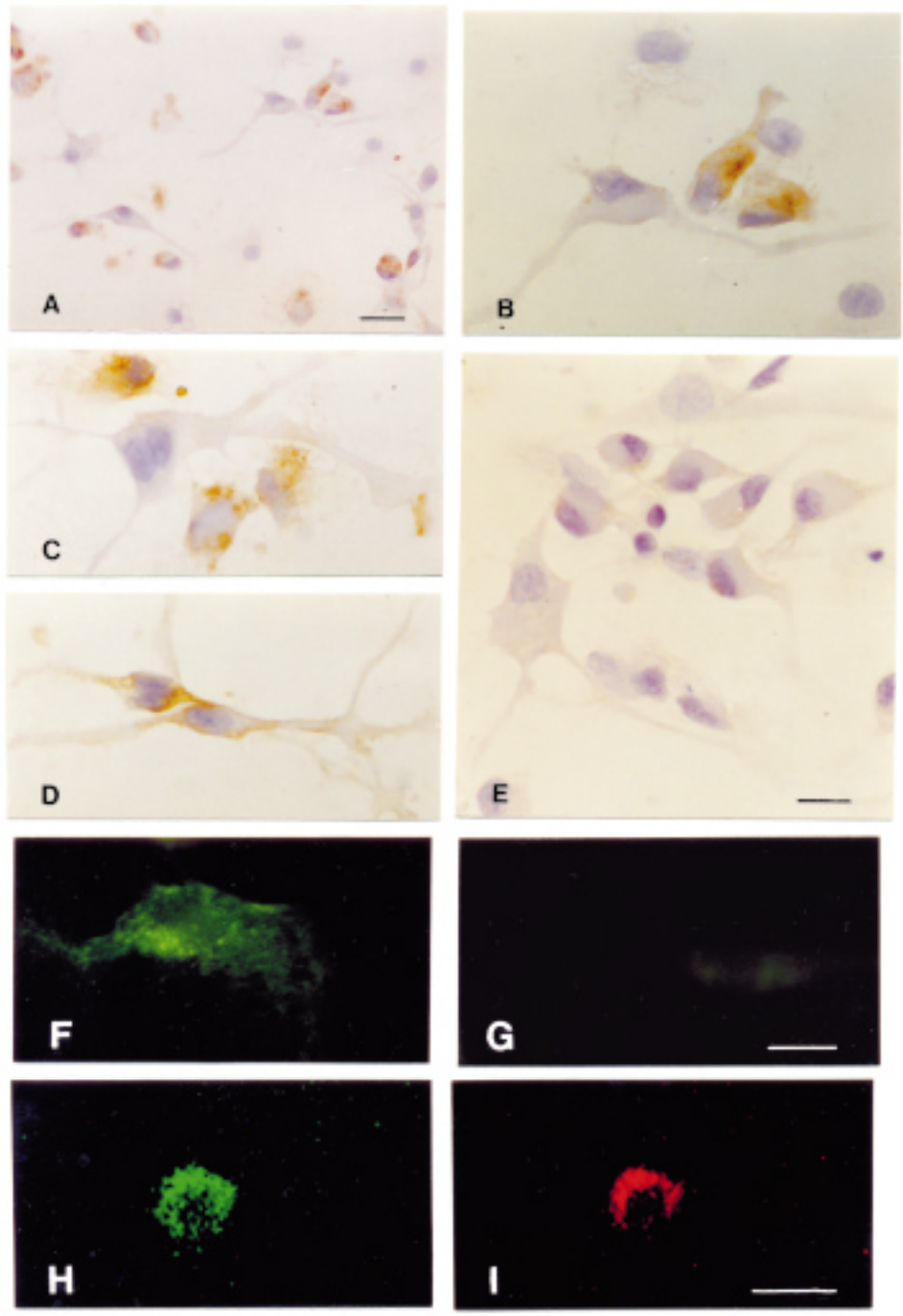

FIGURE 2. ***In vitro*** **expression of caveolin-1 in Leydig cells.** By immunoperoxidase (IP) technique, caveolin-1 was immunolocalized using an anti-caveolin-1 antibody in Leydig cells cultured for 24 h on glass coverslips uncoated (A, B) or coated with laminin-1 (D) or type IV collagen (C, E). Caveolin-1 is observed in numerous Leydig cells (A). At a higher magnification, caveolin-1 expression is observed in Leydig cell cytoplasm with a granular pattern (B, C) or in the cell surface (D). By immunofluorescence (IF), caveolin-1 was identified in the Leydig cell cytoplasm as a bright IF product with a granular pattern (F). No immune reaction was observed in IP (E) and IF (G) controls. Scale bars represent: 20 μm (A), 10 μm (B, C, D, E, F and G).
Localization of caveolin-1 (H) and 3β-hydroxysteroid dehydrogenase (I) is observed in Leydig cells cultured on uncoated glass coverslips using fluorescein isothiocyanate- and rhodamine-conjugated antibodies, respectively. Scale bar represents 10 μm.

observed with an antibody to tyrosine-phosphorylated caveolin-1 in Leydig cells cultured on uncoated or extracellular matrix protein-coated coverslips for 3 and 24 h (data not shown).

A strong positive reaction with a granular pattern was observed by immunofluorescence in the cytoplasm of Leydig cell cultured for 24 h (Fig. 2F), similar to findings obtained by immunoperoxidase technique. Controls were negative (Fig. 2G).

Localization of caveolin-1 and 3β-hydroxysteroid dehydrogenase was detected in the same cells by a double immunofluorescent technique (Fig. 2H and I), confirming the expression of caveolin-1 in Leydig cell.

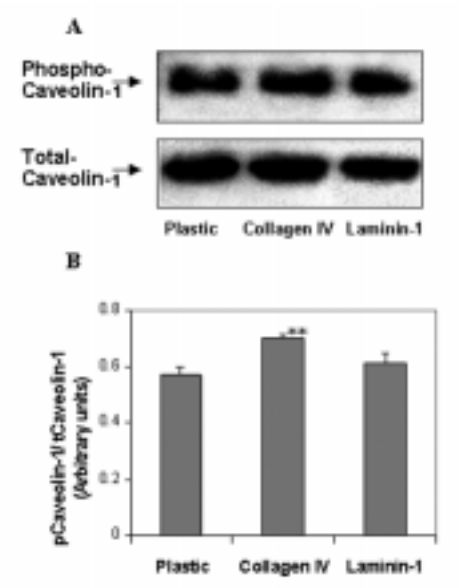

FIGURE 3. Western blot: Expression of caveolin-1 and its phosphorylated form in Leydig cells. A: Leydig cells were cultured for 90 min on plates uncoated or coated with type IV collagen or with laminin-1. Polyvinylidene difluoride membranes were probed with an antibody that recognized the phosphorylated forms of caveolin-1 with a migrating molecular mass around 22 kDa (upper panel). The same membrane was reacted with an antibody that recognizes total caveolin-1 (bottom panel). It is one representative experiment out of three.
B: Each band was quantitated by densitometry using the Scion Image and expressed as arbitrary units (phosphocaveolin-1/total caveolin). Data from three independent experiments. Means±SEM; ** $p<0.01$: collagen IV vs. uncoated plates.

The expression of caveolin-1 was also detected by Western blot analysis in Leydig cells cultured for 90 min on uncoated or laminin-1 or type IV collagen coated plates. Cell lysates reacted with antibodies to caveolin-1; a unique band with an electrophoretic mobility of about 22 kDa was observed (Fig. 3A). Caveolin-1 showed a higher degree of phosphorylation when cells were cultured on type IV collagen than when cultured on uncoated plastic plates (Fig. 3B).

Discussion

Our results show that rat Leydig cells express caveolin-1. Using immunohistochemical techniques, we identified caveolin-1 in the cytoplasm of Leydig cells in testis sections, on the cell surface and in the cytoplasm of Leydig cells cultured on uncoated or extracellular matrix protein-coated glass coverslips. Phosphocaveolin-1 was also detected in Leydig cell cultures. Caveolin-1 immunoreactivity in testis sections was weaker than that observed in Leydig cell cultures. In paraffin-embedded testis sections, caveolin-1 antigen may be partially masked, since without microwave antigen retrieval no reaction product was visible for this molecule. Labelled Leydig cells might represent only those cells with high caveolin-1 expression. The strong caveolin-1 immunoreaction we observed in the lamina propia of seminiferous tubules included peritubular myoid cells as it was recently described by Evans *et al*. (2003). These authors, however did not describe caveolin-1 presence in Leydig cells. This difference could be due to the use of a polyclonal antibody to caveolin-1 and different incubation conditions**.** We did not observe caveolin-1 in Sertoli and germ cells. Similar results were reported by Evans *et al*. (2003) in rats and by Kasahara *et al*. (2002) in human testis sections. In contrast, Travis *et al*. (2001) reported caveolin-1 expression in isolated sperm and meiotic and post-meiotic mouse germ cells. Gillot *et al*. (2005) described *in vitro* expression of caveolin-1 in plasma membranes of 15P-1 cells, a Sertoli-cell-derived line.

By Western blot analysis, we observed a band of about 22 kDa in extracts of Leydig cells when antibodies to caveolin-1 and phospho-caveolin-1 were used. The ratio of phospho-caveolin-1 to caveolin-1 in Leydig cells cultured on type IV collagen was higher than the ratio found in cells cultured on uncoated coverslips. Schwab *et al*. (2000) demonstrated the association of the three caveolin subtypes with β_1-integrin subunit in chondrocytes. Further, caveolin-1 is known to function as a membrane

adaptor linking some integrin subunits to the tyrosine kinase Fyn, resulting in activation of this kinase (Wary *et al*., 1998). Recently, the ability of Fyn to phosphorylate caveolin-1 on tyrosine 14 was demonstrated in response to both oxidative and hyperosmotic stress in fibroblastic cell lines derived from single kinase knockout mice (Sanguinetti *et al*., 2003). We previously showed (Diaz *et al*., 2002) a significant increase in Leydig cell adhesion, and in α_3, α_5 and β_1 integrin subunit expression in cells cultured on extracellular matrix proteins compared to those cultured on uncoated plates. Although no experimental data are currently available for Leydig cells, it is tempting to speculate that the increased expression of integrin subunits and the increased ratio in phospho-caveolin-1/caveolin-1 observed by Western blot in cells cultured on type IV collagen, might be functionally related events. Supporting this hypothesis, we observed that the phospho-caveolin-1/caveolin-1 ratio in cells cultured on type IV collagen decreased when cells were cultured with an antibody to β_1 integrin subunit (ES Diaz, unpublished observations).

While caveolins are clearly involved in signalling events generated at the plasma membrane level, these molecules have also been linked to cholesterol transport. Specifically, it has been reported that transport of the *de novo*-synthesized cholesterol to the plasma membrane follows a caveolin-1 dependent route (Feron *et al*., 1998). The role of caveolin-1 goes beyond cholesterol efflux, also intervening in the influx. The cell surface HDL receptor (class B, type 1 scavenger receptor, SR-B1), responsible for cholesterol uptake from HDL, co-localizes with caveolae. The association of caveolin-1 with SR-B1 in caveolae has been suggested to mediate the influx of cholesterol used for steroidogenesis in an adrenocortical cell line (Babitt *et al*., 1997). Additionally, Frank *et al*. (2002) found that cholesterol plays a direct role in stabilizing caveolin-1.

The demonstration of caveolin-1 in rat Leydig cells suggests that this protein might be involved in maintaining the cellular cholesterol balance. Further studies are needed to clarify the precise role of caveolin-1 in Leydig cell steroidogenesis.

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