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Retinoylation Reaction of Proteins in Leydig (TM-3) Cells[1]

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The covalent incorporation of [^{3}H]all-*trans*-retinoic acid into proteins has been studied in Leydig (TM-3) cells. The maximum retinoylation activity of Leydig cells proteins was 570 ± 27 fmoles/8 × 10^4 cells at 37°C. About 95% of [^{3}H]retinoic acid was trichloroacetic acid-soluble after proteinase-K digestion or after hydrolysis with hydroxylamine. Thus, retinoic acid is most probably linked to proteins as a thiol ester. The retinoylation process was inhibited by 13-*cis*-retinoic acid and 9-*cis*-retinoic acid with IC_{50} values of 0.6 and 1.2 μM respectively. Dibutyryl-cAMP and forskolin increased the retinoylation activity by 75 and 81% at 500 and 25 μM respectively. Also hCG increased the retinoylation binding activity of 110% at 250 ng/mL. After cycloheximide treatment of the Leydig cells the binding activity of [^{3}H]RA was about the same that in the control, suggesting that the bond occurs on proteins in pre-existing cells. Retinoylation was not inhibited by high concentrations of palmitic or myristic acids (500 μM); on the contrary, there was an increase of the binding activity of about 60 and 50% respectively.

KEY WORDS: TM-3; cells; protein; all-*trans*-retinoic acid; retinoylation.

INTRODUCTION

All-*trans*-retinoic acid is an active hormonal form of vitamin A. One mechanism for the activity of RA in a variety of cells involves nuclear retinoic acid receptors, RAR and RXR (Mangelsdorf *et al*., 1994; Kastner *et al*., 1995; Chambon, 1996; Wolf, 2000). The RA effect in development and cell differentiation is mediated by these receptors, by directly activating and repressing transcription of target genes by binding to specific DNA sequences. These receptors have a function similar to other members of the steroid/thyroid hormone receptor family (Evans, 1988), with specific high affinity binding sites for RA and some of its metabolites. However, in addition to binding nuclear retinoid receptors, RA is involved in other biological effects (Bolmer and Wolf, 1982; Smith *et al*., 1989; Crowe, 1993; Varani *et al*., 1996).

Retinoylation (protein acylation by RA) is a post-translational non-genomic mechanism by which RA may act on cells. The retinoylation mechanism involves the formation of a retinoyl-CoA intermediate (Wada *et al*., 2001) and the subsequent transfer and the covalent binding of the retinoyl moiety to protein(s) (Renstrom and DeLuca, 1989).

RA is incorporated into proteins of cells in culture (Takahashi and Breitman, 1989, 1990, 1994; Breitman and Takahashi, 1996; Tournier *et al*., 1996) and into proteins of rat tissues, both in vivo (Myhre *et al*., 1996) and in vitro (Renstrom and DeLuca, 1989; Myhre *et al*., 1998; Genchi and Olson, 2001; Cione and Genchi, 2004).

Several types of lipid modifications occur on many proteins. Some examples are myristoylation, palmitoylation, acetylation, phosphorylation, glycosylation and isoprenylation (Schultz *et al*., 1988; Towler *et al*., 1988). Retinoylation is one of these covalent

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Key to abbreviations: HEPES, *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethane sulphonic acid; db-cAMP, dibutyryl cyclic adenosine monophosphate; hCG, human chorionic gonadotropin; MA, myristic acid; PA, palmitic acid; RA, all-*trans*-retinoic acid; 9-*cis*-RA, 9-*cis*-retinoic acid; 13-*cis*-RA, 13-*cis*-retinoic acid; RAR, retinoic acid nuclear receptor; RXR, 9-*cis*-retinoic acid specific nuclear receptor.

modification reactions occurring on proteins. Biochemical similarities exist between retinoylation, palmitoylation and myristoylation; in fact, RA, PA and MA covalently bind to pre-existing protein via a thioester bond after the formation of a CoA-intermediate (Renstrom and DeLuca, 1989; Wada *et al*., 2001). Such binding of lipids is expected to change the physical properties of the proteins so that hydrophilic proteins can be converted into very hydrophobic ones. These modification reactions will influence the interactions of the proteins with cellular membranes, as well as the interactions with other proteins, lipids or even nucleic acids.

It is known that retinoids play an essential role in spermatogenesis in rodents. A vitamin A-deficient diet caused the cessation of spermatogenesis, loss of mature germ cells and a reduction in testosterone level in mice or rat testes (Wolbach and Howe, 1925; Ganguly *et al*., 1980; Appling and Chytil, 1981). Moreover it has been observed that treatment of adult rat Leydig cells and Leydig tumor cells with RA induced the synthesis of testosterone and progesterone (Chaudhary *et al*., 1989; Chaudhary and Stocco, 1990). The effects of all-*trans*-RA and retinol on the expression levels of genes involved in testicular steroidogenesis have been investigated, showing that the 17 α-hydroxylase/C17-20 lyase (P450 17α) and Steroidogenic Acute Regulatory Protein (StAR) mRNA levels were increased and the 3β-HSD mRNA level was downregulated while P450scc enzyme mRNA level remained constant for a 24 h period (Lefevre *et al*., 1994; Lee *et al*., 1999). These genomic effects are probably due to the action of RARα that is believed to mediate retinoid effects in the testes since RARα knockout mice showed testis degeneration as well as early postnatal lethality (Lufkin *et al*., 1993).

In the present study we describe the retinoylation reaction on protein(s) of TM-3 Leydig cell line by all-*trans*-retinoic acid. This reaction can be modulated positively by hCG through the activation of LH receptor and by activators of cAMP pathway as well as forskolin and db-cAMP; moreover, this reaction is not inhibited by lipids such as MA and PA, while it is inhibited by 13-*cis*-RA and 9-*cis*-RA. These results, demonstrating the presence of an alternative reaction of RA with protein(s) in Leydig cells, opens new perspectives on the studies investigating the mechanisms regulating the effects of retinoids in testicular steroidogenesis.

MATERIALS AND METHODS

Cell Cultures

The TM-3 cell line, derived from testes of immature BALB/c mice, was originally characterized, based on its morphology, hormone responsiveness and metabolism of steroids (Mather, 1980). This cell line was obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM/F12 medium supplemented with 2 mM glutamine, serum [5% horse serum (HS) and 5% foetal calf serum (FCS)] and 1% of a stock solution containing 10000 IU/mL penicillin and 10000 μg/mL streptomycin. Cell cultures were grown on plastic tissue culture dishes (9 cm diameter) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells from exponentially growing stock cultures were removed from the plate with trypsin (0.05% w/v) and EDTA (0.02% w/v). The trypsin/EDTA was inhibited with an equal volume of DMEM/F12 medium supplemented with serum. Cell number has been estimated with a Burker camera and cell viability by trypan blue dye exclusion. The medium was changed twice weekly, and the cells were subcultivated when confluent.

Chemicals

[11-12 ^{3}H] All-*trans*-retinoic acid (50 Ci/mmol) was purchased from Dupont-New England Nuclear. All-*trans*-retinoic acid, 13-*cis*-retinoic acid, 9-*cis*-retinoic acid, palmitic acid, myristic acid, proteinase K, hydroxylamine, db-cAMP, forskolin, hCG and cycloheximide were obtained from Sigma-Aldrich (Milano, Italia); DMEM/F12, FCS, HS, penicillin and streptomycin from Gibco (Invitrogen Life Technologies, Italia); scintillation cocktail from Packard Bioscience (Groningen, The Netherlands). All other chemicals used were of analytical reagent grade.

Incorporation of Radioactive RA

TM-3 cells growing exponentially were removed by trypsin/EDTA, harvested by centrifugation and resuspended at a concentration of 8×10^4 cells/0.5 mL in serum-free DMEM/F12 medium, supplemented with 2 mM glutamine and 1% of a stock solution containing 10000 IU/mL penicillin and 10000 μg/mL streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 h in the presence of 100 nM [^{3}H]RA (about 350000 cpm). Tritiated all-*trans*-retinoic acid and unlabelled retinoic acids (RA, 13-*cis*-RA and 9-*cis*-RA) were dissolved in absolute ethanol and diluted into the growth medium such that the final concentration was no higher than 0.1%. The cells, washed twice in PBS (phosphate-buffered saline, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136.9 mM NaCl, pH 7.2), were collected by scraping in 100 μL ice-cold lysis buffer [50 mM HEPES, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/mL

aprotinin, 50 mM phenyl methyl sulphonyl fluoride and 50 mM sodium orthovanadate]. The lysates were incubated for 30 min on ice with intermittent vortexing, followed by centrifugation in an Eppendorf centrifuge at 14000 rpm/10 min. The supernatants were treated with cold acetone and centrifuged. The pellets were washed twice in PBS, dried, solubilized in 1% SDS, 40 mM Tris, 2 mM EDTA, pH 7.5, and counted in a TriCarb 1600TR liquid scintillation counter (Packard). The counting efficiency was about 70%.

Stability of Bound RA

Evidence of covalently linked RA was obtained from experiments in which the protein of delipidated cell homogenates was dissolved in 1% SDS, containing 2 mM EDTA in 40 mM Tris, pH 7.5 (Genchi and Olson, 2001) and treated with 0.4 mg proteinase K for 60 min at 37°C. Then BSA (50–100 μg/mL) was added followed by trichloroacetic acid (TCA; 5% final concentration at room temperature). After centrifugation in an Eppendorf centrifuge at 14,000 rpm for 10 min, the radioactivity in the supernatant and in the precipitate (dissolved in 1% SDS-EDTA-Tris) was counted in a liquid scintillation counter. The dried delipidated pellet, solubilized in 100 μL 1% SDS-EDTA-Tris, pH 7.5, was incubated with 1 mM NH_2OH and with 1 M NH_2OH (adjusted to pH 10 with NaOH) for 4 and 24 h, either at 25°C or 55°C. BSA and TCA were added, as indicated above, and radioactivity was measured in the supernatant and in the precipitate.

RESULTS

Retinoylation (acylation by RA of protein) is a nongenomic mechanism by which RA may act on cells. All-*trans*-retinoic acid is incorporated into proteins of TM-3 Leydig cells with a binding activity of 570 ± 27 fmoles/8 × 10^4 cells (25 experiments). We have also performed the retinoylation reaction on TM-4 Sertoli cells with very low binding activity (data not shown).

As indicated in Table I, treatment of SDS-solubilized TCA precipitate with proteinase K at 37°C for 1 h or with hydroxylamine (1 M at 55°C for 4 h or 1 M at 25°C for 24 h) released essentially all the radioactivity (93–95%) into the supernatant solution. After 1 mM hydroxylamine treatment at 55°C for 4 h or at 25°C for 24 h, 75% [^{3}H]retinoic acid was released (Stadtman, 1957).

Figure 1 illustrates the time-dependent incorporation of 100 nM [^{3}H]RA into protein from TM-3 Leydig cells.

Table I. Release of Radioactive RA from Proteins Retinoylated by Incubation with Leydig Cells

Treatments	Radioactivity released (%)
Proteinase K (37°C/1 h)	95
NH_2OH (1 mM—55°C/4 h)	75
NH_2OH (1 M—55°C/4 h)	95
NH_2OH (1 mM—25°C/24 h)	75
NH_2OH (1 M—25°C/24 h)	93

Note. The control value of the retinoylation reaction was 575 fmoles/8 × 10^4 cells.

The level of [^{3}H]retinoylated proteins increased linearly in the first hour (170 fmoles/8 × 10^4 cells) and then continued to increase up to 24 h. After 24 h of incubation, the retinoylation was saturated with 597 fmoles of [^{3}H]RA per 8 × 10^4 cells.

The time-course of the retinoylation reaction, as shown in the insert of Fig. 1, represents an exponential approach to isotopic equilibrium, which is demonstrated by the straight line obtained by plotting the natural logarithm of the fraction of equilibrium retinoylation$_{(max)}$/(retinoylation$_{(max)}$ − retinoylation$_{(t)}$) against time. This means that the retinoylation reaction displays first-order kinetics. The first-order constant, k, extrapolated from the slope of the logarithmic plot was 0.24 h^{-1}.

The inhibiting effect of 13-*cis*-RA on the retinoylation reaction is shown in Fig. 2. As the 13-*cis*-RA concentration increased (range among 0.5–10 μM), the graph of

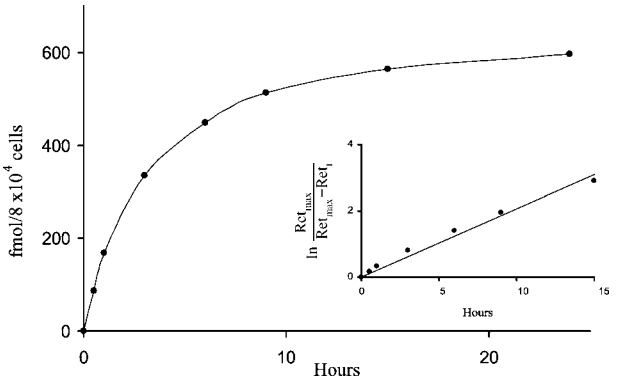

Fig. 1. Time-course of retinoylation reaction on Leydig cell proteins. TM-3 cells were plated at 8 × 10^4 cells/0.5 mL DMEM/F12 supplemented with 2 mM glutamine, 5% horse serum and 5% foetal calf serum. The cells were incubated at indicated times with [^{3}H]RA (100 nM final concentration) at 37°C in a humidified atmosphere of 5% CO_2 in air. The insert represents the logarithmic plot of saturation of retinoylation activity versus time. Retinoylation$_{(max)}$ is the maximum extent of retinoylation binding activity per 8 × 10^4 cells, while retinoylation$_{(t)}$ is the extent of retinoylation binding activity per 8 × 10^4 cells at time t. ln retinoylation$_{(max)}$/(retinoylation$_{(max)}$ − retinoylation$_{(t)}$) = kt.

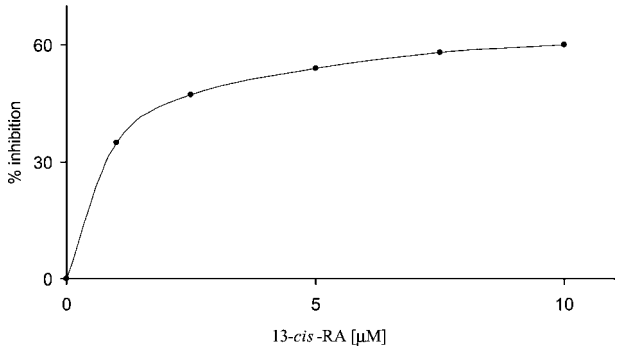

Fig. 2. Effect of 13-*cis*-RA on retinoylation reaction of TM-3 cells. The cells were incubated for 24 h at 37°C with 13-*cis*-RA at indicated concentrations in the presence of 100 nM [^{3}H]RA. Control value was 565 fmoles/8 $\times$ 10^4 cells. Other conditions as in Material and Methods.

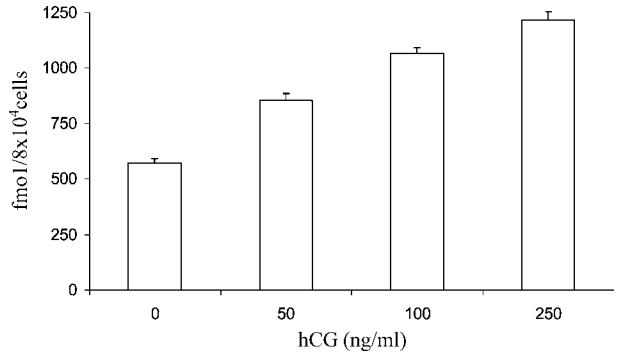

Fig. 4. Effect of hCG on retinoylation reaction. Leydig TM-3 cells (8×10^4/0.5 mL) were incubated for 24 h at 37°C with hCG at indicated concentration in the presence of 100 nM [^{3}H]RA. Control value was 575 fmoles/8 $\times 10^4$ cells. The data are the means of three experiments.

retinoylation activity *vs*. 13-*cis*-RA concentration showed hyperbolic behaviour with a plateau of about 60% inhibition at 5–10 μM 13-*cis*-RA. Similar behaviour has been obtained with 9-*cis*-RA (not shown). The IC_{50} value was 0.6 μM for 13-*cis*-RA and 1.2 μM for 9-*cis*-RA, indicating a higher affinity of 13-*cis*-RA for the retinoylating enzymatic system.

In the presence of db-cAMP (a synthetic analog of cAMP) the incorporation of [^{3}H]RA on the TM-3 proteins increased, suggesting that the retinoylation activity increased with the concentration of the cAMP in the Leydig cells. In fact, as shown in Fig. 3, the retinoylation (570 fmoles/8 $\times$ 10^4 cells in the control) increased with db-cAMP, reaching a maximum level at 250 μM (946 fmoles/8 $\times$ 10^4 cells) with a small increment at 500 μM (961 fmoles/8 $\times$ 10^4 cells).

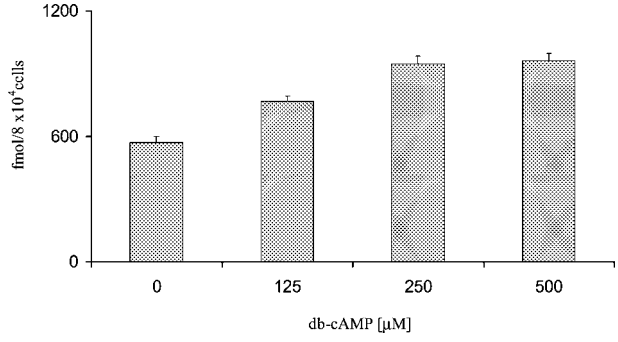

Fig. 3. Effect of db-cAMP on retinoylation reaction. TM-3 cells were incubated for 24 h at 37°C in the absence or in the presence of db-cAMP at indicated concentrations. Other conditions as in Material and Methods. Control value was 570 fmoles/8 $\times$ 10^4 cells. The data are the means of three experiments.

In the same way, forskolin, that activates the adenylate cyclase raising the cAMP intracellular concentration, should increase the incorporation of [^{3}H]RA into the proteins of TM-3 cells. In fact, in the presence of 25 μM forskolin the retinoylation activity was 1021 fmoles/8 $\times$ 10^4 cells, that is 78% higher respect to the control (572 fmoles/8 $\times$ 10^4 cells).

The increase of hCG concentration, that shows similar effects to that of LH in the regulation of reproductive function, has been studied on TM-3 binding activity of retinoic acid. As shown in Fig. 4, the retinoylation activity increased in the presence of hCG (concentration range between 0 and 250 ng/mL) with a maximum binding activity at 250 ng of 1250 fmoles/8 $\times$ 10^4 cells, that means 110% higher than the control.

In order to test whether retinoylation in growing Leydig cultures occurs on the protein in pre-existing cells or on the protein of newly formed cells, we measured the incorporation of [^{3}H]RA into the cellular proteins in the presence of cycloheximide. TM-3 cells (8×10^4/0.5 mL) were suspended in nutrient medium supplemented with 5% foetal bovine serum. Cycloheximide (4 μM final concentration) was added, and the cells were incubated for 45 min before the addition of [^{3}H]RA at 100 nM final concentration. After 24 h of incubation there was a 90% inhibition of protein synthesis and no cell growth (Olsson *et al*., 1982). The level of retinoylation (540 fmoles/8 $\times$ 10^4 cells) in the presence of cycloheximide was about the same as that seen in the control (555 fmoles/8 $\times$ 10^4 cells), suggesting that the [^{3}H]RA bond is formed on protein in pre-existing cells.

Similarities between retinoylation, myristoylation and palmitoylation (Towler *et al*., 1988), including acylation via a thioester bond and acylation of pre-formed proteins (Takahashi and Breitman, 1990), raised the question

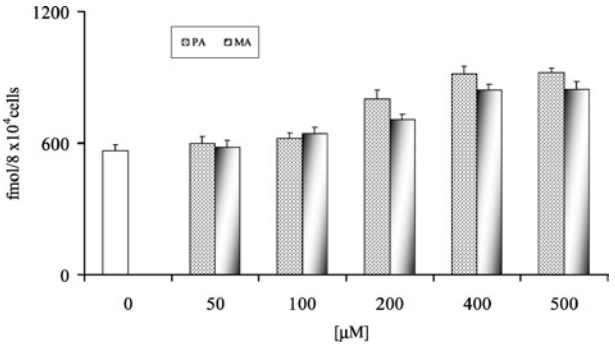

Fig. 5. Level of retinoylated protein in TM-3 cells in the presence of increasing concentrations (50–500 μM) of palmitic and myristic acids. Control value was 565 fmoles/8 $\times$ 10^4 cells. The data are the means of five experiments.

of whether the same protein(s) were substrates of these three lipids. Besides, there is the possibility that RA, PA and MA could share the same metabolic pathway, including competition for the same protein site(s). TM-3 cells were incubated under standard conditions with [^{3}H]RA (100 nM final concentration) in the presence of increasing concentrations of PA and MA (concentration range 50–500 μM). As shown in Fig. 5, at 50 μM PA and MA the retinoylation activity was about the same as that of the control (565 fmoles/8 $\times$ 10^4 cells); but this activity increased by increasing the concentration of PA and MA (100–500 μM) reaching maximum values of 900 fmoles/8 $\times$ 10^4 cells and 845 fmoles/8 $\times$ 10^4 cells at 500 μM palmitic and myristic acids.

DISCUSSION

Acylation of proteins by all-*trans*-retinoic acid (retinoylation) is a mechanism by which retinoids may act on cells non-genomically (Takahashi and Breitman, 1989; Pipkin *et al*., 1991; Takahashi *et al*., 1991a,b). Retinoylation of proteins occurs in HL-60 cells (Takahashi and Breitman, 1989), many other cell types and cellular fractions (Wada *et al*., 2001; Myhre *et al*., 1996, 1998) regulating the activity of enzymes, signaling proteins, suppressor genes, and transcription factors.

In this study, we demonstrated for the first time that the radioactive RA is able to bind covalently to TM-3 cellular proteins (after 24 h of incubation, 570 fmoles of [^{3}H]RA were bound per 8 $\times$ 10^4 cells).

After digestion with proteinase K most of radioactive RA (about 95%) was acid soluble (Table I). Treatment with NH_2OH at different concentrations released about 75–95% of the bound RA in an acid-soluble form (Table I). The sensitivity to NH_2OH is consistent with retinoylation of protein with formation of an ester bond, very probably a thioester bond (Takahashi *et al*., 1991b).

13-*Cis*-RA inhibited the binding of [^{3}H]RA in an exponentially manner until it reached a maximum value of about 60% at 10 μM concentration (Fig. 2). The IC_{50} value of this process was 0.6 μM. A similar behaviour has been obtained with the 9-*cis*-RA isomer with a IC_{50} of 1.2 μM (not shown).

The activation of LH receptors by hCG increased the retinoylation binding activity. Similar results were obtained using activators of eAMP pathway as well as db-cAMP and forskolin. These results suggest that this retinoylation reaction could be regulated by eAMP-activated enzymes. Moreover, these results suggest that the retinoylation reaction has an important physiological role in the regulation of testicular steroidogenesis.

We have found that a high rate of retinoylation occurs on protein(s) in TM-3 cells in the presence of a cycloheximide concentration that inhibits growth completely and inhibits protein synthesis by 90%. This means that retinoylation in TM-3 cells occurs on protein in pre-existing cells.

Several proteins in Leydig cells are regulated by a cAMP-dependent pathway and are involved in steroidogenesis. Among these, particularly interesting is the role of StAR protein that plays an essential role in the delivery of cytosolic cholesterol into the mitochondrial inner membrane, and is an acutely regulated and rate-limiting step for steroid hormone synthesis (Stocco, 2001). It has been demonstrated that the level of StAR mRNA in K28 mouse Leydig tumour cells was induced after the treatment with RA or 9-*cis*-RA in time- and dose-dependent manner (Lee *et al*., 1999). In the same cell line, the treatment of cycloheximide did not inhibit the induction level of StAR mRNA by retinoic acid, which indicated that de novo protein synthesis is not required for the up-regulation of the StAR mRNA levels. This observation is consistent with the report that the induced level of progesterone by RA or retinol in Leydig tumor MA-10 cells was not inhibited by cycloheximide treatment (Chaudhary and Stocco, 1990). The treatment with actinomycin D abolished the effect of 9-*cis* RA, suggesting that 9-*cis*-RA exerts its effect on the transcription of the StAR gene while RA could activated the StAR action also in an alternative way (Lee *et al*., 1999).

Our results, demonstrating the presence of an alternative reaction of RA with protein(s) in steroidogenic cells, open a new perspective on the studies investigating the mechanisms regulating the effects of retinoids in testicular steroidogenesis. Putative protein targets of retinoylation reaction could be factors involved in the complex mechanisms activating StAR protein and regulating the delivery

of cytosolic cholesterol, through other proteins cannot be excluded.

The data in Fig. 5 show that retinoylation is not inhibited by PA and MA in a high concentration range (among 50–500 μM), but on the contrary, there was an increase in the level of retinoylated protein. Therefore, these two acids could be positive effectors of retinoylation reaction. Takahashi and Breitman (1990) also have found higher retinoylation level in the presence of PA on proteins of HL-60 cells. In contrast with these results, in rat testes mitochondria (Cione and Genchi, 2004) we have found that retinoylation reaction is not inhibited by 500 μM myristic and palmitic acids. Thus, the formation of retinoylCoA, palmitoylCoA and myristoylCoA and the transfer of these three lipids to specific protein sites may occur by independent enzymatic pathways.

In conclusion, in the present study we demonstrated that RA is able to influence a retinoylation reaction on protein(s) probably involved on steroidogenesis. At present, the protein that is retinoylated is not known; but further characterization and identification of the retinoylated protein are major objectives for the immediate future.

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