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REGULATION OF EXPRESSION OF 3β-HYDROXYSTEROID DEHYDROGENASE IS MEDIATED BY cAMP IN RAT LEYDIG CELLS AND H540 RAT LEYDIG TUMOR CELLS

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Summary—Leydig cells isolated from testes of adult rats have a relatively high level of expression of 3β-hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4}$isomerase (3βHSD) in primary culture. Agents which increase the intracellular levels of cAMP such as forskolin, dibutyryl cAMP, and LH can positively regulate the expression of 3βHSD in Leydig cells *in vitro*. The effects of these agents are manifest at several levels and include increases in (1) 3βHSD activity, (2) the cellular levels of immunoreactive 3βHSD, (3) the rate of synthesis of 3βHSD, and (4) the cellular levels of 3βHSD mRNA which can be readily translated into 3βHSD *in vitro*. Two rat Leydig tumor cell lines which are steroidogenically active, H540 and R2C cells, also have a relatively high level of expression of 3βHSD. Forskolin can positively regulate the expression of 3βHSD in H540 Leydig tumor cells in which steroidogenesis is responsive to increases in intracellular cAMP, but it has no effect on 3βHSD in R2C Leydig tumor cells in which steroidogenesis is unresponsive to increases in intracellular cAMP. These results clearly support the hypothesis that cAMP mediates transcriptional regulation of 3βHSD in Leydig cells. The implication of these *in vitro* studies is that, *in vivo*, LH is required to maintain optimal levels of expression of the gene encoding testicular 3βHSD.

INTRODUCTION

3β-Hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4}$isomerase (3βHSD) is a critical enzyme in the biosynthesis of testosterone. It catalyzes the dehydrogenation and isomerization of Δ^5-3β-hydroxysteroids which include pregnenolone, an obligatory intermediate in testosterone biosynthesis, to form the more biologically active Δ^4-3-ketosteroids which include androstenedione, the immediate precursor of testosterone [1]. The importance of this enzyme to the study of testicular function is underscored by the fact that historically, 3βHSD has been the classical marker enzyme used to identify steroidogenic cells and to prove that Leydig cells are the steroidogenic cells of the testis [2]. Leydig cells of the rat testis are characterized by a relatively high level of 3βHSD activity which can be readily visualized in testicular tissue or isolated cells by a simple histochemical reaction [3]. This facile assay for 3βHSD activity requires only a 3β-hydroxysteroid as substrate, the cofactor NAD^+, and tetrazolium salts. The seminal study of Samuels and Helmreich [4] showed that testicular 3βHSD activity declines as a function of time following hypophysectomy, to levels that are approximately 25% of control values after 1 to 2 months. The reduced levels of testicular 3βHSD activity resulting from hypophysectomy could be restored to (or exceed) that found in intact control rats by daily injections of hCG. Because this effect was not observed in testicular tissues *in vitro*, and required several days of treatment *in vivo*, these investigators concluded that hCG does not directly activate the enzyme, rather it must increase testicular 3βHSD activity by increasing enzyme synthesis. More recently, it has been shown that testicular 3βHSD activity decreases markedly in rats chronically deprived of LH but not other pituitary hormones such as FSH and prolactin [5], thus confirming a role for the endogenous pituitary hormone LH in the regulation of 3βHSD activity. Although it had been established that Leydig cells specifically

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required LH to maintain normal levels of 3βHSD activity, until recently, studies into the mechanism of action of LH have been limited to those effects on enzymatic activity. The development of specific antibodies [6] and cDNA probes [7, 8] for 3βHSD has enabled new investigations into the regulation of expression of testicular 3βHSD at the molecular level. In this report, we summarize the results of our studies of 3βHSD in primary cultures of rat Leydig cells and rat Leydig tumor cells. We demonstrate that LH and effectors such as forskolin, which act via the intracellular cAMP signal transduction pathway, can positively regulate the expression of 3βHSD in Leydig cells *in vitro*. These results imply that, *in vivo*, LH is required to maintain optimal levels of expression of the gene encoding testicular 3βHSD.

METHODS

Primary cultures of Leydig cells were prepared from testes of adult male Sprague Dawley rats as described previously [9, 10]. Approximately 45% of the cells plated were Leydig cells based on a positive histochemical reaction for 3βHSD activity. Initially attached in serum, the Leydig cell cultures were maintained in serum-free culture medium supplemented with insulin (62.5 ng/ml), transferrin (6.25 μg/ml), selenous acid (6.25 ng/ml), bovine serum albumin (BSA; 1.25 mg/ml), and linoleic acid (5.35 μg/ml) (1:100 mixture of ITS$^+$ and TS$^+$ Premix; Collaborative Research, Bedford, MA, U.S.A.) and 2 mM L-glutamine. Experimental treatments were begun on day 6. Fresh culture medium and hormonal treatments were added daily. When the experimental protocol required Leydig cell cultures to be treated for various periods of time, the initiation of the experimental treatments was staggered such that all treatments were terminated at the same time. The H540 Leydig tumor was obtained from the Mason Research Institute Tumor Bank (Worcester, MA, U.S.A.) and maintained as described [11]. R2C Leydig tumor cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.).

The activity of 3βHSD in intact Leydig cells *in vitro* was measured as the rate of conversion of [3α-^{3}H]dehydroepiandrosterone to androstenedione (and its metabolites) and NAD^{3}H which is oxidized to yield ^3H_2O [12]. Leydig cell cultures were incubated for 2 h in culture medium containing 2×10^5 cpm/ml radiolabeled and 3 μM radioinert dehydroepiandrosterone as described previously [9]. Aliquots of culture medium were extracted with chloroform. Enzymatic activity was calculated from the radioactivity remaining in the aqueous phase and expressed as nmol product/10^6 cells plated per 2 h.

The steady state levels of 3βHSD were quantified by Western analysis and immunoblotting procedures described previously [9]. Equal amounts of protein/lane were size-fractionated by denaturing polyacrylamide (8%) gel electrophoresis (SDS–PAGE), transferred electrophoretically to Immobilon-P membrane (Millipore Corp., Bedford, MA, U.S.A.), and immunoblotted with a polyclonal antiserum against human placental 3βHSD [6] and [^{125}I]protein A. Newly synthesized 3βHSD was quantified in Leydig cells radiolabeled with [^{35}S]L-methionine as described previously [9]. Leydig cell cultures were incubated for 2 h in methionine-free culture medium containing [^{35}S]L-methionine (60 μCi/ml). A volume of cellular lysate representing 2×10^6 cpm that were specifically incorporated into protein was immunoprecipitated with 3βHSD antiserum. The immunoprecipitates were size-fractionated by SDS–PAGE and transferred electrophoretically to Immobilon-P membrane.

The steady state levels of 3βHSD mRNA were quantified by Northern analysis of cellular RNA and hybridization with a radiolabeled cDNA (*Eco*RI–*Hind*III restriction fragment) encoding testicular 3βHSD (rat type I) [7] using methods described previously [9]. Total RNA was size-fractionated on agarose (1%)/formaldehyde gels and transferred by capillarity to Zeta-probe membrane (Bio-Rad, Richmond, CA, U.S.A.). The amount of RNA in each lane of a gel was similar and showed no evidence of degradation based on visual inspection of ethidium bromide-stained RNA. The ability of the 3βHSD mRNA to be translated into protein was quantified using nuclease-treated, rabbit reticulocyte lysates (Promega Corp., Madison, WI, U.S.A.) as described previously [9]. 10 μg of total RNA and 40 μCi of [^{35}S]L-methionine were added to the cell-free protein synthesizing system according to the manufacturer's instructions. A volume of lysate representing 2×10^6 incorporated cpm was immunoprecipitated with 3βHSD antiserum, size-fractionated by SDS–PAGE, and transferred electrophoretically to Immobilon-P membrane. Radioactive membranes were exposed to autoradiographic film at

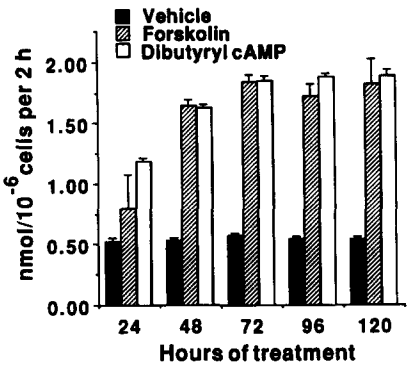

Fig. 1. Effects of forskolin and dibutyryl cAMP on 3βHSD activity in primary cultures of rat Leydig cells. Leydig cells were treated with 1 μM forskolin, 1 mM dibutyryl cAMP, or vehicle beginning on day 6 of primary culture. After 24, 48, 72, 96, and 120 h of treatment, Leydig cells were incubated an additional 2 h with [3α-^{3}H]dehydroepiandrosterone (3 μM). The data are expressed as nmol product formed/10^6 cells plated per 2 h. The bars represent the mean and SD of four observations for one pool of cells and are representative of four independent experiments.

−80 C and were also analyzed using an AMBIS Radioanalytic Imaging System (AMBIS Systems Inc, San Diego, CA, U.S.A.). The resulting data are expressed as net counts which is the total counts in the area overlying a radioactive band minus background counts in the same area.

EXPRESSION OF 3βHSD IN PRIMARY CULTURES OF RAT LEYDIG CELLS

3βHSD activity

We investigated the effects of forskolin and dibutyryl cAMP on 3βHSD activity in Leydig cells *in vitro*. These agents, which increase the intracellular levels of cAMP, increase 3βHSD activity. Leydig cells were isolated from testes of adult rats, cultured in a serum-free medium, and treated with forskolin (1 μM) or dibutyryl cAMP (1 mM) for various periods of time. At the end of the experimental treatment, Leydig cells were incubated in culture medium containing [3α-^{3}H]dehydroepiandrosterone (3 μM) to measure 3βHSD activity. The results are shown in Fig. 1. Forskolin and dibutyryl cAMP increased 3βHSD activity by 3- to 4-fold compared with vehicle-treated control cells, after 48 h of treatment. This increased level of activity was maintained from 48 through 120 h of treatment.

Immunoreactive 3βHSD

The increase in 3βHSD activity in Leydig cells treated with forskolin or dibutyryl cAMP (Fig. 1) can be accounted for quantitatively by an increase in the cellular levels of immunoreactive 3βHSD. Cellular lysates were size-fractionated on an 8% polyacrylamide gel, electroblotted onto a membrane, and immunoblotted with a polyclonal antiserum against human placental 3βHSD [6]. This antiserum recognizes a protein in the cellular lysates which appears as a single band of approx. 45 kDa when imaged on autoradiographic film. Radioactivity on the immunoblots was quantified using an AMBIS Radioanalytic Imaging System. The results shown in Fig. 2 represent Leydig cells treated with 1 μM forskolin, but

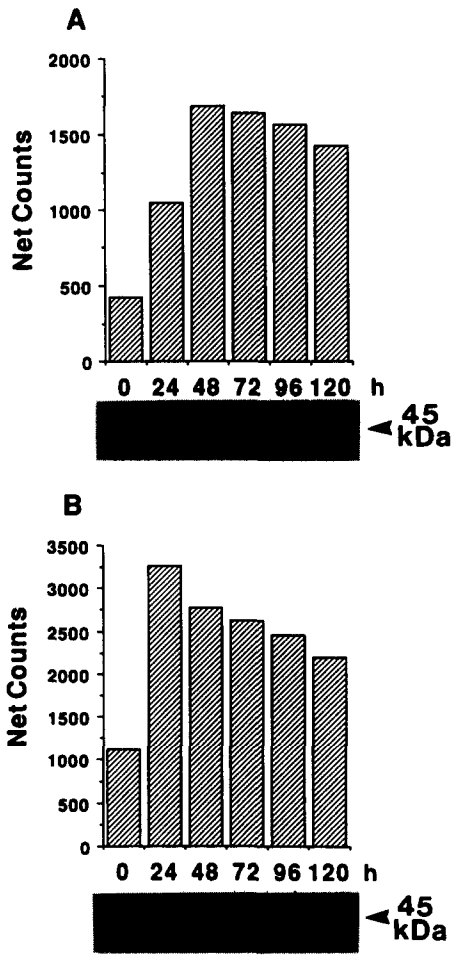

Fig. 2. Effects of forskolin on the cellular levels of immunoreactive 3βHSD in primary cultures of rat Leydig cells. Leydig cells were isolated from intact (A) or hypophysectomized (Hypox) (B) rats and treated with 1 μM forskolin for 0, 24, 48, 72, 96, and 120 h, beginning on day 6 of primary culture. The cellular lysates were size-fractionated on a polyacrylamide gel (60 μg protein/lane) and immunoblotted with a 3βHSD antiserum and [^{125}I]protein A. The bars represent the radioactivities on the immunoblots associated with 3βHSD. An autoradiographic image of the immunoblot is shown below each plot. Arrowheads indicate the apparent molecular size (45 kDa) of 3βHSD.

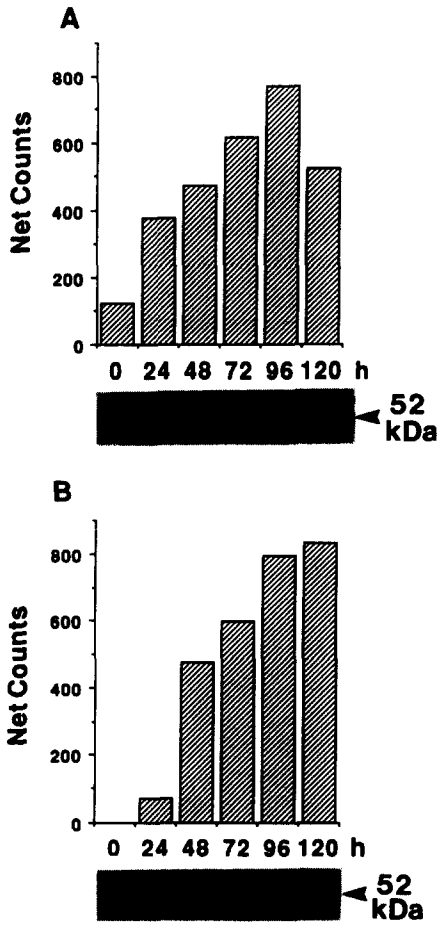

Fig. 3. Effects of forskolin on the cellular levels of immunoreactive $P450_{scc}$ in primary cultures of rat Leydig cells. Leydig cells were isolated from intact (A) or hypophysectomized (Hypox) (B) rats and treated with 1 μM forskolin for 0, 24, 48, 72, 96, and 120 h, beginning on day 6 of primary culture. These results were obtained by reprobing the immunoblots in Fig. 2 with a $P450_{scc}$ antiserum and [^{125}I]protein A. The bars represent the radioactivities on the immunoblots associated with $P450_{scc}$. Counts in the zero (control) lane were undetectable in B. An autoradiographic image of the immunoblot is shown below each plot. Arrowheads indicate the apparent molecular size (52 kDa) of $P450_{scc}$.

similar results were obtained using dibutyryl cAMP [9]. Forskolin increased the levels of immunoreactive 3βHSD in Leydig cells by 3- to 4-fold, compared with control cells. This result was obtained when the Leydig cells were isolated from testes of intact control rats [Fig. 2(A)] or rats that had been hypophysectomized 4 weeks previously [Fig. 2(B)]. In both cases, the cellular levels of immunoreactive 3βHSD increased maximally after 24 to 48 h, and remained elevated through 120 h, of treatment. Although the levels of 3βHSD appear greater in Leydig cells of hypophysectomized compared with intact rats (Fig. 2), such comparisons between experiments cannot be made confidently because (1) differences in the density of Leydig cells among cell preparations, i.e. from testes of intact versus hypophysectomized rats, can affect the apparent concentration of 3βHSD and (2) differences in the duration of detection and activity of the isotope affect the number of counts detected on a given membrane. We have shown previously that the increase in immunoreactive 3βHSD in Fig. 2 can be explained by an increase in the apparent rate of synthesis of 3βHSD [9]. This was demonstrated by measuring the rate of synthesis of 3βHSD in Leydig cells using the experimental paradigm in Fig. 2. At the end of the treatment period, Leydig cells were incubated an additional 2 h with [^{35}S]L-methionine, and the cellular lysates were immunoprecipitated with the 3βHSD antiserum. The cellular pool of newly synthesized 3βHSD increased by 5- to 9-fold in Leydig cells treated with forskolin or dibutyryl cAMP, compared with control cells [9].

The effects of forskolin and dibutyryl cAMP to increase the cellular levels and the rates of

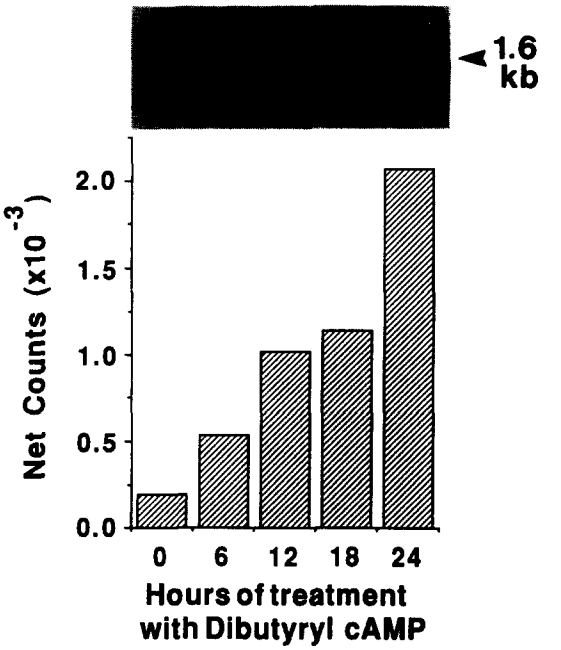

Fig. 4. Effect of dibutyryl cAMP on the cellular levels of 3βHSD mRNA in primary cultures of rat Leydig cells. Leydig cells were treated with 1 mM dibutyryl cAMP for 0, 6, 12, 18, and 24 h, beginning on day 6 of primary culture. Cellular RNA was isolated, size-fractionated on an agarose/formaldehyde gel (6 μg/lane), and hybridized with a radiolabeled cDNA encoding testicular 3βHSD (rat type I). The amount of RNA in each lane of the gel was similar and showed no evidence of degradation based on visual inspection of ethidium bromide-stained RNA. The bars represent the radioactivities on the Northern blot associated with 3βHSD mRNA. An autoradiographic image of the blot is shown above the plot. The arrowhead indicates the apparent molecular size (1.6 kb) of 3βHSD mRNA.

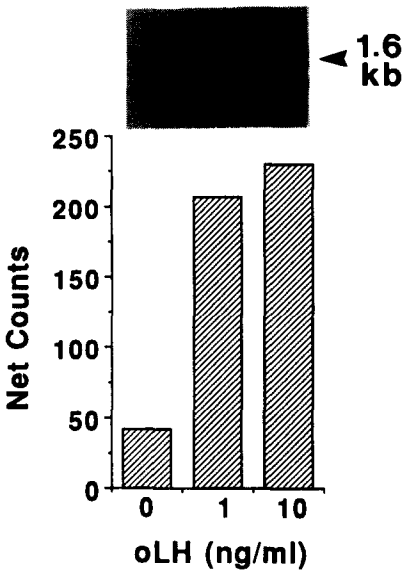

Fig. 5. Effects of oLH on the cellular levels of 3βHSD mRNA in primary cultures of rat Leydig cells. Leydig cells were treated with 0, 1, or 10 ng/ml oLH for 24 h, beginning on day 6 of primary culture. Cellular RNA was isolated, size-fractionated on an agarose/formaldehyde gel (10 μg/lane), and hybridized with a radiolabeled cDNA encoding testicular 3βHSD (rat type I). The amount of RNA in each lane of the gel was similar and showed no evidence of degradation based on visual inspection of ethidium bromide-stained RNA. The bars represent the radioactivities on the Northern blot associated with 3βHSD mRNA. An autoradiographic image of the blot is shown above the plot. A band appeared in the zero (control) lane when the blot was overexposed to autoradiographic film. The arrowhead indicates the apparent molecular size (1.6 kb) of 3βHSD mRNA.

synthesis of 3βHSD can be attributed to the ability of these agents to activate the intracellular cAMP signal transduction pathway. This signalling pathway is involved in regulating the expression of other steroidogenic enzymes, namely those containing cytochrome *P*450 [13]. If these effects on 3βHSD are mediated by cAMP, then other steroidogenic enzymes should be affected as well. Since it had been reported that dibutyryl cAMP and hCG increase the rate of synthesis of cholesterol side chain cleavage cytochrome *P*450 ($P450_{scc}$) in rat Leydig cells [10], the immunoblots in Fig. 2 were reprobed using a $P450_{scc}$ antiserum. The results in Fig. 3 show that the basal levels of immunoreactive $P450_{scc}$ were low in Leydig cells of intact control rats [Fig. 3(A)] and undetectable in those of hypophysectomized rats [Fig. 3(B)]. The levels of immunoreactive $P450_{scc}$ increased markedly in Leydig cells treated with forskolin (Fig. 3) or dibutyryl cAMP (not shown).

3βHSD mRNA

Several agents which increase the intracellular levels of cAMP, including forskolin, dibutyryl cAMP, cholera toxin, and LH, increase the levels of 3βHSD mRNA in primary cultures of rat Leydig cells [9]. We have shown previously [9] that these effects are dose-and time-dependent using Northern blot analysis in which cellular RNA was hybridized with a radiolabeled cDNA encoding testicular 3βHSD (rat type I) [7]. The Northern blot in Fig. 4 shows that dibutyryl cAMP (1 mM) increased by 10-fold 3βHSD mRNA in Leydig cells after 24 h of treatment. Luteinizing hormone is the endogenous trophic hormone required by Leydig cells to maintain their fully differentiated structure and function [14]. Leydig cells in primary culture respond to LH with an 8- to 10-fold increase in the amount of testosterone secreted into the culture medium (not shown). This response, which is mediated by the second messenger cAMP [15], indicates that the Leydig

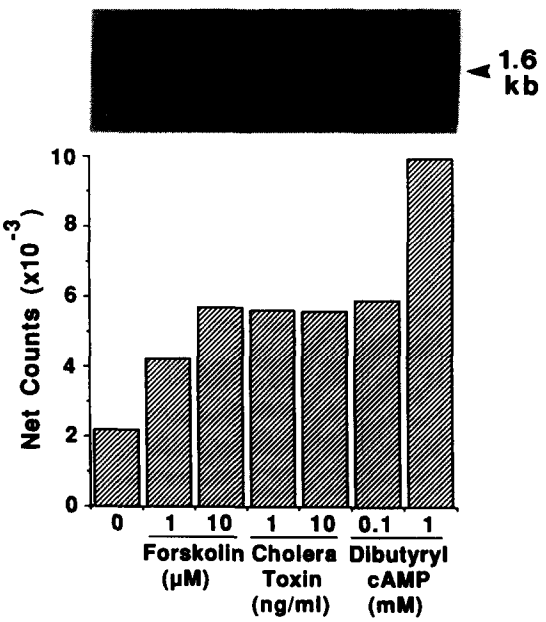

Fig. 6. Effects of forskolin, cholera toxin, and dibutyryl cAMP on the cellular levels of 3βHSD mRNA in H540 rat Leydig tumor cells. H540 Leydig tumor cells were passaged *in vitro* and treated with 1 or 10 μM forskolin, 1 or 10 ng/ml cholera toxin, and 0.1 or 1 mM dibutyryl cAMP for 12 h. Cellular RNA was isolated, size-fractionated on an agarose/formaldehyde gel (10 μg/lane), and hybridized with a radiolabeled cDNA encoding testicular 3βHSD (rat type I). The amount of RNA in each lane of the gel was similar and showed no evidence of degradation based on visual inspection of ethidium bromide-stained RNA. The bars represent the radioactivities on the Northern blot associated with 3βHSD mRNA. An autoradiographic image of the blot is shown above the plot. The arrowhead indicates the apparent molecular size (1.6 kb) of 3βHSD mRNA.

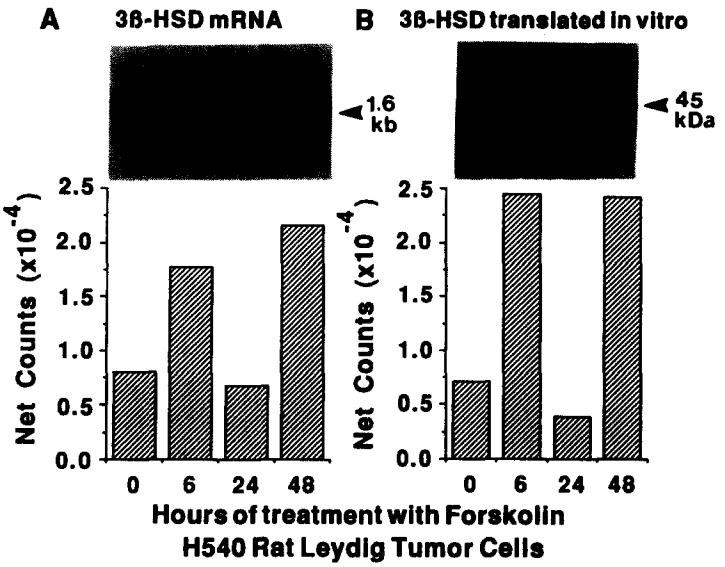

Fig. 7. Effects of forskolin on 3βHSD mRNA in H540 rat Leydig tumor cells, and its translation *in vitro*. H540 Leydig tumor cells were passaged *in vitro* and treated with 1 μM forskolin for 0, 6, 24, and 48 h. (A) Cellular RNA was isolated, size-fractionated on an agarose/formaldehyde gel (15 μg/lane), and hybridized with a radiolabeled cDNA encoding testicular 3βHSD (rat type I). (B) Rabbit reticulocyte lysates were programmed with 10 μg RNA (same as in A) in the presence of [^{35}S]L-methionine. The translation products (2×10^6 cpm) were immunoprecipitated with a 3βHSD antiserum and size-fractionated on a polyacrylamide gel. The bars represent the radioactivities associated with 3βHSD mRNA on the Northern blot (A) and immunoreactive 3βHSD translated *in vitro* (B). Autoradiographic images are shown above the plots; arrowheads indicate the apparent molecular size of 3βHSD mRNA (1.6 kb) (A) and immunoreactive 3βHSD (45 kDa) (B).

cells have functional LH receptors which can activate the cAMP signalling pathway. In Leydig cells treated with oLH, the cellular levels of 3βHSD mRNA increased 5-fold compared with control cells (Fig. 5). We have shown previously that in a cell-free, *in vitro* translation system, 3βHSD mRNA was readily translated into immunoreactive 3βHSD [9]. Leydig cell RNA was used to program rabbit reticulocyte lysates in the presence of [^{35}S]L-methionine. In that study, forskolin increased 3βHSD mRNA in cultured Leydig cells and the magnitude of the increase was comparable to the increase in immunoreactive 3βHSD which was immunoprecipitated from the *in vitro* translation products of forskolin-treated Leydig cells. Thus, the increase in 3βHSD mRNA in response to LH and effectors such as forskolin and dibutyryl cAMP and the ability of the 3βHSD mRNA to be translated *in vitro* support the hypothesis that cAMP can mediate transcriptional regulation of 3βHSD in Leydig cells *in vitro*.

EXPRESSION OF 3βHSD IN RAT LEYDIG TUMOR CELLS

We investigated the expression of 3βHSD in Leydig tumor cells that are steroidogenically active: H540 and R2C rat Leydig tumor cells. H540 Leydig tumor cells are similar to their normal counterparts in that steroidogenesis in these cells is positively regulated by the cAMP signal transduction pathway [16]. A major difference is that they express high levels of 5α-reductase activity and thus, secrete principally 5α-androstan-3,17-dione instead of testosterone [11]. R2C Leydig tumor cells are highly steroidogenic, but they differ from normal Leydig cells in two respects: (1) they have a high constitutive level of steroid secretion which is not stimulated by cAMP, and (2) they secrete principally progesterone and 20α-hydroxyprogesterone [17]. Despite these differences, both Leydig tumor cells have relatively high levels of 3βHSD activity and provide useful models to study the regulation of 3βHSD.

H540 rat Leydig tumor cells retain 3βHSD activity when passaged *in vitro* in an equal mixture of Dulbecco's Modified Eagle's and Ham's F12 culture media containing 5% bovine calf serum. The expression of 3βHSD in these cells is responsive to agents that increase intracellular cAMP. Figure 6 shows that forskolin, cholera toxin, and dibutyryl cAMP increase the cellular levels of 3βHSD mRNA by 2- to 5-fold after 12 h of treatment. In a timecourse study,

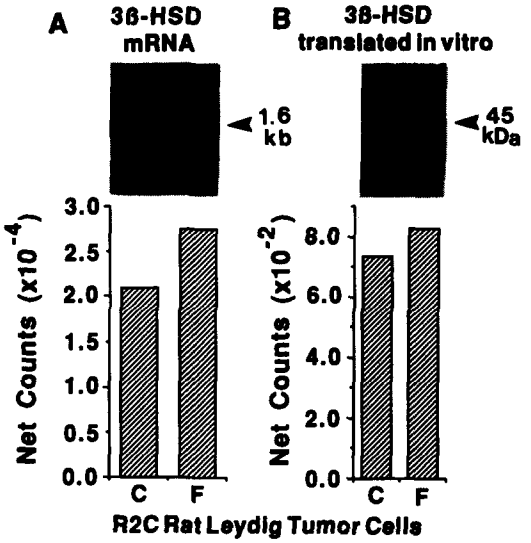

Fig. 8. Effects of forskolin on 3βHSD mRNA in R2C rat Leydig tumors cells, and its translation *in vitro*. R2C Leydig tumor cells were treated with 25 μM forskolin (F) for 24 h; Control cells (C) were treated with vehicle. (A) Cellular RNA was isolated, size-fractionated on an agarose/formaldehyde gel (30 μg/lane), and hybridized with a radiolabeled cDNA encoding testicular 3βHSD (rat type I). (B) Rabbit reticulocyte lysates were programmed with 10 μg RNA (same as in A) in the presence of [^{35}S]L-methionine. The translation products (2×10^6 cpm) were immunoprecipitated with a 3βHSD antiserum and size-fractionated on a polyacrylamide gel. The bars represent the radioactivities associated with 3βHSD mRNA on the Northern blot(A) and immunoreactive 3βHSD translated *in vitro* (B). Autoradiographic images are shown above the plots; arrowheads indicate the apparent molecular size of 3βHSD mRNA (1.6 kb) (A) and immunoreactive 3βHSD (45 kDa) (B).

forskolin had a biphasic effect on the expression of 3βHSD (Fig. 7). The significance of this biphasic effect is unclear, although it was repeatable and independent of whether the culture medium was changed after 24 h. The increased levels of 3βHSD mRNA in the forskolin-treated cells [Fig. 7(A)] paralleled the increased levels of immunoreactive 3βHSD produced when the RNA was translated *in vitro* [Fig. 7(B)]. These results for H540 Leydig tumor cells contrast with those obtained for R2C Leydig tumor cells in which steroidogenesis is unresponsive to increases in cAMP. Forskolin had little effect on the cellular levels of 3βHSD mRNA in R2C cells after 24 h of treatment [Fig. 8(A)]. Similar results were obtained when R2C cells were treated with cholera toxin for 12 h or with forskolin for various periods of time up to 48 h (not shown). When RNA from the R2C cells [Fig. 8(A)] was translated *in vitro*, the amount of immunoreactive 3βHSD in the translation products was similar for control and forskolin-treated cells [Fig. 8(B)]. Thus, the expression of 3βHSD is positively regulated by forskolin in H540 Leydig tumor cells in which steroidogenesis is responsive to increases in intracellular cAMP and apparently unregulated (at least by forskolin) in R2C Leydig tumor cells in which steroidogenesis is unresponsive to increases in intracellular cAMP. These results for Leydig tumor cells and those described for rat Leydig cells in primary cultures clearly support a key role for cAMP in the transcriptional regulation of testicular 3βHSD. Further proof, however, requires the identification of regulatory sequences in the gene encoding testicular 3βHSD that are responsible for these cAMP-mediated effects.

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