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THE TRANSMETHYLATIONS INHIBITOR 3-DEAZAADENOSINE, INHIBITS *IN VITRO* TESTOSTERONE PRODUCTION BY RAT TESTIS INTERSTITIAL CELLS STIMULATED WITH HCG

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Summary—3-Deazaadenosine (3-DZA), an inhibitor of somatic cell transmethylations, inhibited *in vitro* HCG-stimulated testosterone synthesis by rat testis interstitial cells. A maximal inhibition of 50% was observed with 100 μM 3-DZA; in addition homocysteine-thiolactone (Hcy) enhanced the inhibitory effect of 3-DZA. On the other hand, when cells were stimulated with dibutyryl cyclic AMP $(Bt)_2$-cAMP, 3-DZA did not exert any effect on the stimulation. The presence of 3-DZA in the incubation medium neither modified HCG K_d values nor the number of its binding sites to Leydig cells. These results demonstrate that inhibitors of transmethylation reactions interfere with hormone-stimulated testosterone synthesis, suggesting that those reactions (presumably phospholipid methylation) at the plasma membrane level are involved in hormone-stimulated testosterone synthesis by rat Leydig cells.

INTRODUCTION

Testosterone synthesis by rat testis Leydig cells is stimulated by LH/HCG and cAMP [1]. LH/HCG acts via a plasma membrane receptor-mediated mechanism which involves adenylate cyclase activation. cAMP analogs are able to by-pass this membrane mechanism [2]. It has been suggested that external signal transduction through biological membranes, involves phospholipid methylation reactions [3–5]. In this pathway *S*-adenosyl-methionine (SAM) donates the methyl groups for the enzymatic formation of phosphatidylcholine from phosphatidylethanolamine via synthesis of phosphaditil-*N*-monomethylethanolamine and phosphadidyl-*N*-*N*-dimethylethanolamine. It is also known that SAM-dependent transmethylations may be specifically inhibited with 3-deazaadenosine (3-DZA) [6–7]; and the effect of 3-DZA may be potentiated by homocysteine-thiolactone (Hcy) [8].

Notwithstanding that SAM-dependent transmethylations have been related to a wide range of membrane associated phenomena [3, 5], it is still undefined whether those reactions are involved in the transduction of the LH/HCG signal through the plasma membranes of target cells. Here we present evidence obtained by analyzing the effect of 3-DZA on isolated interstitial cells of rat testis under HCG or dibutiryl cyclic AMP ($(Bt)_2$-cAMP) stimulation, that relates transmethylation reactions to testicular steroidogenesis.

EXPERIMENTAL

Materials

Medium 199 (M-199) was obtained from Grand Island Biological Corporation, (Grand Island, NY). [^{125}I]HCG (49 μCi/μg) was purchased from New England Nuclear Corp. (Boston, MA). 3-DZA was from Southern Research Institute (Birmingham, AL). Collagenase was obtained from Worthington Biochemical Co. (Freehold, NJ). The testosterone RIA kit was supplied by the WHO. Other chemicals were obtained from Sigma Chemical Co. (St Louis, MO), and were of analytical reagent grade.

Glass distilled water, further purified by a Milli Q-3 System (Millipore) was utilized throughout the experiments, and had a specific resistance of 18 megohms/cm.

Preparation of interstitial cells

Decapsulated testes of adult Wistar rats were dispersed with collagenase according to the method of Dufau *et al.*[9]. After treatment, interstitial cells were finally suspended in Medium 199–0.1% Bovine serum albumin (M-199–0.1% BSA). Total nucleated cell concentration was determined by counting aliquots in a haemocytometer and viability was evaluated before and after the incubation period by the Trypan blue exclusion method; the cell viability was 88–90% after the incubation period.

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Testosterone production measurement

Approximately 2×10^6 cells were incubated in 1 ml final volume of M-199–0.1% BSA at 34°C, in a humidified atmosphere of 5% CO_2–95% O_2. Incubations were carried out in siliconized plastic vials under continuous shaking at 60 cycles/min. The effect of transmethylation reaction inhibitors was tested as follows: cells were preincubated for 10 min in 0.95 ml of M-199–0.1% BSA in the absence or the presence of appropriate concentrations of inhibitor (3-DZA and/or Hcy). After that period, cells were stimulated to produce testosterone by addition of either 10 mIU HCG (in 50 μl of M-199–0.1% BSA) or 20 mM $(Bt)_2$-cAMP 2 mM 1-methyl-3-isobutyl-xanthine (MIX) [in 50 μl of M-199–0.1% BSA]. After 3 h incubation, cell suspensions were quantitatively transferred to glass tubes and placed in a boiling water bath for 3 min; tubes were then centrifuged 10 min at 1000 *g* and supernatants stored at −20°C until assaying testosterone by radioimmunoassay; the sensitivity of this assay was less than 12 pg and the interassay coefficient of variation of the standard samples was 13.5%.

[^{125}I]hCG binding measurements

Cells (5×10^5 in 0.2 ml final incubation volume) were incubated with 0.1–8 ng of [^{125}I]HCG in the presence or absence of 100 μM 3-DZA. After 90 min incubation using the conditions described above, 1.5 ml of cold M-199–0.1% BSA was added, and cells were separated by centrifugation. Pellets were washed once and separated and counted in a gamma radiation spectrometer. Non-specific binding for each [^{125}I]HCG concentration was measured from parallel incubations in the presence of a 500-fold excess of non-labeled HCG. Maximal binding capacity and dissociation constants were calculated using the Scatchard method [10]. Data were corrected according to the method of Rosenthal [11].

RESULTS

Effect of 3-DZA on testosterone production by interstitial cells

Since transmethylation reactions of membrane phospholipids have been involved in the transduction of membrane–receptor interactions; we analyzed this possibility indirectly by studying the effect of an inhibitor of those reactions: 3-deazaadenosine. However, since this compound eventually could act through an adenosine receptor, it was necessary to exclude the possibility that adenosine modifies HCG action. In our *in vitro* system 100 μM adenosine did not modify either basal or HCG stimulated testosterone production (0.91 ± 0.2 ng/10^6 cells and 4.1 ± 0.25 ng/10^6 cells, respectively, $n = 3$).

The presence of 3-DZA during the incubation, inhibited HCG-stimulated testosterone production and this effect of 3-DZA was dose-dependent (Fig. 1). It may be observed that a 50% inhibition of HCG-stimulated testosterone synthesis is obtained with doses higher than 50 μM ($P < 0.01$). Basal testosterone production (cells not stimulated) was 0.5 ng/10^5 cells for controls and 0.43 ng/10^6 cells for 3-DZA treated cells. When cells were stimulated with HCG, testosterone production increased to 6.13 ± 0.79 ng/10^6 cells in control cells, and only to 3.4 ± 0.81 ng/10^6 cells in 3-DZA treated cells, ($n = 3$).

In Table 1 the potentiating effect of Hcy on the inhibitory effect of 3-DZA is shown. One-hundred μM Hcy alone caused a very low inhibition; a 100% inhibition was observed, however, with higher Hcy concentrations (1 mM, data not shown).

Action of 3-DZA on the stimulation of steroidogenesis

Figure 2 compares the effect of the inhibitor on testosterone synthesis stimulated either by HCG or by the intracellular mediator of the hormonal signal.

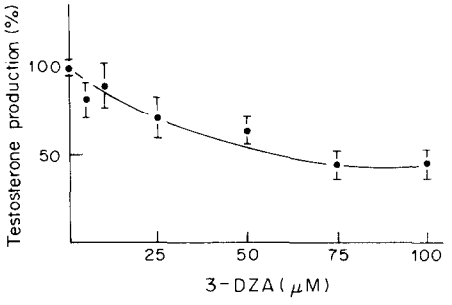

Fig. 1. Dose dependent 3-DZA inhibition of HCG-stimulated testosterone production by rat testis interstitial cells. Aliquots of approx 2×10^6 cells were preincubated in the presence of various 3-DZA concentration as described in the Experimental section. After preincubation, cells were incubated with 10 mIU of HCG during 3 h. Results are the mean ± SEM of 3 experiments.

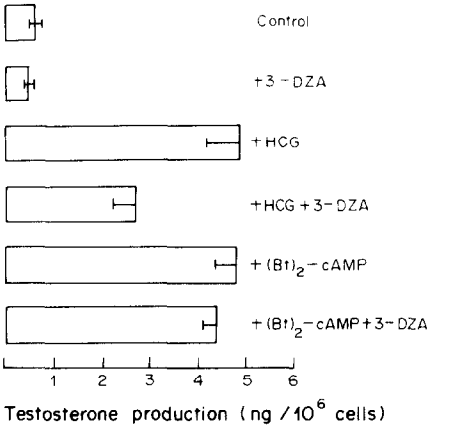

Fig. 2. Comparison of 3-DZA-effect on testosterone synthesis stimulated either by 10 mIU of HCG or 20 mM $(Bt)_2$-cAMP, 2 mM MIX, by interstitial cells. Aliquots of approx 2×10^6 cells were treated as described in the Experimental section. Results are the mean ± SEM of 4 experiments.

Table 1. Effect of 3-DZA and Hcy on HCG-stimulated testosterone production by interstitial cells

Incubation condition*	Testosterone production ng/10^6 cells	Inhibition (%)
HCG (10 mIU)	4.1 ± 0.33	0.0
HCG (10 mIU) + 3-DZA (100 μM)	2.15 ± 0.23†	47.6 ± 5.6
HCG (10 mIU) + 3-DZA (100 μM) + Hcy (100 μM)	1.6 ± 0.1†	61 ± 2.4
HCG (10 mIU) + Hcy (100 μM)	3.7 ± 0.16	9.8 ± 3.9

*Cells were incubated for 3 h with the indicated additions. Basal testosterone production was 0.66 ± 0.22 ng/10^6 cells and was not affected by incubation with Hcy, 3-DZA or Hcy + 3-DZA. Results are the mean ± SEM of 3 experiments.
†Significantly lower ($P < 0.01$) than HCG (10 mIU) value.

As in the former experiment 3-DZA clearly inhibited HCG-stimulated steroidogenesis, however the inhibitor did not prevent the stimulatory action of $(Bt)_2$-cAMP on testosterone synthesis.

Effect of 3-DZA on HCG binding parameters

To analyze whether 3-DZA modifies the interaction of HCG with its membrane receptors, dissociation constant and the number of binding sites were analyzed both in the absence and in the presence of the inhibitor.

The values of K_d and binding sites (n) were not significantly different for both experimental conditions. ($K_d = 3.8 \pm 0.04 \times 10^{-10}$ M; $n = 4.76 \pm 0.36$ fmol/10^6 cells for control cells and $K_d = 3.91 \pm 0.5 \times 10^{-10}$ M; $n = 3.6 \pm 0.2$ fmol/10^6 cells, for 3-DZA treated cells). A characteristic [^{125}I]HCG binding analysis is shown in Fig. 3.

DISCUSSION

Rat Leydig cells increase testosterone synthesis subsequently to LH/HCG or cAMP analog stimulation [1]; the former stimulators act through a plasma membrane receptor-mediated mechanism involving adenylate cyclase activation, while cAMP analogs by-pass the membrane events [2].

In Leydig cells, membrane phenomena involving interactions between hormone, receptors and adenylate cyclase have been exhaustively analyzed, however the relationship of membrane phospholipid to those events has not been studied in any detail. In other cellular systems, it has been suggested that SAM-dependent phospholipid transmethylation reactions may be involved in the transduction of external signals through the plasma membrane [3, 4]. In this paper we infer the involvement of transmethylation reactions with membrane HCG-signal-transduction, by analyzing the effect of inhibitors of those reactions on testosterone synthesis.

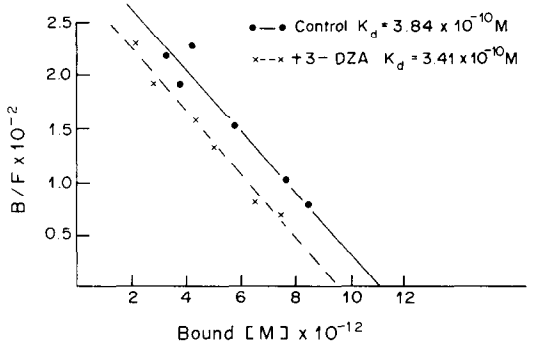

Fig. 3. Scatchard plot of [^{125}I]HCG binding to interstitial cells. Aliquots of approx 5 × 10^5 cells were incubated with increasing concentrations of [^{125}I]HCG as described in the Experimental section, in the presence (× --- ×) or in the absence (○——○) of 100 μM 3-DZA.

In our experimental system we observed in the presence of 3-DZA, a clear dose-dependent inhibition of HCG-stimulated steroidogenesis. In addition, Hcy enhanced the inhibitory effect of 3-DZA. 3-DZA is a particularly effective inhibitor of phospholipid methylation and both, 3-DZA and Hcy inhibit somatic cell methylation of proteins, nucleic acids and phospholipids by more than one mechanism involving competitive inhibition [6, 7].

The fact that the inhibition attained was around 50% of the stimulated value, notwithstanding that 3-DZA dose ranged from 50 to 100 μM, could be explained by the competitive nature of the inhibitor, although further investigations are needed to clarify this assumption. With regard to this, similar observations have been reported by Milvae *et al.*[12], who observed in bovine luteal cells, that the addition of 3-DZA inhibited by 50% the stimulatory effect of LH on progesterone production.

On the other hand, we excluded the possibility that 3-DZA would act through an adenosine receptor, which has been described in gonadal tissues [13–15], although not in Leydig cells [14]. Our results demonstrate that adenosine itself did not exert any effect either on basal nor on HCG-stimulated testosterone production. These results agree with the observations of Rommerts *et al.*, in cultured Leydig cells. They observed that freshly prepared Leydig cells cannot be stimulated by adenosine; suggesting that development of an adenosine responsiveness can be due to prolonged *in vitro* conditions [15].

The differential effect of 3-DZA on steroidogenesis stimulated either by HCG or $(Bt)_2$-cAMP, strongly suggests that the inhibitory effect of 3-DZA would be related to an early membrane associated event, involving enzymatic transmethylations; those events would be by-passed by the cAMP analog. Although we cannot exclude an inhibitory effect of 3-DZA on protein and/or nucleic acid transmethylations, this

possibility seems unlikely, since a full steroidogenic response is attained in the presence of the inhibitor, by stimulation with the intracellular hormone mediator analog. Notwithstanding, recently it has been reported that 3-DZA and Hcy inhibited steroidogenesis in murine interstitial cells, and that such inhibition was not by-passed by Bt_2cAMP [16]. Since these observations are in an abstract form, the experimental conditions are not fully specified which make it difficult to compare those results with ours.

From another point of view it has been shown in lymphocytes that 3-DZA increases cAMP levels, pointing out that a direct inhibitory effect of 3-DZA on adenylate cyclase is highly unlikely [17].

Finally from the analysis of HCG binding parameters, we conclude that the inhibition induced by 3-DZA on HCG-stimulated steroidogenesis does not involve a hindrance to hormone–receptor interaction.

At the present time it is difficult to define the role of phospholipid transmethylation reactions in hormone action. In Leydig cells, Nieto and Catt demonstrated that phospholipid methyltransferase (PMT) activity may be stimulated by HCG, cholera toxin and 8-Br-cAMP, concluding that a cAMP-dependent mechanism would be involved in the stimulation of the enzyme [18]. On the other hand, Hirata *et al.*, suggested a relationship between PMT activity and β-adrenergic receptor state (occupied or unoccupied). Moreover, phospholipid methylation increased the lateral mobility of the hormone–receptor complex, facilitating the coupling of the complex to adenylate cyclase [19]. The latter interpretation is in accordance with our results, which strongly suggest that SAM-dependent transmethylation reactions at the plasma membrane level (most likely phospholipid methylations), are an important early step related to the mechanism by which HCG stimulates adenylate cyclase and subsequent testosterone production in Leydig cells.

Note added in proof—While this work was under revision, Moger reported similar findings in murine interstitial cells [16]. Furthermore he demonstrated that 3-DZA inhibited methyl incorporation into phospholipids of rat Leydig cells [20].

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