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Proliferative Effect of Histamine on MA-10 Leydig Tumor Cells Mediated Through HRH2 Activation, Transient Elevation in cAMP Production, and Increased Extracellular Signal-Regulated Kinase Phosphorylation Levels[1]

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ABSTRACT

Mast cells (MC) occur normally in the testis with a species-specific distribution, yet their precise role remains unclear. Testicular MC express histidine decarboxylase (HDC), the unique enzyme responsible for histamine (HA) generation. Evidence to date supports a role for HA as a local regulator of steroidogenesis via functional H_1 and H_2 receptor subtypes (HRH1 and HRH2, respectively) present in Leydig cells. Given that HA is a well-known modulator of physiological and pathological proliferation in many different cell types, we aimed in the present study to evaluate whether HA might contribute to the regulation of Leydig cell number as well as to the control of androgen production. Herein, we demonstrate, to our knowledge for the first time, that MA-10 Leydig tumor cells, but not normal immature Leydig cells (ILC), exhibit a proliferative response upon stimulation with HA that involves HRH2 activation, transient elevation of cAMP levels, and increased extracellular signal-regulated kinase (ERK) phosphorylation. Our results also reveal that MA-10 cells show significantly heightened HDC expression compared to normal ILC or whole-testicular lysate and that inhibition of HDC activity decreases MA-10 cell proliferation, suggesting a possible correlation between autocrine overproduction of HA and abnormally increased proliferation in Leydig cells. The facts that germ cells are also both source and target of HA and that multiple testicular cells are susceptible to HA action underline the importance of the present study, which we hope will serve as a first step for further research into regulation of non-MC-related HDC expression within the testis and its significance for testicular function.

cyclic adenosine monophosphate (cAMP), histamine, reproductive immunology, signal transduction, testis

INTRODUCTION

Mast cells (MC) occur normally in the testis with a species-specific distribution [1–4], yet knowledge about their function and precise repertoire of secretory products remains limited [5]. Apart from the proteases chymase and tryptase [6–8], testicular MC express histidine decarboxylase (HDC), the unique enzyme responsible for histamine (HA) generation [5]. Several studies over the past few years have provided evidence supporting a role for HA as a local regulator of Leydig cell steroidogenesis. First, Mayerhofer et al. [2] reported that HA stimulates steroid biosynthesis and potentiates the effects of luteinizing hormone (LH) in golden hamster testicular parenchyma. Our group further described a dual concentration-dependent effect of HA on acute steroidogenesis in MA-10 murine Leydig cells and in purified rat Leydig cells [9, 10], and a similar biphasic action was later demonstrated by Khan and Rai [11] in Leydig cells of the wall lizard (*Hemidactylus flaviviridis*). Accordingly, functional H_1 and H_2 histamine receptor subtypes (HRH1 and HRH2, respectively) are present in Leydig cells of several species, including human [2, 5, 9–11].

Of particular interest, Gaytan et al. [12] have described simultaneous proliferation and differentiation of MC and Leydig cells in the rat testis, suggesting that dynamic interactions might occur between the two cell types during testicular development. Also, findings by Zieher [13] indicate that the normal HA concentration in the adult rat testis is significantly lower than that in the neonatal gonad. In line with this evidence, HDC-deficient mice (homozygous for the $Hdc^{tm1Nagy}$ allele) show reduced testis weight and altered steroidogenesis compared with wild-type mice [14]. On the basis of these reports and considering the well-documented role of HA as modulator of physiological and pathological cell proliferation in diverse experimental models [15], we speculated that testicular HA might contribute to the regulation of Leydig cell number as well as to the control of androgen production. Herein, we describe, to our knowledge for the first time, that MA-10 Leydig tumor cells show HRH2-mediated proliferative response to HA and overexpress HDC, as opposed to normal Leydig cells, which have a very low level of HDC expression and manifest proliferative unresponsiveness to HA. Taken collectively, our results will surely arouse interest in a potential role of HA as an autocrine-paracrine regulator of Leydig cell proliferation under testicular pathological conditions.

MATERIALS AND METHODS

Materials

Purified human chorionic gonadotropin (hCG; CR-127; 14 900 IU/mg) was a gift from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of

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Health (NIH). [^{3}H]thymidine (20 Ci/mmol) and Na^{125}I (600 Ci/mmol) were purchased from New England Nuclear. Histamine dihydrochloride, HRH2-selective agonist amthamine (AMTH), HRH1 agonist 2-(3-trifluoromethylphenyl)histamine (FMPH), 2′-*O*-monosuccinyladenosine-3′,5′-cyclic monophosphate tyrosyl methyl ester (TME-cAMP), dibutyryl adenosine 3′,5′-cyclic monophosphate (db-cAMP), 3-isobutyl-1-methylxanthine (IBMX), forskolin (FSK), epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), Hepes, PMSF, sodium orthovanadate, leupeptin, aprotinin, pepstatin A, spermidine, spermine, sodium fluoride (NaF), 1,4-diamino-2,3-dicyano-1,4-*bis*-(*o*-aminophenylmercapto)butadiene ethanolate (U0126), and mouse monoclonal anti-β tubulin antibody were purchased from Sigma-Aldrich Chemical Co. Collagenase type II (CLS-2; 125 IU/mg) was from Worthington Biochemical Co. Cell-culture supplies were obtained from Gibco-BRL, and plasticware was obtained from Corning and BD Falcon. TME-cAMP was radiolabeled with Na^{125}I in our lab by the method of chloramine-T as described by Piroli et al. [16]. The specific antibody for cAMP was provided by Dr. A.F. Parlow (National Hormone and Pituitary Program, NIDDK). Rabbit polyclonal anti-HDC antibody was commercially obtained from Santa Cruz Biotechnology. Mouse monoclonal anti-phosphorylated extracellular signal-regulated kinase (pERK) and rabbit polyclonal anti-extracellular signal-regulated kinase (ERK) antibodies were a gift from Dr. Marta Tesone (Ovarian Physiology Laboratory, Institute of Biology and Experimental Medicine, Buenos Aires, Argentina). Secondary antibodies, peroxidase-labeled goat anti-rabbit immunoglobulin (Ig) G and peroxidase-labeled horse anti-mouse IgG, were purchased from Vector Laboratories. Other reagents used were of the best grade available and were obtained from commonly used suppliers.

Culture of MA-10 Leydig Tumor Cells

The MA-10 mouse Leydig tumor cell line [17, 18] was a generous gift from Dr. Mario Ascoli (University of Iowa, Iowa City, IA). MA-10 cells were grown in Dulbecco modified Eagle medium/Ham F-12 (DMEM/F12) containing 4.76 g/L of Hepes, 1.2 g/L of sodium bicarbonate, 1 ml/L of Gentamicin Reagent Solution (Gibco, Invitrogen), and 15% horse serum (growth medium) at 37°C and 5% CO_2 incubator. All experiments described herein were performed in triplicate, with at least three different cell line batches or passage numbers of cells, and similar results were obtained each time.

Preparation of Progenitor and Immature Rat Leydig Cells

Progenitor Leydig cells (PLC) and immature Leydig cells (ILC) were isolated from a pool of 16 testes obtained from eight Sprague-Dawley rats (age, 18–20 days for PLC and 35 days for ILC; Charles River descendants; Animal Care Laboratory, Institute of Biology and Experimental Medicine) by collagenase dispersion and Percoll gradient centrifugation as previously described [9, 19, 20]. The procedures used were approved by the Institutional Animal Care and Use Committee (Institute of Biology and Experimental Medicine–CONICET), which follows NIH guidelines. The purity of the Leydig cells obtained was greater than 91% as assessed by histochemical staining for 3β-hydroxysteroid dehydrogenase activity [21]. All experiments were repeated with at least three different preparations of Leydig cells obtained from different sets of animals. The concentration of hCG or insulin-like growth factor-1 (IGF-1) used (100 ng/ml) was chosen empirically, as the lowest concentration that elicits the maximum proliferative effect (data not shown).

[^{3}H]Thymidine Incorporation into DNA

Cell proliferation was assessed as a function of [^{3}H]thymidine incorporation as described previously [22]. MA-10 cells (2.5×10^4 cells/well) were seeded and cultured in 96-well plates for 24 h, and then growth medium was replaced with starvation medium (serum-free medium). Cells were incubated in these conditions for 24 h and then treated with HA, AMTH, FMPH, FSK, db-cAMP, or EGCG as indicated in each figure. Twenty hours after initiation of treatment, [^{3}H]thymidine (0.25 μCi/ml) was added, and the incubation was continued for another 4 h. PLC or ILC (7×10^4 cells/well) were seeded and cultured in M199 containing 1.2 g/L of $NaHCO_3$, 20 mM Hepes, 1 ml/L of Gentamicin Reagent Solution, and 0.5% fetal bovine serum in 96-well plates coated with gelatin for 18 h, after which medium was changed to fresh medium and cells were treated with hCG, IGF-1, HA, AMTH, or db-cAMP as indicated in each figure. Eight hours after initiation of treatment, [^{3}H]thymidine (0.25 μCi/ml) was added, and the incubation was continued for another 16 h. For all cell types (MA-10 cells, PLC, and ILC), each treatment was terminated by washing the cells twice with ice-cold PBS (15 sec/wash). Subsequently, cells were trypsinized and harvested by vacuum aspiration onto glass-fiber filters, which were washed (5 sec/wash), dried, and transferred to glass vials containing scintillation cocktail (Optiphase HiSafe 3 scintillation liquid; Wallac) for measurement of radioactivity (TriCarb 1600TR; Packard). All data points are the average of three experiments, and each experiment was performed in octuplicate.

MTS Cell Proliferation Assay

The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] assay, a colorimetric method for determining viable (metabolically active) cells, was used to assess proliferation in HA- or AMTH-treated MA-10 cells compared to controls. Cell proliferation was determined spectrophotometrically by measuring the conversion of the MTS substrate to the formazan product, which is directly proportional to the number of viable cells. In brief, following treatment with the corresponding stimuli as described above, MTS reagent (20 μl/well; CellTitre 96 Aqueous One Solution Cell Proliferation Assay kit; Promega Corporation) was added to control and treated cells, and the optical density (OD) at 490 nm was recorded after 1 h of incubation at 37°C using a microplate reader. Relative cell numbers were determined based on the OD of the samples. Blank values were subtracted from each well of the treated cells and controls; and the mean and SEM for each treatment were calculated relative to the control as follows:

$$\%\ \text{of viable cells} = \frac{A_T - A_B}{A_C - A_B} \times 100,$$

where A_C = absorbance of control cells (mean value), A_T = absorbance of treated cells (mean value), and A_B = absorbance of blank wells (mean value).

Determination of Intracellular cAMP in MA-10 Leydig Cells

The method for the determination of intracellular cAMP in the MA-10 cell line has been previously published [9, 23, 24]. For the time course of cAMP accumulation assays performed herein, MA-10 cells were incubated in DMEM/F12 containing 4.76 g/L of Hepes, 1.2 g/L of sodium bicarbonate, and 1 ml/L of Gentamicin Reagent Solution, in the absence of IBMX, and exposed to the indicated stimuli for various periods of time.

Western Blot Analysis and Immunodetection of Proteins

Total cellular protein was obtained by placing MA-10 cells, PLC, or ILC in lysis buffer (10 mM Tris-HCl [pH 8], 1% Triton X-100, and 0.5 mM ethyleneglycoltetra-acetic acid) containing a protease inhibitor cocktail (5 μg/ml of leupeptin, 5 μg/ml of pepstatin A, 5 μg/ml of aprotinin, 25 mM NaF, 400 μM PMSF, 0.15 mM spermine, 0.5 mM spermidine, and 25 mM sodium orthovanadate), followed by brief sonication (10 cycles of 10 sec). Protein concentrations were measured using the Bradford assay (Bio-Rad Laboratories, Inc.) for total protein. Equal amounts of protein per sample (30–40 μg) were then boiled for 5 min in sample buffer (0.06 M Tris-HCl [pH 6.8], 25% v/v glycerol, 2% w/v SDS, 0.01% w/v bromophenol blue, and 5% v/v β-mercaptoethanol), subjected to SDS-PAGE, and transferred to nitrocellulose (Bio-Rad Laboratories). Membranes were blocked in 5% non-fat dried milk in PBS-T and incubated overnight with either polyclonal anti-HDC antibody or monoclonal anti-pERK antibody at 4°C. After three washes (10 min at room temperature) in PBS-T (PBS + 0.1% Tween-20), membranes were subjected to a 2-h incubation with the appropriate secondary antibody. Detection was performed with ECL Plus Western Blotting Detection Reagents (Amersham, GE Healthcare BioScience Corporation). The intensity of immunospecific bands was quantified using ImageJ software (NIH) [25]. To correct for equal loading, HDC blots were stripped and reprobed for β-tubulin, and pERK blots were stripped and reprobed for total ERK.

Statistical Analysis

All experiments performed herein were repeated at least three times, and the data were pooled. If heterogeneity of variance was detected by Bartlett test, this was reduced by logarithmic transformation of the data before analysis. These data were then subjected to either Student *t*-test or one-way ANOVA followed by Newman-Keuls test for multiple-range comparisons. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

HA Stimulates MA-10 Cell Proliferation via HRH2

MA-10 Leydig tumor cells were incubated with increasing concentrations of HA (0.1 nM to 10 μM) for 24 h, and [^{3}H]thymidine incorporation was determined as a measure of cell proliferation. As shown in Figure 1A, HA stimulated MA-

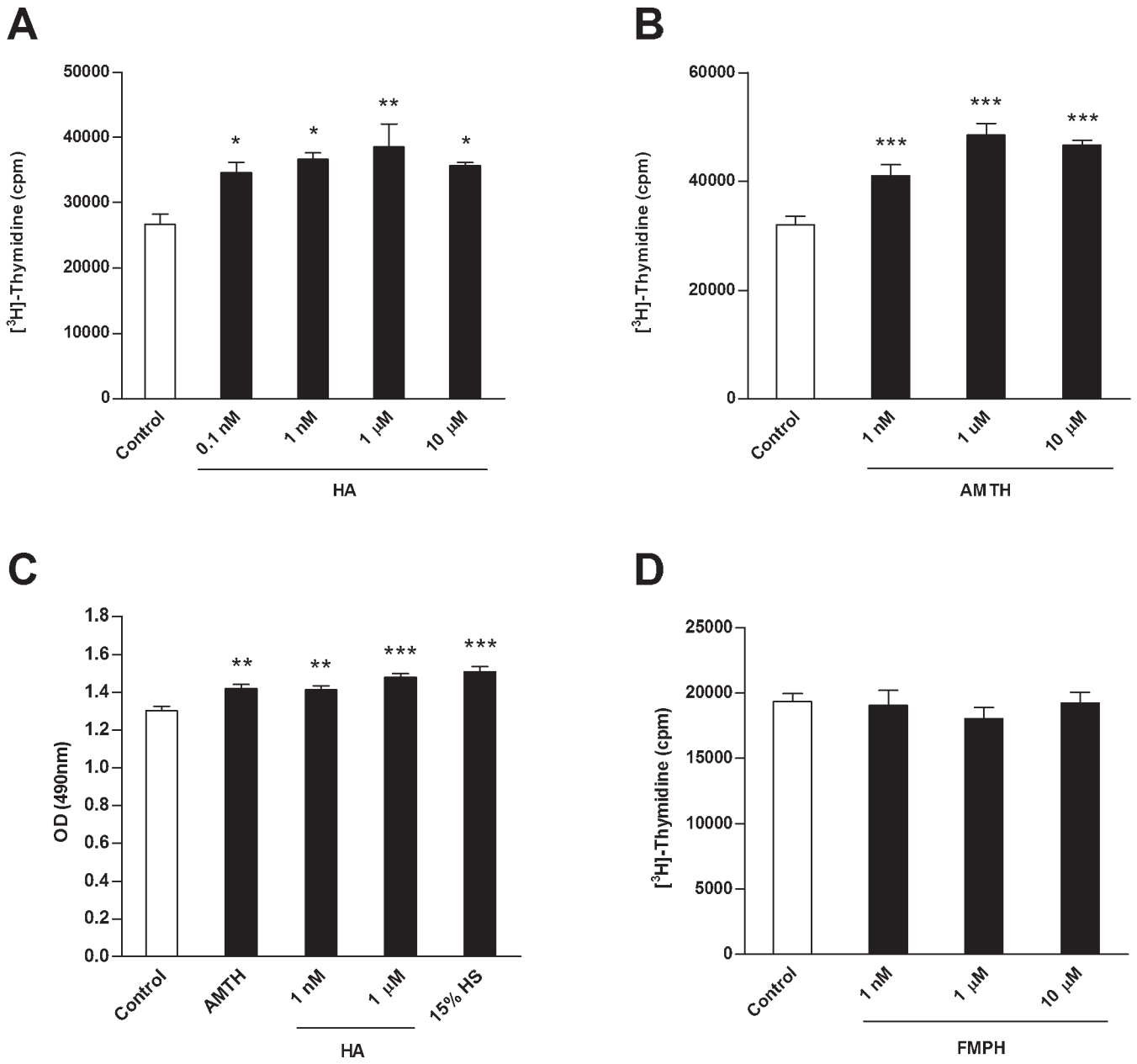


FIG. 1. Effects of HA and AMTH on MA-10 cell proliferation. **A**) MA-10 cells were incubated with increasing concentrations of HA (0.1 nM to 10 μM) for 24 h as indicated. The cells were labeled with [^{3}H]thymidine during the last 4 h of this incubation, and the radioactivity incorporated into DNA was measured as described in *Materials and Methods*. Each bar represents the mean ± SEM of three independent experiments. **B**) MA-10 cells were incubated with increasing concentrations of AMTH (1 nM to 10 μM) for 24 h as indicated. The cells were labeled with [^{3}H]thymidine during the last 4 h of this incubation, and the radioactivity incorporated into DNA was measured as described in *Materials and Methods*. Each bar represents the mean ± SEM of three independent experiments. **C**) MA-10 cells were incubated with HA (1 nM or 1 μM) or AMTH (1 μM) for 24 h as indicated. MTS reagent (20 μl/well) was then added, and the OD at 490 nm was recorded after 1 h of incubation at 37°C using a microplate reader. Horse serum at 15% (15% HS) was used as positive control. The percentage of viable cells is shown. **D**) MA-10 cells were incubated with increasing concentrations of FMPH (1 nM to 10 μM) for 24 h as indicated. The cells were labeled with [^{3}H]thymidine during the last 4 h of this incubation, and the radioactivity incorporated into DNA was measured as described in *Materials and Methods*. Each bar represents the mean ± SEM of three independent experiments. No significant differences were observed between control and treated cells at any FMPH concentration evaluated. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. control.

10 cell proliferation in a concentration-dependent manner, with a maximum effect observed in the presence of 1 μM HA (44% stimulation on average). In a previous publication [9], we demonstrated the involvement of HRH2 in the mechanism of HA-mediated acute stimulation of steroidogenesis in Leydig cells. Thus, to evaluate the participation of such receptor subtype in the induction of MA-10 cell proliferation by HA, cells were incubated with increasing concentrations (1 nM to 10 μM) of the specific HRH2 agonist AMTH for 24 h, and [^{3}H]thymidine incorporation was determined. As observed for HA, AMTH significantly stimulated MA-10 cell proliferation. Maximum stimulation (51% on average) was seen in the presence of 1 μM AMTH (Fig. 1B). Consistent results were obtained when assessing cell proliferation indirectly with the MTS colorimetric assay (Fig. 1C). Finally, to explore the possibility that HA-mediated stimulation of MA-10 cell

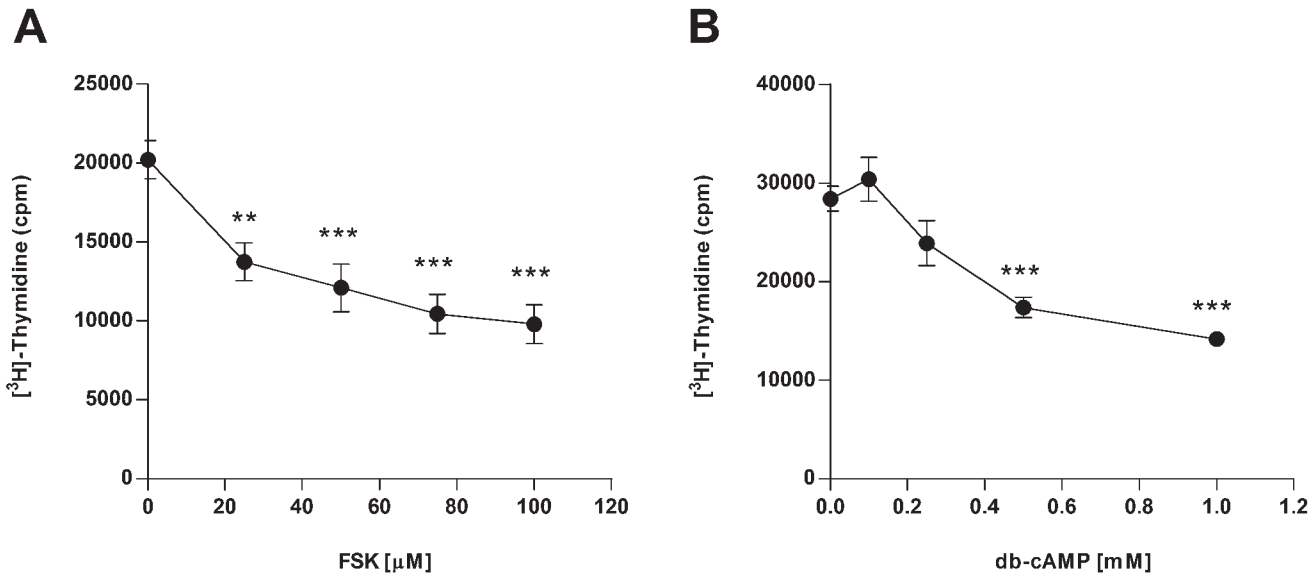


FIG. 2. Effects of FSK and db-cAMP on MA-10 cell proliferation. **A**) MA-10 cells were incubated with increasing concentrations of FSK (25 μM to 100 μM) for 24 h as indicated. The cells were labeled with [^{3}H]thymidine during the last 4 h of this incubation, and the radioactivity incorporated into DNA was measured as described in *Materials and Methods*. **B**) MA-10 cells were incubated with increasing concentrations of db-cAMP (0.1 mM to 1 mM) for 24 h as indicated. The cells were labeled with [^{3}H]thymidine during the last 4 h of this incubation, and the radioactivity incorporated into DNA was measured as described in *Materials and Methods*. Each data point represents the mean ± SEM of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

proliferation might also involve HRH1 activation, cells were incubated with increasing concentrations (1 nM to 10 μM) of the specific HRH1 agonist FMPH for 24 h, and [^{3}H]thymidine incorporation was determined. FMPH had no effect on the proliferation of MA-10 cells (Fig. 1D).

HA-Induced Stimulation of MA-10 Leydig Cell Proliferation via HRH2 Is Mediated by a Transient Increase in Intracellular cAMP Levels

The HRH2 couples to adenylyl cyclase (AC) via the GTP-binding protein G_s in a wide variety of cell types [26–29], including MA-10 cells and rat Leydig cells [10, 17]. Therefore, it was of interest to evaluate the involvement of AC, and its product cAMP, in HA-induced stimulation of MA-10 cell proliferation. Cells were incubated with FSK (direct stimulator of AC, 25–100 μM) or db-cAMP (a membrane-permeable cAMP analog, 0.1–1 mM) for 24 h at 37°C, and [^{3}H]thymidine incorporation was measured. Intriguingly, both FSK and db-cAMP inhibited MA-10 cell proliferation in a concentration-dependent manner (Fig. 2). Thus, in subsequent experiments, we compared the time course of AMTH- versus FSK-elicited cAMP accumulation. Both AMTH and FSK raised cAMP levels rapidly and significantly (Fig. 3). However, cAMP accumulation stimulated by AMTH was transient and promptly declined to basal level (within 15 min), whereas cAMP accumulation induced by FSK was more sustained. We then evaluated the effect of a short-term treatment (5–60 min) with 0.5 mM db-cAMP on MA-10 cell proliferation, this time observing a significant increase in [^{3}H]thymidine incorporation after 24 h, corresponding to a 10- to 15-min pulse of db-cAMP stimulation (Fig. 4A). In accordance, further experiments in which cAMP degradation was prevented by addition of the phosphodiesterase inhibitor IBMX (0.1 mM) to the cells 15 min after initiation of HA or AMTH treatment converted the proliferative effects of both HA and AMTH to anti-proliferative ones (Fig. 4, B and C, respectively).

HRH2 Activation Increases pERK 1/2 Levels in MA-10 Leydig Cells

Considering that the ERK 1/2 pathway has been implicated as a critical component of the proliferation and survival of Leydig cells [30, 31], we evaluated the capacity of AMTH to

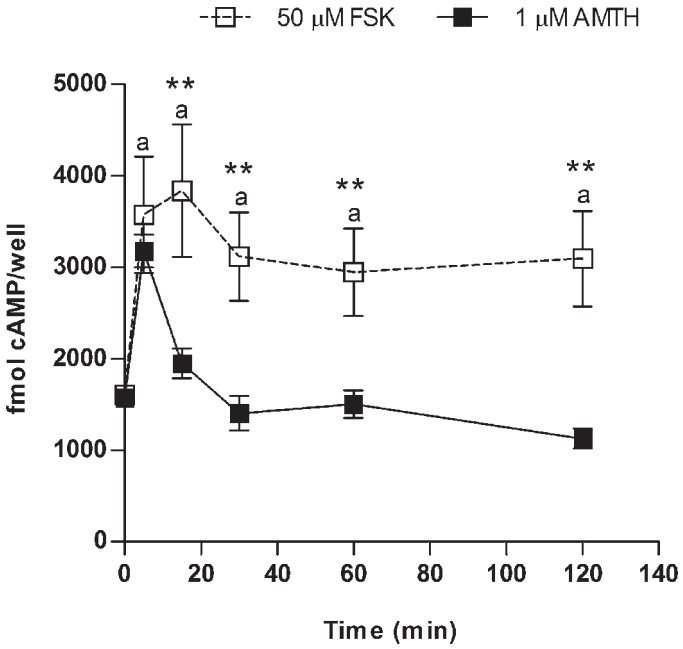
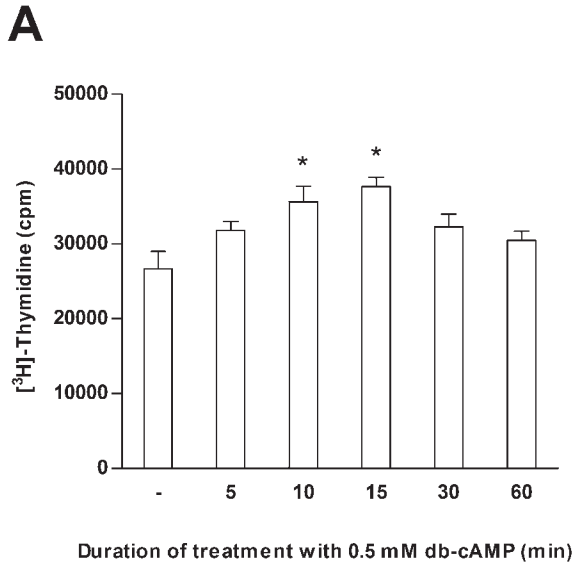
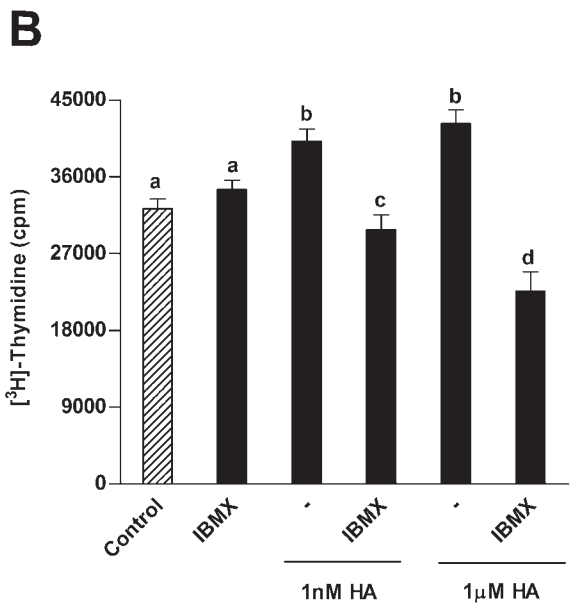
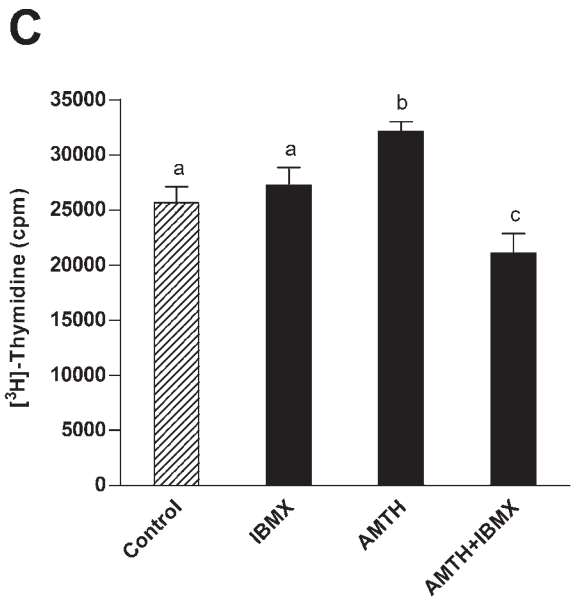


FIG. 3. Time course of AMTH- and FSK-induced cAMP production in MA-10 cells. MA-10 cells were incubated with 1 μM AMTH or 50 μM FSK in the absence of IBMX for the indicated periods of time, and cAMP levels were determined as described in *Materials and Methods*. Each data point represents the mean ± SEM of three independent experiments performed with triplicate samples. The same letter above the data points within the curve for FSK denotes that the values are not significantly different. $^{**}P < 0.01$ vs. corresponding values for AMTH treatment.







modulate ERK 1/2 phosphorylation levels. To examine the time course of ERK 1/2 activation, MA-10 cells were subjected to AMTH stimulation for various times, and cell lysates were collected for immunoblot analysis using specific anti-pERK 1/2 and anti-ERK 1/2 antibodies as described in *Materials and Methods*. Figure 5, A and B, shows that AMTH induced a rapid and transient increase in the phosphorylation of ERK 1/2. To confirm the participation of these kinases in the mechanism of HA action via HRH2, MA-10 cells were incubated with the MEK (mitogen-activated protein kinase kinase) 1/2 inhibitor U0126 (10 μM) 10 min before AMTH stimulation, and pERK 1/2 levels were assayed by Western blot analysis after 15 min. The increase in pERK 1/2 levels induced by AMTH was completely abrogated by pretreatment with U0126 (Fig. 5, C and D).

Neither HA nor AMTH Modulates Rat ILC Proliferation

In an attempt to evaluate if the observed effect of HA on the proliferation of MA-10 cells could be extrapolated to their normal counterpart, we performed a series of experiments using a proliferating population of ILC in culture that were isolated from 35-day-old rats as described in *Materials and Methods*. ILC were incubated with either 100 ng/ml of hCG, 100 ng/ml of IGF-1, 0.5 mM db-cAMP, or increasing concentrations of HA (10 pM to 10 μM) or AMTH (1 nM to 10 μM) for 24 h, and [^{3}H]thymidine incorporation was determined. Both hCG and IGF-1 promoted ILC proliferation significantly (28% and 98%, respectively), whereas db-cAMP exhibited an antiproliferative action (Fig. 6A). In contrast, neither HA nor AMTH had any significant effect (Fig. 6B). Subsequently, to rule out the possibility that our results showing a lack of effect of HA on ILC might be due to the fact that the rats from which we isolated the cells were too old, we repeated the aforementioned proliferation experiments using PLC isolated from 18- to 20-day-old rat testes as described in *Materials and Methods*. Again, neither HA nor AMTH showed any significant effect on cell proliferation (Fig. 6C).

Differential HDC Expression Between MA-10 Leydig Cells and Normal ILC

Both MA-10 cells and normal rat Leydig cells express functional HRH2 membrane receptors and exhibit an acute steroidogenic response to HA and AMTH [9]. Thus, in an attempt to find a possible explanation for the differential effects of HA on the proliferation of MA-10 cells versus normal ILC, and considering the well-documented correlation between

FIG. 4. **A**) Effect of a short-term db-cAMP treatment on MA-10 cell proliferation. MA-10 cells were incubated with 0.5 mM db-cAMP for the times indicated, after which the medium was aspirated and replaced with fresh medium. In all cases, incubation continued in this medium up to a total incubation time of 24 h. The cells were labeled with [^{3}H]thymidine during the last 4 h of this incubation, and the radioactivity incorporated into DNA was measured as described in *Materials and Methods*. Each bar represents the mean ± SEM of three independent experiments. *$P < 0.05$ vs. control. **B** and **C**) Effect of the phosphodiesterase inhibitor IBMX on HA- or AMTH-induced MA-10 cell proliferation. MA-10 cells were incubated with 1 nM HA or 1 μM HA (**B**) or with 1 μM AMTH (**C**), and 0.1 mM IBMX was added after 15 min. Incubation continued in these conditions up to a total incubation time of 24 h. The cells were labeled with [^{3}H]thymidine during the last 4 h of this incubation, and the radioactivity incorporated into DNA was measured as described in *Materials and Methods*. Each bar represents the mean ± SEM of three independent experiments. Different letters above the bars indicate that the groups differ significantly at $P \leq 0.05$.

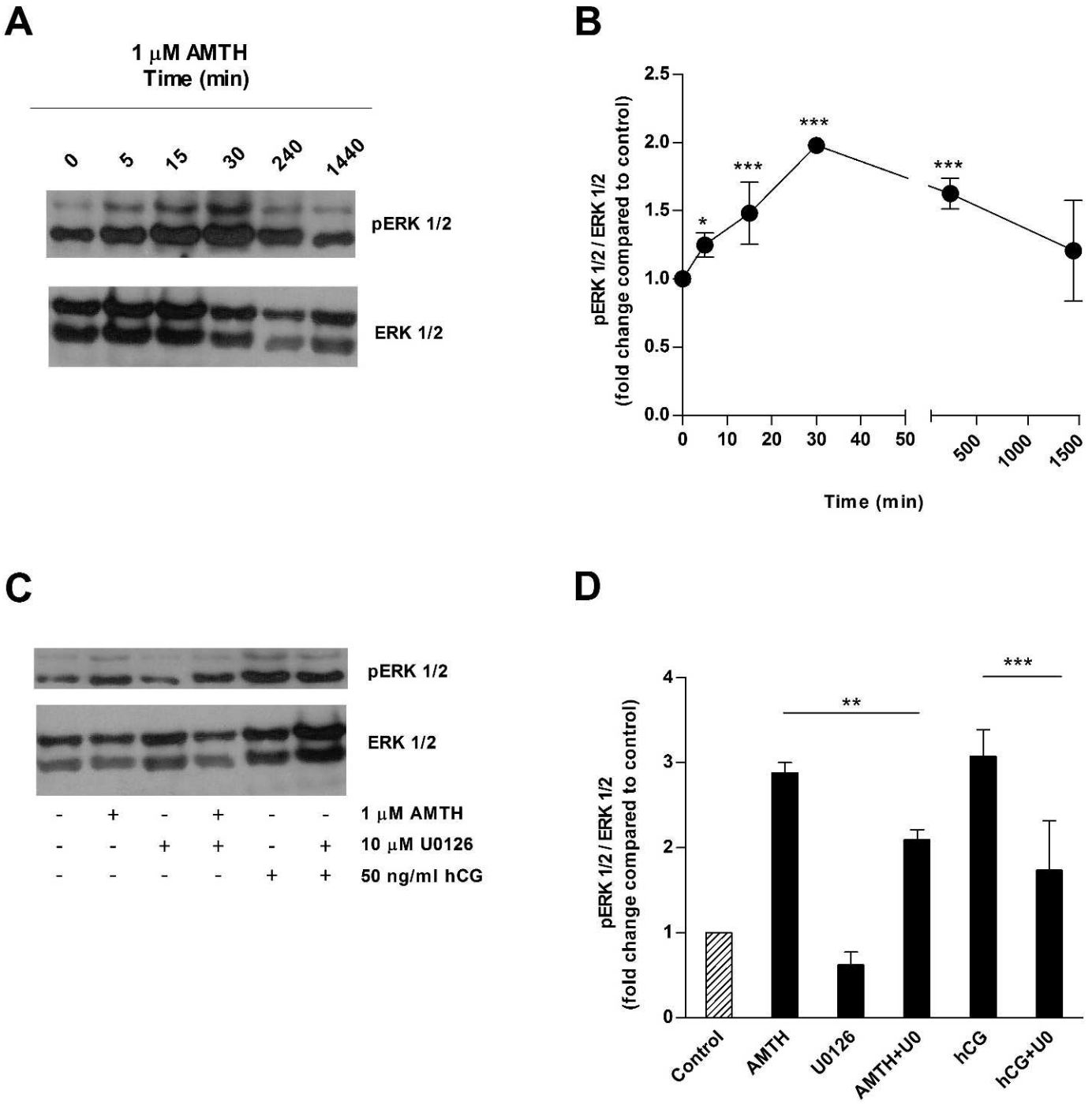
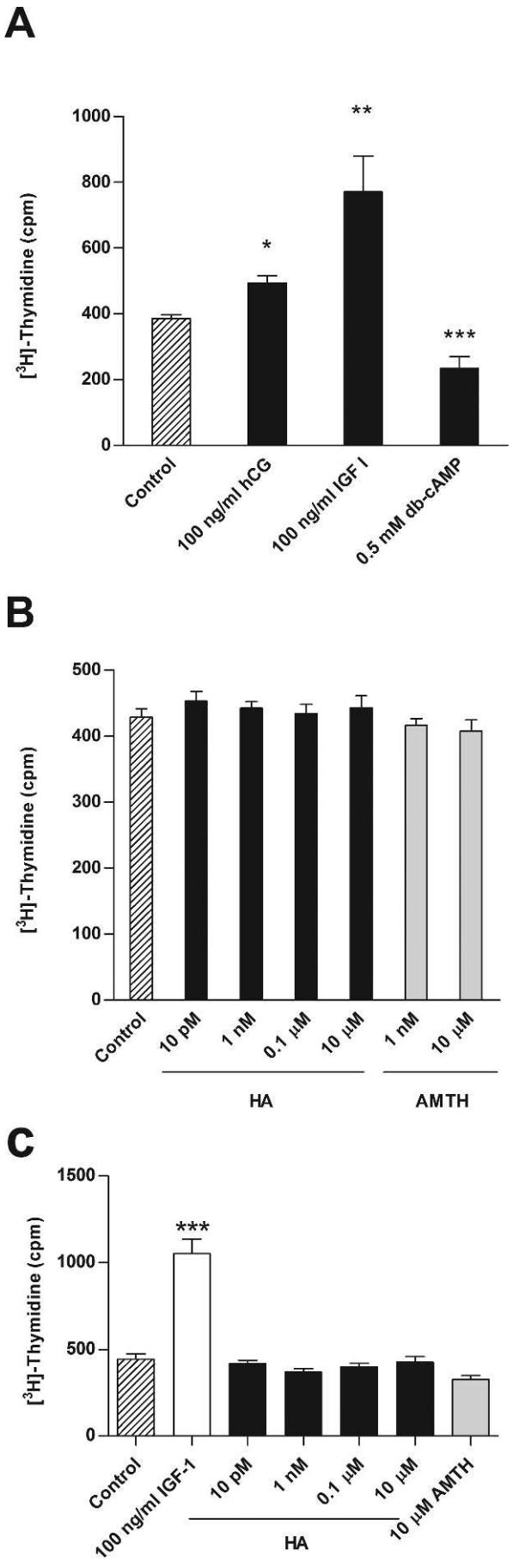


FIG. 5. **A** and **B**) Effect of AMTH on the phosphorylation of ERK 1/2 in MA-10 cells. Cells were incubated with 1 μM AMTH for the times indicated. The levels of pERK 1/2 as well as total ERK 1/2 were ascertained by Western blot analysis with the appropriate antibodies and quantitated as described in *Materials and Methods*. **A**) Representative immunoblot. **B**) Quantitation of the effect of AMTH on ERK 1/2 phosphorylation. The phosphorylation signal was corrected for the total protein signal, and this value was defined as one for the zero time-point (i.e., no agonist added). All other points are expressed relative to this basal level of phosphorylation. Each point represents the mean ± SEM of three independent experiments. $*P < 0.05$, $***P < 0.001$ vs. control. **C** and **D**) Effect of U0126 on AMTH-induced phosphorylation of ERK 1/2 in MA-10 cells. Cells were preincubated for 10 min with or without 10 μM U0126 as indicated. The phosphorylation of ERK 1/2 (pERK 1/2) was measured at the end of a 15-min incubation with 1 μM AMTH or 50 ng/ml of hCG as positive control. **C**) Representative immunoblot. **D**) Quantitation of protein levels by scanning densitometry. The phosphorylation signal was corrected for the total protein signal, and this value was defined as one for the zero time point (i.e., no agonist added). All other points are expressed relative to this basal level of phosphorylation. Each point represents the mean ± SEM of three independent experiments. $**P < 0.01$, $***P < 0.001$ vs same treatment in the presence of U0126.

HDC expression and cell proliferation in several experimental models [15], we compared the expression levels of HDC enzyme in the MA-10 cell line versus ILC by Western blot analysis. The active form of the HDC enzyme (53–55 kDa) is expressed at significantly higher levels in MA-10 cells compared to normal ILC, and even compared to whole-testicular lysate (WTL) of 35-day-old rats (Fig. 7). We then evaluated HDC expression in WTL of rats ranging from 7 to



240 days of age. HDC levels decreased significantly from Day 7 to Day 21 and then remained relatively low and constant (Fig. 8). Finally, as a first approach to demonstrating more directly the critical role of HDC and MA-10 cell-derived HA regulation of cell proliferation, we evaluated the effect of the green tea catechin EGCG, known to directly and potently inhibit HDC activity [32, 33], on the proliferation of MA-10 cells. We also experimentally compared such an effect with that of EGC, a catechin that is structurally related to EGCG but shows weaker activity as HDC inhibitor [33]. As expected, EGCG inhibited MA-10 cell proliferation in a concentration-dependent manner (Fig. 9), whereas EGC was less effective ($\leq$20% inhibition at all concentrations evaluated, data not shown).

DISCUSSION

The existence of a functional histaminergic system in the testis has been widely demonstrated [5, 9, 10, 13, 14, 16, 34], and it is well-established that HA plays a role as a modulator of cell proliferation in the most diverse experimental models [15, 35]. Until now, however, the possibility that HA may regulate Leydig cell proliferation has not been deeply explored. In this regard, the only evidence available came from a study by Kahn and Rai [11], in which it was shown that HA does not affect follicle-stimulated hormone-stimulated Leydig cell proliferation in the wall lizard (*H. flaviviridis*).

Herein, we report, to our knowledge for the first time, the proliferative effect of HA on MA-10 Leydig cells, mediated via HRH2 activation, increased cAMP production, and increased ERK phosphorylation. Accordingly, in a previous publication [18], we described the coupling of HRH2 to the AC/cAMP/protein kinase A pathway for stimulation of acute Leydig cell steroidogenesis. Moreover, both cAMP and ERK have been demonstrated to participate in LH/hCG-dependent mitogenic signal transduction in Leydig cells [30, 31, 36] and in HA-stimulated proliferation via HRH2 in other cell types [35, 37–39]. The potential contribution of HRH1 to the induction of MA-10 cell proliferation by HA may be ruled out on the basis of our findings that specific stimulation of HRH2 by AMTH can fully account for the proliferative effect of HA and that the HRH1 agonist FMPH has no effect on cell proliferation.

FIG. 6. **A**) Effects of hCG, IGF-1, and db-cAMP on ILC proliferation. ILC were incubated with 100 ng/ml of hCG, 100 ng/ml of IGF-1, or 0.5 mM db-cAMP for 24 h as indicated. The cells were labeled with [^{3}H]thymidine during the last 16 h of this incubation, and the radioactivity incorporated into DNA was measured as described in *Materials and Methods*. Each bar represents the mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control. **B**) Effects of HA and AMTH on ILC proliferation. ILC were incubated with increasing concentrations of HA (10 pM to 10 μM) or AMTH (1 nM to 10 μM) for 24 h as indicated. The cells were labeled with [^{3}H]thymidine during the last 16 h of this incubation, and the radioactivity incorporated into DNA was measured as described in *Materials and Methods*. Each bar represents the mean ± SEM of three independent experiments. No significant differences were observed between control and treated cells at any HA or AMTH concentration evaluated. **C**) Effects of IGF-1, HA, and AMTH on PLC proliferation. PLC were incubated with 100 ng/ml of IGF-1, increasing concentrations of HA (10 pM to 10 μM), or 10 μM AMTH for 24 h as indicated. The cells were labeled with [^{3}H]thymidine during the last 16 h of this incubation, and the radioactivity incorporated into DNA was measured as described in *Materials and Methods*. Each bar represents the mean ± SEM of three independent experiments. No significant differences were observed between control and treated cells at any HA or AMTH concentration evaluated. ***$P < 0.001$, IGF-1 vs. control.

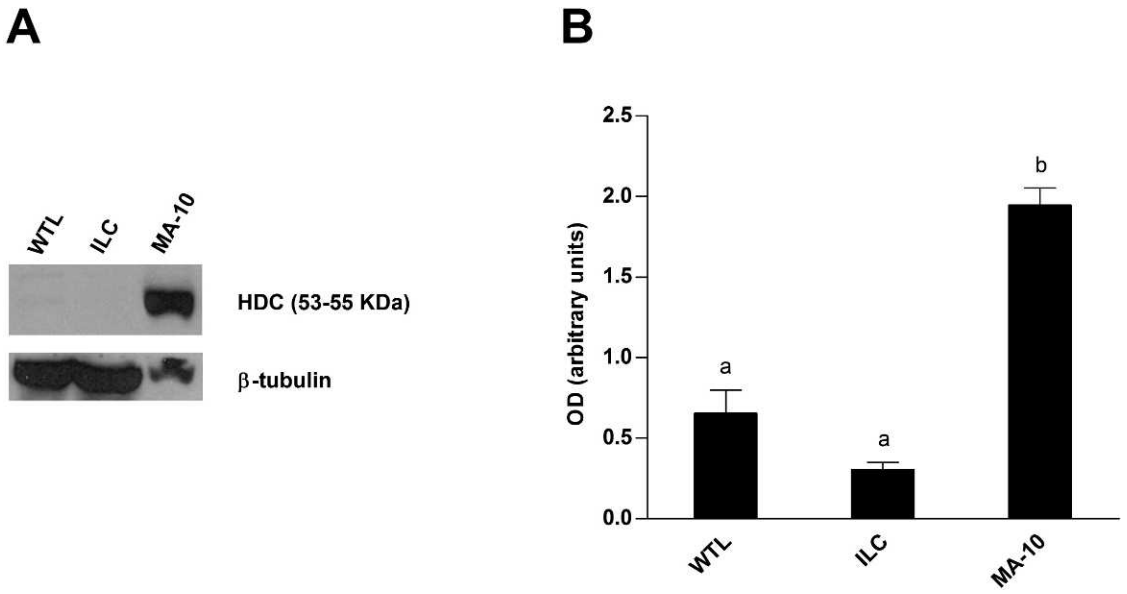


FIG. 7. HDC protein expression in MA-10 cells versus normal ILC. MA-10 cells and ILC were lysed and subjected to Western blot analysis as described in *Materials and Methods*. Data were normalized to the 55-kDa internal control, β-tubulin. **A**) Representative Western blot of HDC protein. **B**) Quantitation of protein levels by scanning densitometry. Each bar represents the mean ± SEM of three independent experiments performed with triplicate samples. Different letters above the bars indicate that the groups differ significantly at $P \leq 0.05$.

Interestingly, treatment of MA-10 cells with AMTH induced a transient increase in the production of cAMP, in contrast to the sustained elevation of cAMP levels elicited by FSK. Considering that AMTH and FSK exerted opposing effects on MA-10 cell proliferation, it could be speculated that the time course of cAMP signaling may determine the proliferative fate of MA-10 cells. A relevant observation in this regard is that prevention of cAMP degradation by addition of IBMX shortly after initiation of AMTH treatment converted the stimulatory effect of AMTH on MA-10 cell proliferation to an inhibitory one. Consistently, LH/hCG, an important proliferative signal for Leydig cells during postnatal development, induces a potent, but transient, increase in cAMP levels [40, 41]. Our present findings are in agreement with those reported by Shayo et al. [29] for the promonocytic U-937 cell line, in which AMTH and FSK trigger similar maximum cAMP responses after 5 min of incubation, but whereas FSK causes a persistent increase in cAMP levels that promotes U-937 cell differentiation, AMTH-mediated cAMP response is transient and fails to induce cell maturation. Published evidence also documents a dual time-dependent effect of cAMP on estrogen receptor (ER) mRNA levels in MA-10 cells,

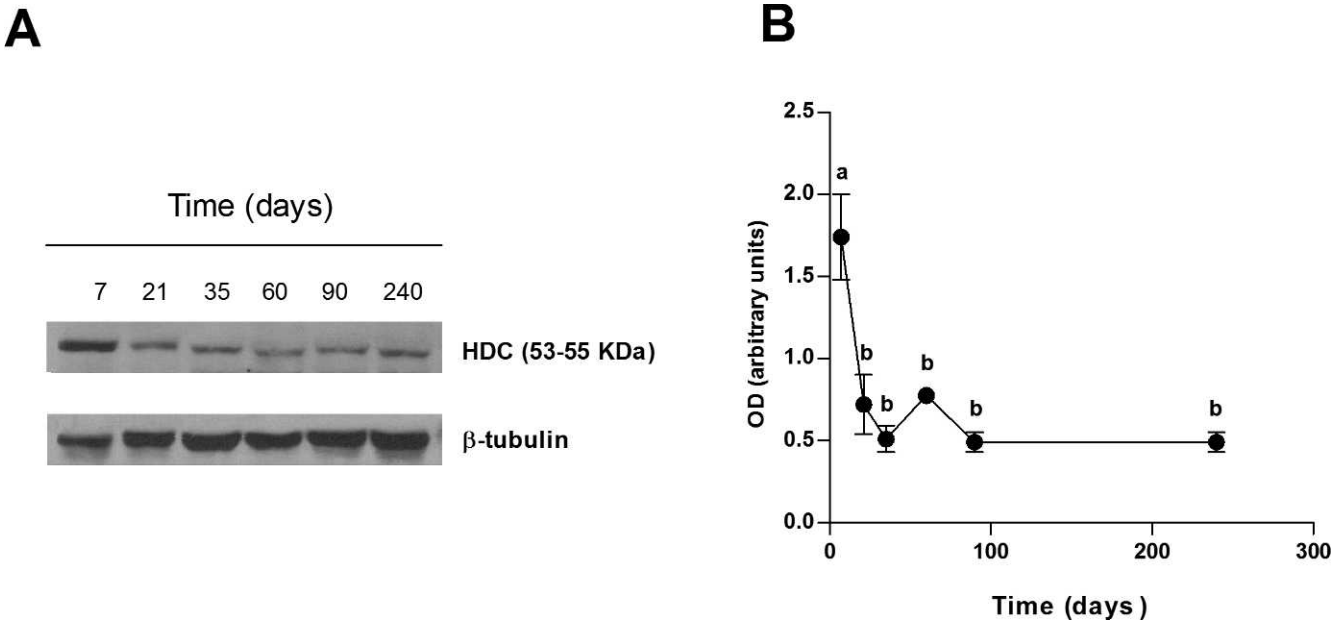


FIG. 8. HDC protein expression in WTL of rats from 7 to 240 days of age. Testicular lysates were subjected to Western blot analysis as described in *Materials and Methods*. Data were normalized to the 55-kDa internal control, β-tubulin. **A**) Representative Western blot of HDC protein. **B**) Quantitation of protein levels by scanning densitometry. Each dot represents the mean ± SEM of three independent experiments performed with triplicate samples. Different letters above the dots indicate that the groups differ significantly at $P \leq 0.05$.

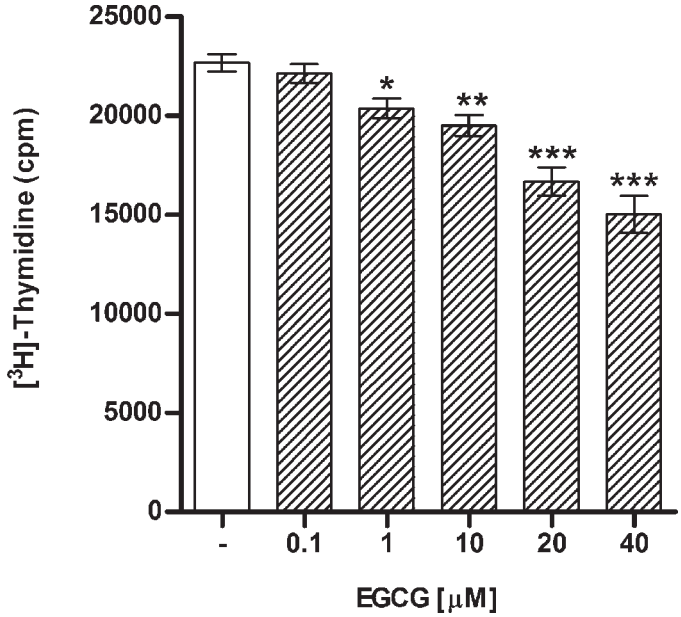


FIG. 9. Effect of EGCG treatment of MA-10 cell proliferation. MA-10 cells were incubated with increasing, nontoxic concentrations of EGCG (0.1–40 μM) for 24 h as indicated. The cells were labeled with [^{3}H]thymidine during the last 16 h of this incubation, and the radioactivity incorporated into DNA was measured as described in *Materials and Methods*. Each bar represents the mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control.

in which a short-time incubation (0–2 h) with the cAMP analog 8-bromo-cAMP enhances the expression of ER mRNA (2-fold) whereas longer incubations (6 h) have the opposite effect (the level of ER mRNA is reduced by 60%–70%) [42]. Moreover, the strict control of the intensity and spatiotemporal distribution of the cAMP signal by phosphodiesterases is crucial for proper regulation of steroidogenesis in different endocrine tissues, including Leydig cells [43–45]. Thus, on the basis of our current findings and previous evidence, it seems most likely that the temporal pattern of cAMP signaling plays a significant role in the orchestration and coordination of MA-10 cell responses to different stimuli.

Given the tumor origin of MA-10 cells, it was pertinent to evaluate the proliferative effect of HA in normal rat Leydig cells. These assays were performed in cultured ILC obtained from 35-day-old rat testes, taking into consideration that adult Leydig cells present minimal proliferative capacity [46, 47]. Of note, ILC have been used successfully as an experimental model to study proliferative effects of various mitogenic factors, such as human chorionic gonadotropin (hCG), IGF-1, and epidermal growth factor [36, 48]. Indeed, the present results show that both hCG and IGF, but not HA or AMTH, can induce ILC proliferation, suggesting that HA exerts a differential effect in the tumor MA-10 cell line versus normal ILC in terms of regulation of cell proliferation. This is reinforced by our observation that a 24-h db-cAMP treatment of ILC leads to inhibition of cell proliferation, as occurs in MA-10 cells, implying that ILC and MA-10 cells respond differently to HA stimulation but not to hCG or db-cAMP treatment. Moreover, PLC, which show a higher proliferative capacity compared to ILC [46, 47], were also unresponsive to HA. Our results are in line with those of Kahn and Rai [11], who described the lack of effect of HA on normal Leydig cell proliferation in *H. flaviviridis*. Interestingly, our present findings also reveal significantly increased HDC protein expression in MA-10 cells compared to ILC, or even compared to normal WTL. Overexpression of HDC correlates with augmented cell proliferation in a wide range of tumor derived-cell lines and tumors, including colon, breast, stomach, lung cancer, and leukemia [15, 49]. In these tumors, HA would act as an autocrine growth factor [15, 49]. It is shown herein that treatment of MA-10 cells with EGCG, a known potent and direct inhibitor of HDC activity, significantly decreases proliferation. Also, our findings indicate that testicular expression of the active form of HDC enzyme declines significantly from Day 7 onward in the rat, then remains relatively low and constant. There could then be some association between the differential effect of HA on MA-10 cells versus ILC and the overexpression of HDC in MA-10 cells. Clearly, further experiments are needed to conclusively verify this hypothesis. Moreover, although both MA-10 cells and rat Leydig cells have been reported to express HRH2, we cannot discard the possibility of a differential regulation between the two experimental models at the level of HRH2 desensitization.

In conclusion, the present study demonstrates, to our knowledge for the first time, that MA-10 Leydig tumor cells, but not normal ILC or PLC, exhibit a proliferative response upon stimulation with HA that involves HRH2 activation, transient elevation of cAMP levels, and increased ERK phosphorylation. Most tellingly, we reveal herein that MA-10 cells show significantly heightened HDC expression compared to normal ILC or WTL and that inhibition of HDC activity decreases MA-10 cell proliferation, suggesting that autocrine overproduction of HA might be somewhat linked to abnormally increased proliferation in Leydig cells, as occurs in numerous cell types [15, 35, 49]. The facts that germ cells are also both source and target of HA [50] and that multiple testicular cells are susceptible to HA action [5, 9, 11] underline the importance of the present study, which we hope will serve as a first step for further research into regulation of non-MC-related HDC expression within the testis and its significance for testicular function.

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