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Dual Role of Histamine in Modulation of Leydig Cell Steroidogenesis via HRH1 and HRH2 Receptor Subtypes[1]

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ABSTRACT

Although several reports indicate effects of histamine (HA) on female reproductive functions, scant literature exists to suggest a physiological role of HA in the male gonad. In the present study, we report a dual concentration-dependent effect of HA on steroidogenesis in MA-10 murine Leydig cells and purified rat Leydig cells. Although 1 nM HA can stimulate steroid production and significantly increase the response to LH/hCG in these cells, 10 μM HA exerts an inhibitory effect. We also provide confirming evidence for the existence of functional HRH1 and HRH2 receptors in both experimental models. The use of HRH1 and HRH2 selective agonists and antagonists led us to suggest that HRH2 activation would be largely responsible for stimulation of steroidogenesis, while HRH1 activation is required for inhibition of steroid synthesis. Our results regarding signal transduction pathways associated with these receptors indicate the coupling of HRH2 to the adenylate cyclase system through direct interaction with a Gs protein. Moreover, we show HRH1 activation mediates increases in inositol phosphate production, possibly due to coupling of this receptor to Gq protein and phospholipase C activation. The data compiled in this report clearly indicate that HA can modulate Leydig cell steroidogenesis in the testis and suggest a possible new physiological site of action for HA. Given that many drugs binding to HRH1, HRH2, or both, are widely prescribed for the treatment of diverse HA-related pathologies, it seems necessary to increase the knowledge regarding histaminergic regulation of testicular functions, to avoid possible unexpected side effects of such substances in the testis.

cyclic adenosine monophosphate, Leydig cells, signal transduction, testis, testosterone

INTRODUCTION

Although testicular steroidogenesis has long been recognized to be dependent on LH, numerous reports have clearly indicated that locally produced factors may exert autocrine or paracrine effects (or both) on Leydig cells [1, 2]. In this respect, histamine (HA) has been proved to be synthesized in the testis [3]. However, histaminergic regulation of Leydig cell function has not been extensively studied, possibly because mast cells are not frequently found in the testicular interstitium of the adult rat [4]. However, numerous mast cells are located beneath the tunica albuginea, around subcapsular blood vessels [4, 5], suggesting they may be linked, by way of their paracrine secretions, to local regulation of the testis function.

Histamine is a monoamine neurotransmitter synthesized exclusively by L-histidine decarboxylase (HDC) in most mammalian tissues. It plays a role in a variety of physiological processes [6]. The presently known HA receptors (HRH1, HRH2, and HRH3) are all G protein-coupled molecules, which transduce extracellular signals via Gq, Gs, and Gi/o, respectively [7]. A fourth HA receptor subtype (HRH4) with a unique expression profile has been recently discovered [8]. Of interest, *Hrh4* mRNA has been documented to be expressed in an unpurified rat testis cell preparation [8]. Also, Nemetallah et al. [9] have suggested the presence of HRH1 in both rabbit and rat testicular capsules. Finally, Fitzsimons et al. [10] have reported prolonged HA deficiency in HDC-deficient mice (homozygous for the Hdc^{tm1Nag} allele, herein denoted Hdc^{-}) maintained on an HA-free diet induces expression of *Hrh2* mRNA in the testis. However, *Hrh2* mRNA levels are nondetectable in $Hdc^{-/-}$ mice maintained on a normal diet, or in wild-type mice [10]. So far, these constitute the only data available to support the existence of HA receptors in the male gonad.

Previously, HA was reported to exert effects on female reproductive functions. In the rat and chicken, ovarian HA appears to play a role in follicular development and ovulatory processes [11, 12]. In this respect, HA induces maximum relaxation of the rat ovarian artery just before ovulation [13]. Embryo-uterine interactions have also been associated with HA in the mouse. Uterine-derived HA interacts with embryonic HRH2 in a paracrine fashion to initiate the process of implantation [14]. In ovarian steroidogenesis, HA has been reported to have a direct stimulatory effect on progesterone and estradiol secretion in human granulosa cells, suggesting a physiological role in the regulation of granulosa cell function during the menstrual cycle [15]. Moreover, HA, acting via HRH2, can stimulate progesterone synthesis by isolated rat preovulatory follicles [16].

Literature documenting HA-induced effects on male reproductive function is less abundant. In this respect, HA has been reported to have a role in human penile erection [17]. Also, Safina et al. [18] have suggested HA synthesis by mouse male germ cells. Their observations indicate that HA can be produced in and from the acrosomes. However, the role of this germ cell-related HA remains unclear. Even

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fewer data are available regarding HA regulation of testicular steroid synthesis. So far, HA has been shown to stimulate steroidogenesis and to potentiate the effects of LH in testicular parenchyma of the golden hamster [5].

Taken together, the reports referred to above led us to speculate that HA might have a physiological role in the male gonad. Specifically, we identified HA receptors on Leydig cells using binding assays and studied the signal transduction pathways coupled to these receptors, as well as the effects of HA on steroidogenesis. Collectively, our results suggest a possible new physiological site of action for HA.

MATERIALS AND METHODS

Materials

Purified 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 (Bethesda, MD). [^{3}H]-pyrilamine ([^{3}H]-P), [^{3}H]-tiotidine ([^{3}H]-T), Na^{125}I, and [^{3}H]-inositol were purchased from New England Nuclear (Boston, MA). Histamine dihydrochloride, HRH2 selective antagonist famotidine, cAMP, 2′-O-monosuccinyladenosine-3′,5′-cyclic monophosphate tyrosyl methyl ester (TME-cAMP) were purchased from Sigma Chemical Co. (St. Louis, MO). HRH1 agonist HTMT-dimaleate, HRH1 antagonist mepyramine maleate (pyrilamine), and HRH2 agonist dimaprit dihydrochloride were purchased from Tocris Cookson Inc. (Ellisville, MO). (For relative potencies, K_B values of HA receptor agonists and antagonists, or both, see [19].) Collagenase was from Worthington (Freehold, NJ). Cell culture supplies and plastic ware were obtained from Gibco-BRL (Gaithersburg, MD) and Corning (Corning, NY), respectively. AG-I-X8 (formate form) was from Bio-Rad (Hercules, CA). TME-cAMP was radiolabeled with Na^{125}I in our laboratory by the method of chloramine-T (specific action 600 Ci/mmol), described by Birnbaumer [20]. The specific antibody for cAMP was provided by Dr. A.F. Parlow (National Hormone and Pituitary Program). The specific antibodies for progesterone and testosterone were a gift from Dr. G.D. Niswender (Animal Reproduction and Biotechnology Lab, Colorado State University, Fort Collins, CO). Other reagents used were of the best grade available and were obtained from commonly used suppliers.

Cellular Culture of MA-10 Leydig Cells

The MA-10 line (kindly provided by Mario Ascoli, University of Iowa, Ames, IA) is a clonal strain of Leydig tumor cells that secrete progesterone rather than testosterone as a major steroid, and provides a suitable model system for the study of gonadotropin actions and regulation of differentiated functions of Leydig cells. The origin and handling of MA-10 cells has already been described [21, 22]. Cells were plated in 24 × 16-mm well plates on Day 0 at a density of 1.25×10^5 cells/well, and in a total volume of 1 ml of growth medium (Waymouth MB752/1, modified to contain 1.1 g/L $NaHCO_3$, 20 mM Hepes, 50 μg/ml gentamycin, and 15% horse serum pH 7.4). The cells were maintained in a humidified atmosphere containing 5% CO_2 and were used on Day 3. At this time, the cell density was approximately 5×10^5 cells/well. On this day, the cells were washed twice with 1 ml of warm serum-free medium supplemented with 1 mg/ml of BSA (assay medium). Incubations were performed in a total volume of 0.5 ml assay medium at 37°C with the corresponding additions as described in each figure. After 5 h, media were removed and progesterone was measured by radioimmunoassay (RIA), as described [23]. The intraassay and interassay variations were 8.0% and 14.2%, respectively. To study the reversibility of the inhibitory effect of HA on steroid synthesis, MA-10 cells were incubated in the absence or presence of HA (10^{-5} M), with or without hCG (10 ng/ml). After 5 h, media were collected for progesterone determination (Day 1). Cells were washed and incubated with HA-free fresh medium for an additional 24 h. By the end of this incubation period, cells were stimulated with dibutyryl cAMP (dbcAMP) (0.1 mM) for 5 h (Day 2).

Preparation of Rat Leydig Cells

Leydig cells were isolated from testis of 60-day-old male Sprague-Dawley rats (200–250 g; Charles River descendants, Animal Care Lab, IBYME, Buenos Aires, Argentina) as previously described [24]. Animals were housed in groups in an air-conditioned room, with lights-on from 0700 to 1900 h. They were given free access to laboratory chow and tap water. Animals were killed by decapitation according to protocols for animal use approved by the institutional animal care and use committee (IBYME-CONICET), which follows National Institutes of Health guidelines.

Briefly, testes were decapsulated and dispersed by shaking (20 min, 80 cycles/min, 34°C) in a tissue-culture flask containing 0.05% collagenase, in Medium-199 (M199) supplemented with 0.1% BSA (1 ml/testis). The crude cell suspension was filtered twice through Nitex monofilament. The cells were pelleted by centrifugation at 800 × *g* for 5 min, and washed twice. Interstitial cells were resuspended in 5 ml of a 1.7 mM Tris, 140 mM NH_4Cl solution pH 7.2, as described [25] and incubated for 10 min at 37°C. Incubation was terminated by dilution with medium and centrifuged. This procedure eliminates any interference due to the presence of red blood cells and does not affect the cell response to gonadotropin stimulation [25]. Interstitial cell preparations were then resuspended in 10 ml of M199-0.1% BSA. Testicular macrophages were separated by differential attachment to plastic culture plates (34°C, 15 min). The plates were washed five times with PBS to remove unattached cells. These were centrifuged at 800 × *g* for 5 min. The cell pellet was resuspended in M199-0.1% BSA and purified by fractionation on a three-layer Percoll density gradient (26%, 34%, and 60%), which was centrifuged at 800 × *g* for 30 min. The purity of Leydig cells obtained was greater than 87%, as assessed by histochemical staining for 3β-hydroxysteroid dehydrogenase activity [26]. Cells were collected and resuspended in M199 containing 1.2 g/L $NaHCO_3$, 20 mM Hepes, 0.1 mM 1-methyl-3-isobutylxanthine (MIX), and 0.5% BSA. Incubations were performed in plastic tubes in a volume of 500 μl (5×10^5 cells/tube) at 34°C with shaking for 5 h. The samples were then centrifuged at 800 × *g* for 5 min and the supernatants were used to determine testosterone by RIA [25]. The intraassay and interassay variations were 7.3% and 13.2%, respectively.

Ligand-Binding Assays on Intact Cells

[^{3}H]-P (HRH1 antagonist) and [^{3}H]-T (HRH2 antagonist) were employed as radioligands.

MA-10 Cells

MA-10 cells grown in 24-well plates were assayed at a density of 10^5 cells per well. The cells were incubated for 40 min at 4°C in 200 μl of 50 mM Tris/HCl containing increasing concentrations of [^{3}H]-P (1 to 100 nM) or [^{3}H]-T (0.5 to 40 nM). Nonspecific binding was defined with 100 μM cold pyrilamine for HRH1 receptor assays and 1 mM HA for HRH2 receptor assays. All samples were analyzed in triplicate. After incubation, cells were washed three times with ice-cold PBS, then scraped to remove them from the wells; radioactivity was determined by liquid-scintillation counting.

Rat Leydig Cells

Rat Leydig cells were incubated with 1 to 100 nM [^{3}H]-P or 0.5 to 65 nM [^{3}H]-T in 200 μl of 50 mM Tris/HCl. Nonspecific binding was defined with 100 μM cold pyrilamine for HRH1 assays and 1 mM HA for HRH2 assays. After a 40-min incubation at 4°C, assays were vacuum-filtered through Whatman GF/B filters presoaked in 0.3% polyethylenimine. Dry filters were then placed into scintillation fluid and quantified by liquid scintillation spectrometry.

Specific binding data were analyzed for the determination of kinetic parameters (dissociation constant, K_D and maximum number of binding sites, B_{max}) by using GraphPad Prism program (GraphPad Prism Software, San Diego, CA).

Determination of Intracellular cAMP in MA-10 Leydig Cells

The methodology for the determination of intracellular cAMP in the MA-10 cell line has been previously published [27]. After a 20-min incubation of the cells in the presence of the corresponding additions, the plates were placed on ice and the medium was aspirated. Then, 0.5 ml of cold distilled water was added to each well, and the cells were scraped and disrupted by ultrasonic oscillation. The samples were heated for three periods of 1 min in boiling water to destroy endogenous protein kinase. After centrifugation in an Eppendorf microfuge (Brinkmann Instruments, Westbury, NY) for 3 min, samples were diluted using 50 mM sodium acetate buffer (pH 6.0). Unknown samples and standards were acetylated and assayed by RIA using the protocol described by Steiner et al. [28]

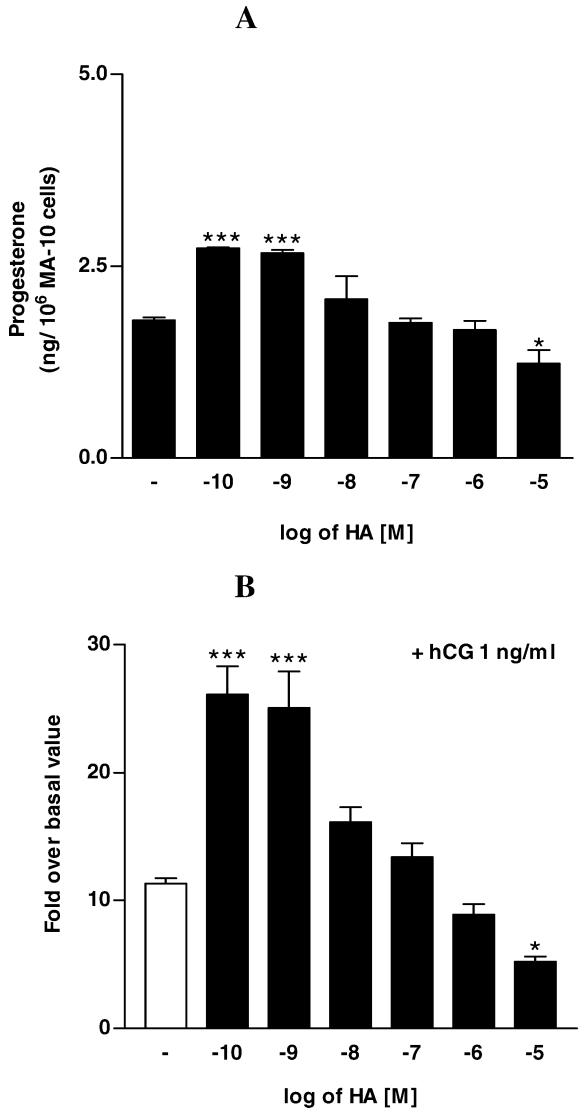

FIG. 1. MA-10 cells. **A**) Effect of HA on basal progesterone production. $*P < 0.05$ vs. control. $***P < 0.001$ vs. control. **B**) Effect of HA on progesterone production stimulated by 1 ng/ml hCG. $*P < 0.05$ vs. hCG alone. $***P < 0.001$ vs. hCG alone. Bars represent means, and error lines the SEM.

modified as follows [27]: At the end of the incubation, the antigen-antibody complexes were precipitated by the addition of 50 μl of 2% BSA and 2 ml of cold ethanol (95%), and centrifuged at 2000 × *g* for 20 min. Supernatants were then aspirated, and radioactivity of the pellets was determined in a Packard Auto-Gamma counter (Packard Instrument Co., Downers Grove, IL). The interassay and intraassay coefficients of variation were less than 10%.

Determination of Total [³H]-Inositol Phosphates

Inositol phosphates (IP) were measured as described previously [22]. Cells were incubated in warm assay medium A (Waymouth MB752/1, containing 1.1 g/L $NaHC0_3$, 20 mM Hepes, and 1 mg/ml albumin pH 7.4) with 4–5 μCi/ml [³H]-inositol for 24 h before the experiment. At the end of the labeling period, the cells were washed five times with 2-ml aliquots of warm assay medium B (Waymouth MB752/1 without $NaHC0_3$, but containing 20 mM Hepes, and 1 mg/ ml albumin pH 7.4). Cells were then preincubated for 15 min in 2 ml of warm assay medium B containing 20 mM lithium chloride (LiCl). HA agonists were added at the end of this period. After a 30-min incubation, the wells were placed on ice and the medium was quickly aspirated and replaced with 0.5 ml of cold 0.5 M perchloric acid ($HClO_4$). The cells were scraped with a rubber policeman and transferred to tubes. The wells were then washed with 0.7 ml of cold $HClO_4$, and this wash was combined with the previous extract. After a 30-min incubation on ice, the extracts were centrifuged. Collected supernatants were neutralized by the addition of 0.72 M KOH/0.6 M $KHCO_3$. The precipitated $KClO_4$ was removed by centrifugation, and supernatants were mixed with 0.5 ml of 100 mM inositol. The volume was adjusted to 5 ml with H_2O, and supernatants were chromatographed on 0.5 × 3.0 cm Dowex (Bio-Rad) columns (formate form). The columns had been prewashed with 10 ml of 10 mM inositol. After adding the samples, the columns were sequentially washed with 15 ml of 10 mM inositol (to wash residual [³H]-inositol), 5 ml of 5 mM sodium borate/60 mM sodium formate (to elute glycerophosphoinositol), and 5 ml of 0.1 M formic acid/1.0 M ammonium formate (to elute total IP). Eluted fractions were transferred to vials containing scintillation solution and radioactivity was determined by liquid scintillation counting. Results are expressed as the ratio obtained when total [³H]-inositol phosphate activity was normalized to total [³H]-inositol recovered from the initial water wash of the columns corresponding to intracellular [³H]-inositol pool.

TABLE 1. Recovery of MA-10 Leydig cell steroidogenesis after 10^{-5} M HA treatment.

Day 1		Day 2	
Treatment	Progesterone (ng/ml·5h)[a]	Treatment	Progesterone (ng/ml·5h)[a]
Control	1.8 ± 0.1	dbcAMP	72.6 ± 18.9
HA	1.2 ± 0.1*	dbcAMP	60.8 ± 20.3
hCG 10	124.6 ± 4.9	dbcAMP	75.5 ± 11.6
hCG + HA	78.5 ± 1.1**	dbcAMP	76.2 ± 31.6

[a] Data represent mean ± SEM of three independent experiments, each performed using three culture wells per condition.
* $P < 0.05$ vs Control; ** $P < 0.01$ vs hCG.

Statistical Analysis

All experiments reported herein were repeated at least three times, and the data were pooled. If heterogeneity of variance was detected by the Barlett test, this was reduced by logarithmic transformation of the data before analysis. These data were then subjected to one-way analysis of variance followed by the Newman-Keuls test for multiple range comparisons. *P* values < 0.05 were accepted as significant. Error bars represent the SEM.

RESULTS

Effect of HA on MA-10 Leydig Cell Progesterone Production

MA-10 Leydig tumor cells were incubated with increasing concentrations of HA (10^{-10} M to 10^{-5} M) for 5 h. HA showed a dual effect on basal steroidogenesis (Fig. 1A), being stimulatory at nanomolar concentrations and inhibitory at a concentration of 10^{-5} M. The inhibitory effect was not due to cytotoxicity. Trypan blue dye-exclusion assay showed the percentages of viable cells per dish were similar between 10^{-5} M HA-treated cells (94%) and nontreated cells (95%) after a 5-h incubation. The dual effect of HA was also observed in the presence of submaximal concentrations of hCG (1 ng/ml), suggesting HA might be involved in modulating LH/hCG actions (Fig. 1B). Lower HA concentrations than the ones reported herein had no significant effect on either basal or hCG-stimulated steroidogenesis.

To further confirm HA 10^{-5} M was not causing damage to the cells, we also examined the reversibility of the inhibitory effect. Table 1 shows the inhibitory effect of HA 10^{-5} M on basal and hCG-stimulated steroid synthesis (30% and 48% inhibition after a 5-h HA treatment, respectively; Day 1) was completely reversed 24 h after the removal of HA. MA-10 cells fully recovered their capacity to respond to a potent steroidogenic stimulus (Table 1, Day 2). We used dbcAMP to bypass any possible decrease in the steroidogenic response caused by down-regulation of

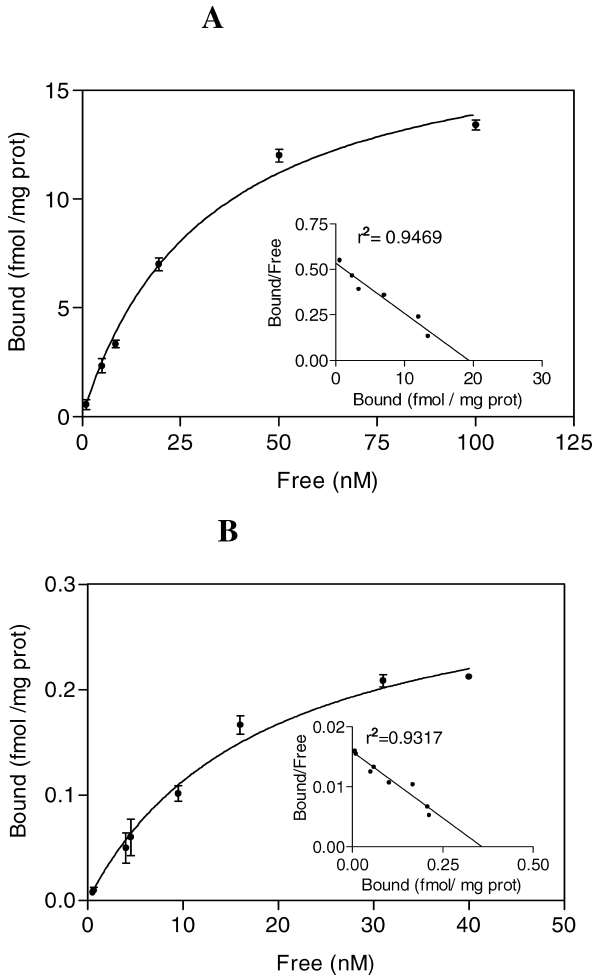

FIG. 2. **A**) Binding of [^{3}H]-P to intact MA-10 cells. Saturation analysis reveals a single and saturable binding site. Inset, Scatchard plot of [^{3}H]-P-specific binding. **B**) [^{3}H]-T binding to intact MA-10 cells. Saturation analysis reveals a single and saturable binding site. Inset, Scatchard plot of [^{3}H]-T-specific binding. Shown are typical results of experiments replicated at least three times, with data representing mean of duplicate determinations. Bars = SEM.

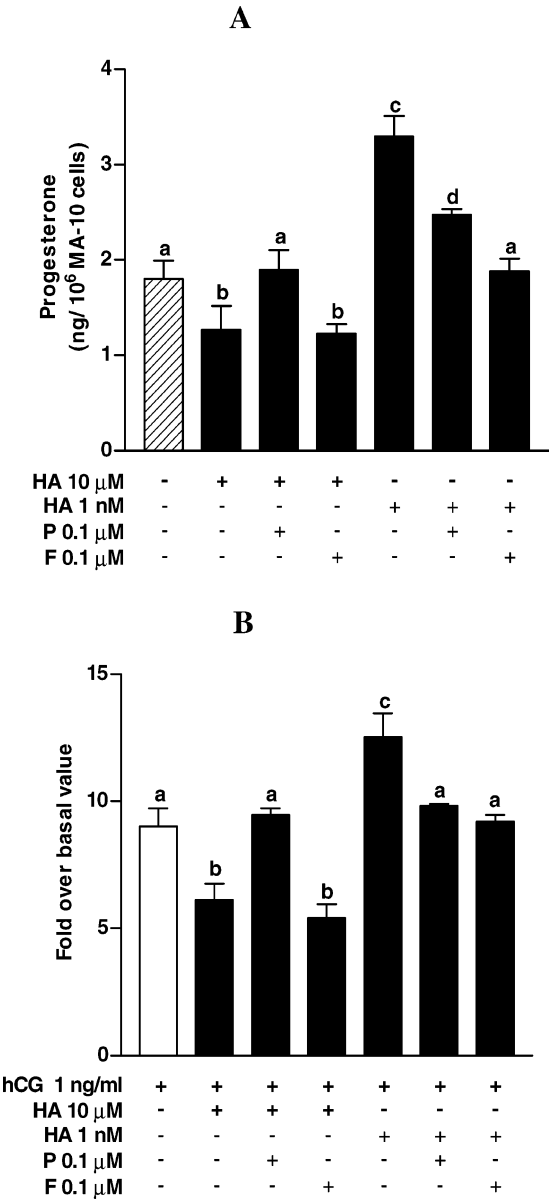

FIG. 3. MA-10 cells. Effects of pyrilamine (P) and famotidine (F) on HA modulation of basal (**A**) and hCG-stimulated (**B**) progesterone production. Each bar and vertical line represent the mean ± SEM. Different letters above the bars indicate that the groups differ significantly at least at $P < 0.05$.

hCG receptors in the cells that had been incubated with this factor the first day.

[^{3}H]-P and [^{3}H]-T Binding Assays

Both for [^{3}H]-P and [^{3}H]-T, binding to intact MA-10 cells fitted best a one-site model, suggesting the presence of a single class of sites (Fig. 2, A and B). In each case, an analysis of three separate experiments, performed in triplicate, yielded the values for K_D and B_{max}. ([^{3}H]-P: K_D [nM] = 36.5 ± 2.5; 95% confidence interval [CI], 33.3 to 40.0; B_{max} [fmol/mg prot] = 19.5 ± 0.8; 95% CI, 17.9 to 20.9; [^{3}H]-T: K_D [nM] = 22.9 ± 1.5; 95% CI, 21.2 to 24.4; B_{max} [fmol/mg prot] = 0.36 ± 0.02; 95% CI, 0.34 to 0.38.)

Effects of HRH1 and HRH2 Antagonists on HA Modulation of Basal and hCG-Stimulated Progesterone Production in MA-10 Cells

Specific HRH1 and HRH2 antagonists (pyrilamine, P; and famotidine, F, respectively) were tested for the ability to antagonize the effects of HA on basal (Fig. 3A) or hCG-induced (Fig. 3B) progesterone production. MA-10 cells were incubated with 10^{-5} M or 10^{-9} M HA, in the presence or absence of submaximal concentrations of hCG (1 ng/ml), with or without 0.1 μM P or 0.1 μM F. Both in basal conditions and in the presence of hCG, P completely reversed the inhibitory effect of 10^{-5} M HA, whereas F had no significant effect. On the other hand, F completely antagonized 10^{-9} M HA-induced increases in basal and hCG-induced progesterone production. In addition, P partially reversed the stimulatory effect of the amine on basal steroidogenesis, but could completely antagonize the effects observed in the presence of hCG. No detectable changes in progesterone levels were observed for P or F alone (data not shown).

Effects of HRH1 and HRH2 Agonists on MA-10 Leydig Cell Progesterone Production

We investigated the effects of selective HRH1 and HRH2 agonists (HTMT-dimaleate, HT; and dimaprit, DIM,

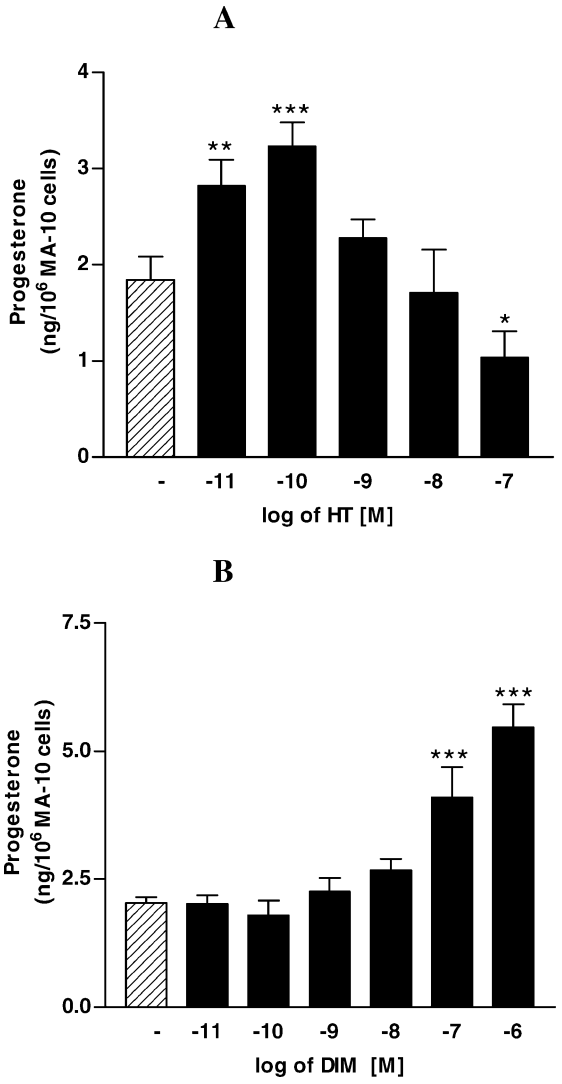

FIG. 4. MA-10 cells. **A**) Effects of HTMT-dimaleate (HT) on basal progesterone production. $*P < 0.05$ vs. control; $**P < 0.01$ vs. control; $***P < 0.001$ vs. control. **B**) Effects of dimaprit (DIM) on basal progesterone production. $***P < 0.001$ vs. control. Bars represent means, and error lines the SEM.

respectively) on progesterone production in MA-10 cells. Incubations were carried out in the presence of increasing concentrations of HT (10^{-11} to 10^{-7} M) or DIM (10^{-11} to 10^{-6} M) for 5 h. Higher concentrations of HT than 10^{-7} M were not used because of possible nonspecific effects of the agonist. As shown in Figure 4A, HT showed a dual concentration-dependent effect on basal progesterone production, as previously observed for HA. In contrast, DIM induced a concentration-dependent increase in basal progesterone production (Fig. 4B), reaching a maximum of 2.7-fold above basal level at the concentration of 10^{-6} M.

When we examined the effect of each agonist on hCG-induced progesterone production, we observed a similar profile to the one observed in basal conditions (Fig. 5). DIM potentiated the response of MA-10 cells to hCG, producing an average 6-fold increase in hCG-induced progesterone secretion. Moreover, statistical analysis revealed the stimulatory effect observed in the presence of DIM was significantly higher than that observed for HT, both in basal conditions and in the presence of hCG. Concentrations of HT or DIM with no effect on basal progesterone production

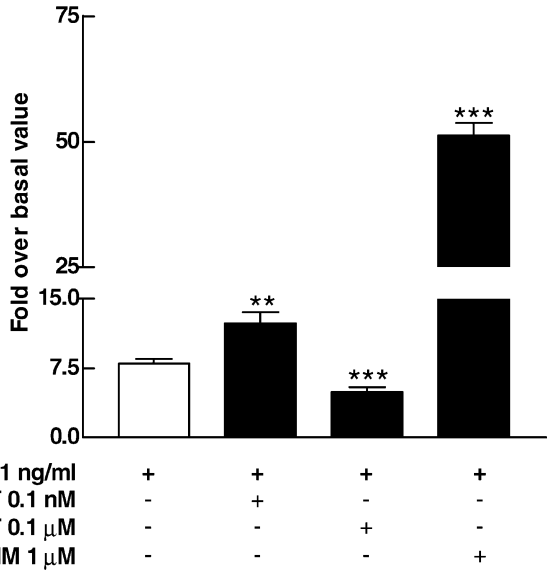

FIG. 5. MA-10 cells. Effects of HT and DIM on hCG-stimulated progesterone production. $**P < 0.01$ vs. hCG alone; $***P < 0.001$ vs. hCG alone. Bars represent means, and error lines the SEM.

had no significant effect on hCG-stimulated steroidogenesis (data not shown).

Effects of HRH1 and HRH2 Agonists on Basal and hCG-Induced Intracellular cAMP Accumulation in MA-10 Cells

To start studying the signal transduction pathways associated with HRH1 and HRH2, both HT and DIM were tested for their ability to modulate intracellular cAMP accumulation. MA-10 cells were incubated for 20 min with 10^{-10} M or 10^{-7} M HT, or 10^{-6} M DIM, in the absence or presence of a maximal hCG concentration (10 ng/ml). For HT, effects on basal cAMP levels were nondetectable. DIM, however, induced a stimulatory effect (control, 1.94 ± 0.10; 10^{-6} M; DIM, 3.104 ± 0.41 pmol of cAMP/10^6 cells; $P < 0.05$, $n = 6$). With regard to hCG-induced intracellular cAMP accumulation, DIM enhanced the response of MA-10 cells to the hormone, increasing cAMP levels by 3-fold (Fig. 6). HT 10^{-7} M elicited a significant reduction in hCG-stimulated cAMP levels, whereas lower concentrations of this agonist failed to produce any change.

Effects of HRH1 and HRH2 Agonists on Total [^{3}H]-Inositol Phosphates Accumulation in MA-10 Cells

To further characterize the signal transduction pathways associated with HRH1 and HRH2, both HT and DIM were tested for their ability to increase total IP. MA-10 cells were incubated for 30 min with 10^{-10} M or 10^{-7} M HT, or 10^{-6} M DIM. Sodium fluoride (NaF, 10 mM) was used as a positive control. Figure 7 shows that NaF increased the total IP content of MA-10 cells to more than 4-fold above basal level. HT at 10^{-7} M produced a 2.3-fold increase in the levels of IP; 10^{-10} M HT also elicited a significant augmentation of IP, although less pronounced. DIM, however, had no significant effect.

Effect of HA on Purified Rat Leydig Cell Testosterone Production

To show the dual effect of HA on steroidogenesis was not exclusive for the MA-10 cell line and could be extrap-

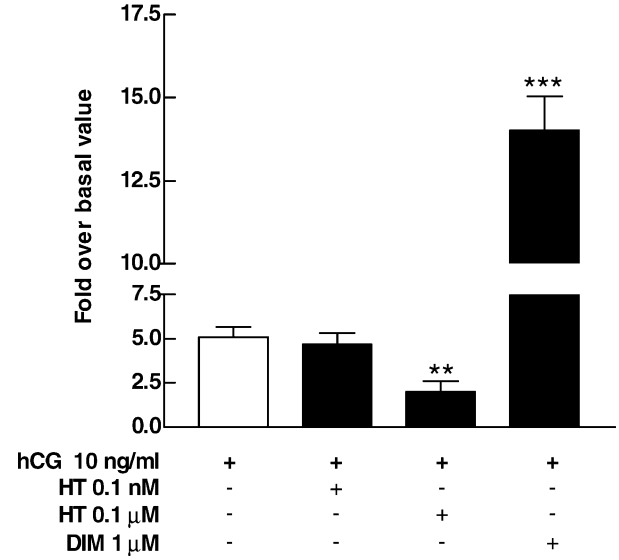

FIG. 6. MA-10 cells. Effects of HT and DIM on hCG-stimulated cAMP production. Intracellular cAMP levels were determined as described in *Materials and Methods.* Basal levels were 1.94 ± 0.10 pmol cAMP/10^6 cells. $^{**}P < 0.01$ vs. hCG alone; $^{***}P < 0.001$ vs. hCG alone. Bars represent means, and error lines the SEM.

olated to normal Leydig cells, we studied the effect of HA on testosterone biosynthesis in a suspension of highly purified rat Leydig cells. Figure 8A shows that HA also exerted a dual effect on basal steroidogenesis in these cells. At concentrations of 10^{-10} M to 10^{-8} M, HA stimulated testosterone production, whereas 10^{-5} M HA had an inhibitory effect. Moreover, the dual effect was observed in the presence of submaximal doses of hCG (0.2 ng/ml) (Fig. 8B).

Binding of [³H]-P and [³H]-T to Rat Leydig Cells

As observed for MA-10 cells, saturation analysis using intact rat Leydig cells showed one single class of site for

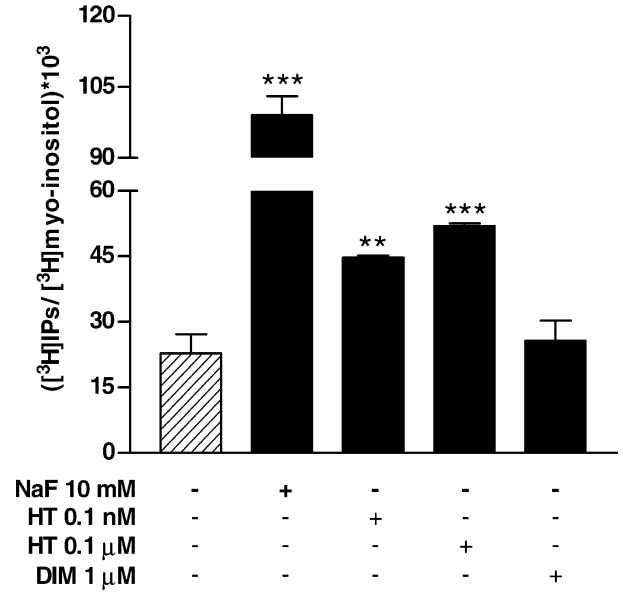

FIG. 7. MA-10 cells. Effects of HT and DIM on total [³H]-inositol phosphates accumulation. Total IP were determined as described in *Materials and Methods.* $^{**}P < 0.01$ vs. control; $^{***}P < 0.001$ vs. control. Bars represent means, and error lines the SEM.

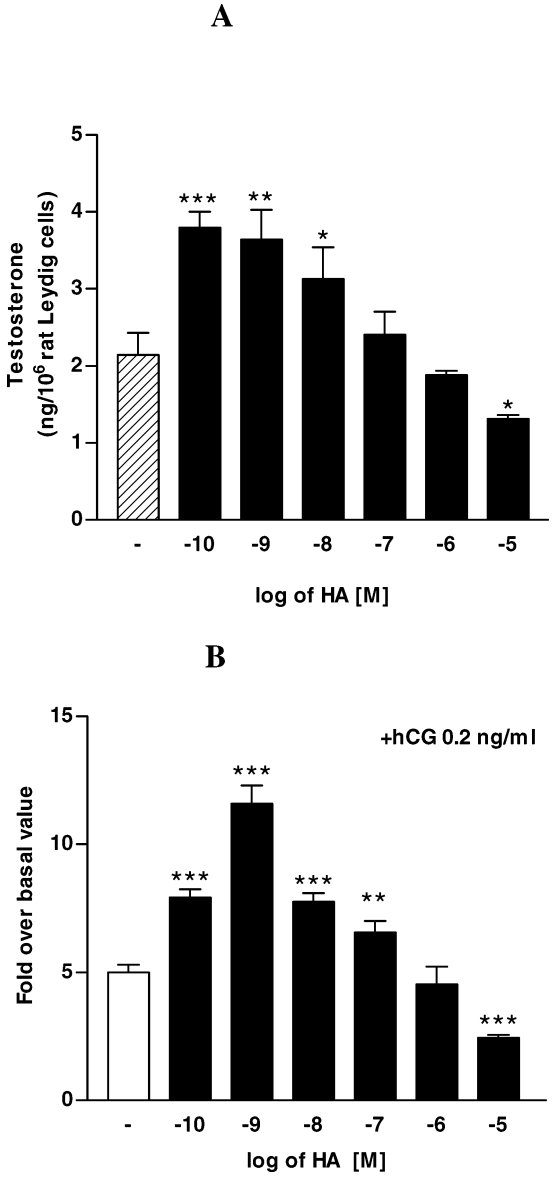

FIG. 8. Rat Leydig cells. **A**) Effect of HA on basal testosterone production. $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control; $^{***}P < 0.001$ vs. control. **B**) Effect of HA on testosterone production stimulated by hCG. $^{**}P < 0.01$ vs. hCG alone. $^{***}P < 0.001$ vs. hCG alone. Bars represent means, and error lines the SEM.

[³H]-P and [³H]-T binding (Fig. 9, A and B). In each case, an analysis of three separate experiments performed in triplicate yielded the values for K_D and B_{max} ([³H]-P: K_D [nM] = 31.8 ± 1.6; 95% CI, 29.6 to 34.2; B_{max} [fmol/mg prot] = 578.1 ± 14.6; 95% CI, 541.9 to 614.3; [³H]-T: K_D [nM] = 22.0 ± 1.6; 95% CI, 20.2 to 23.9; B_{max} [fmol/mg prot] = 100.4 ± 4.8; 95% CI, 90.47 to 110.25).

Effects of HRH1 and HRH2 Agonists on Rat Leydig Cell Testosterone Production

HT exerted opposing effects on basal testosterone production, as previously observed for MA-10 cells (Fig. 10A). In contrast, far lower concentrations of DIM were needed to elicit stimulation of steroidogenesis in these cells (Fig. 10B), possibly due to the much higher number of HRH2 binding sites compared to MA-10 cells. Both ago-

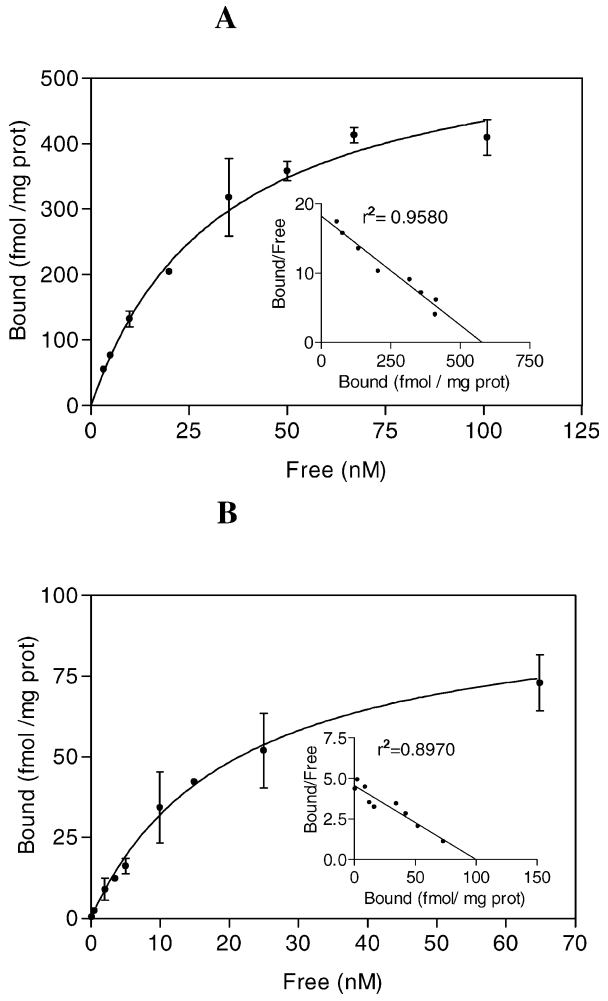

FIG. 9. **A**) Binding of [^{3}H]-P to rat Leydig cells. Saturation analysis reveals a single and saturable binding site. Inset, Scatchard plot of [^{3}H]-P-specific binding. **B**) [^{3}H]-T binding to rat Leydig cells. Saturation analysis reveals a single and saturable binding site. Inset, Scatchard plot of [^{3}H]-T-specific binding. Shown are typical results of experiments replicated at least three times, with data representing mean of duplicate determinations. Bars = SEM.

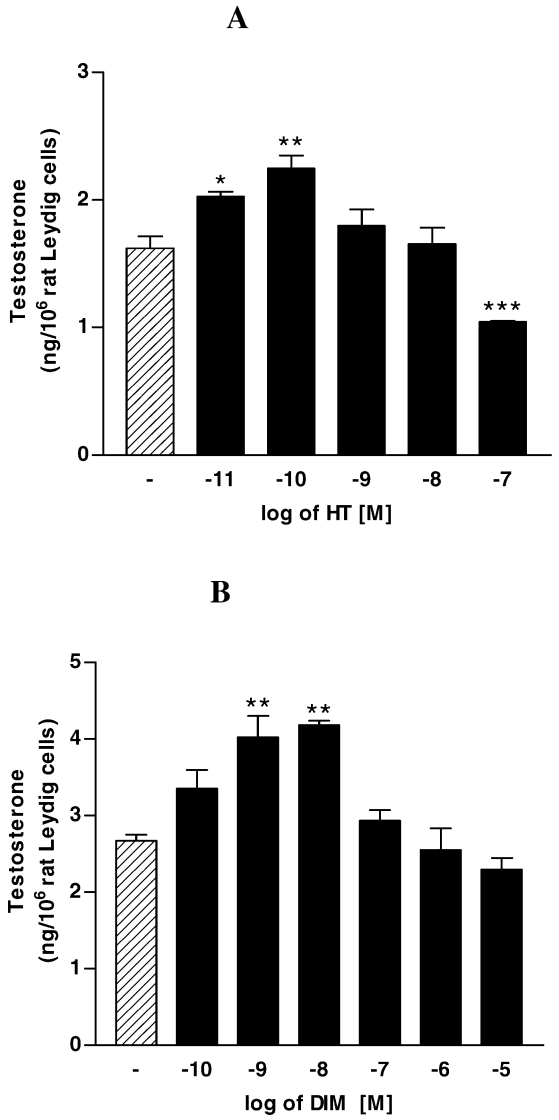

FIG. 10. Rat Leydig cells. **A**) Effect of HT on basal testosterone production. $*P < 0.05$ vs. control; $**P < 0.01$ vs. control; $***P < 0.001$ vs. control. **B**) Effect of DIM on basal testosterone production. $**P < 0.01$ vs. control. Bars represent means, and error lines the SEM.

nists showed similar profiles to those under basal conditions in the presence of hCG (data not shown).

DISCUSSION

To our knowledge, this is the first report of a direct effect of HA on Leydig cell steroidogenesis. Mayerhofer et al. [5] had previously suggested that HA can stimulate Leydig cell function, especially in the not fully functional gonad of the golden hamster. However, it remained unclear whether Leydig cells could be target cells of HA or whether other testicular cell types expressing HA receptors might be involved, by paracrine actions, in modulation of testicular steroidogenesis. In the present study, we show that HA can exert opposing effects on Leydig cell function, depending upon its concentration. Our results appear to be at variance with the reported effects of HA in the hamster [5], in which low concentrations had no effect on steroidogenesis, whereas high concentrations were stimulatory. Experiments carried out using hamster testicular parenchyma may not be directly comparable to those in isolated Leydig cells, given the presence of cell types other than Leydig cells in the first case. Moreover, K_D and B_{max} values for HA receptors in hamster Leydig cells have never been determined. However, it could be speculated that hamster Leydig cells may have a lower sensitivity to HA than mouse or rat Leydig cells and thus respond to higher concentrations of the amine. In agreement with our results, HA is known to exert biphasic effects on other, nonsteroidogenic target cells [29, 30]. From a physiological perspective, having opposing effects on steroidogenesis, HA could possibly contribute to the homeostatic control of steroid levels within the testis. Also, there is evidence for an in vivo modulation of testicular steroidogenesis by HA. Pap et al. [31] have reported that HA deficiency in *Hdc*$^{-/-}$ mice leads to altered testicular and serum steroid levels compared to that in wild-type mice, with no differences in hypothalamic *Gnrh* mRNA expression.

On the basis of our findings, the response to stimulatory HA concentrations would be primarily mediated by HRH2. Moreover, HRH2 agonist-induced augmentation of cAMP production indicates HRH2 couples to the adenylate cyclase (AC) system in Leydig cells. Such increase in cAMP concentration explains the potent stimulatory effect of this

agonist on steroid formation. These results are in agreement with previous reports documenting HA-induced enhancement of ovarian steroidogenesis via HRH2 activation and increased cAMP production [16]. Our data also indicate a minor but significant contribution of HRH1 to stimulation of steroid synthesis, not mediated via increased cAMP levels. HRH1 agonist-induced IP production suggests coupling to Gq protein, as occurs in a variety of tissues [32]. On the basis of our observations, IP might have a positive role in modulating steroid production in Leydig cells, either directly or by regulating Ca^{2+} release to activate some Ca^{2+}/calmodulin-dependent protein with positive effects on steroidogenesis. In this respect, IP_3 can increase progesterone levels in porcine ovarian granulosa cells by regulating intracellular Ca^{2+} levels [33]. Themmen et al. [34] have suggested that LH actions involve calcium fluxes through the plasma membrane, in addition to activation of protein kinase A. Moreover, Rossato et al. [35] have recently demonstrated that an influx of Ca^{2+} from the external medium can stimulate testosterone production in Leydig cells. It is well known that G protein-coupled cascades have extensive modulatory interactions with each other. Thus, it could be speculated the stimulatory effects of HA on basal and LH/hCG-stimulated steroidogenesis result from positive interactions between Gs and Gq signaling pathways.

Reduction of steroid levels by HA at 10 μM would be solely mediated via HRH1 activation. Inhibitory HRH1 agonist concentrations increase IP production but decrease cAMP levels in the presence of hCG. These observations indicate a negative cross-talk with the LH/hCG-activated cAMP/protein kinase A signaling pathway, and explain the negative modulation of stimulated steroid synthesis. A role for IP as an inhibitor of AC activity has been previously suggested, although Ca^{2+} release seems necessary to mediate this effect. Dyer et al. [36] have recently reported long-lasting inhibition of AC mediated by inositol 1,4,5-trisphosphate-evoked Ca^{2+} release. Moreover, Pereira et al. [37] have reported that Ca^{2+} decreases the rate of cAMP accumulation induced by hCG in MA-10 cells. Collectively, our observations suggest that HRH1 activation would lead to positive or negative interactions with the LH/hCG signaling pathway. Clearly, further studies are required to investigate the physiological implications of these opposing effects. According to the findings by Zieher et al. [3], the normal HA concentration in an adult rat testis is in the nanomolar range, whereas micromolar HA concentrations are typically found in neonatal testes. Thus, it could be speculated the stimulatory effect of HA on Leydig cell steroidogenesis has physiological significance in the adult male gonad. In contrast, inhibition of steroid synthesis by HA might have physiological importance in neonatal testes.

With regard to other HA receptor subtypes, Nguyen et al. have documented expression of *Hrh4* mRNA in rat testicular homogenate [8]. However, we point out that the testis is composed of numerous cell types that are strikingly different. Moreover, *Hrh4* mRNA has been found to be primarily expressed in eosinophils [8], which might reside transiently in the testis. The data reported herein show complete reversion of HA-induced stimulatory and inhibitory effects in the presence of HRH2 and HRH1 selective antagonists, respectively. Clearly, this evidence strongly supports the interpretation that HRH1 and HRH2 are the main mediators of HA-induced effects on Leydig cell steroidogenesis.

A physiological role for testicular mast cells has not yet been described. However, there are reports indicating simultaneous proliferation and differentiation of mast cells and Leydig cells in the rat testis, suggesting there might be interactions between the two cell types [38]. Given that LH can cause ovarian mast cell degranulation in the female [39], a similar situation might exist in the male gonad as well. Thus, testicular mast cell-related HA would act in a paracrine way to modulate LH actions on Leydig cells. HA production has been reported in macrophages [40], as well as in germ cells [18]. Because these cell types reside in the vicinity of Leydig cells, they might be sources of HA to regulate Leydig cell function. If this were the case, it would in part resemble the situation in the female mammary glands and uterus, in which two pools of HA modulate physiological functions: mast cell-related and epithelial cell-related HA [41, 42].

Finally, although mast cells are not normally found in the testicular interstitium of the adult rat, they are frequently encountered in the interstitial spaces of the human testis [43]. Given that many drugs used in medicine bind to HRH1 or HRH2 receptors, this study will surely attract more attention to possible unexpected side effects of such drugs, which might alter the local balance and in turn enhance or decrease androgen production. In this respect, cimetidine, a potent histaminic HRH2 antagonist extensively prescribed for ulcers has been found to be a reproductive toxicant in male rats [44]. In conclusion, the presented evidence strongly supports the interpretation that HA might have a role in the regulation of male reproductive function, which deserves to be studied more thoroughly.

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