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HISTONES INHIBIT HUMAN CHORIONIC GONADOTROPHIN-STIMULATED BUT NOT ATRIAL PEPTIDE-STIMULATED TESTOSTERONE PRODUCTION AND CYCLIC NUCLEOTIDE FORMATION BY ISOLATED MOUSE LEYDIG CELLS

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Summary—Recently it has been reported that histone type H2A can inhibit gonadotrophin-stimulated cAMP formation and steroidogenesis by ovarian cells. In the present study we have investigated if similar antigonadotrophic effects of commercially available histones can also be demonstrated on testicular steroidogenic cells. Using percoll-purified mouse Leydig cells, we have demonstrated that several types of histones could almost completely inhibit hCG-stimulated testosterone production and cAMP formation. The inhibition was dose-dependent and could be reversed by the addition of excess of hCG. The most potent histone types were H2AS and H8S, both of which could inhibit hCG-stimulated cAMP formation half-maximally at concentrations of 4–5 μg/ml. Forskolin-stimulated cAMP formation was not affected by histones. When the cells were stimulated with either db-cAMP or rAP-II, histone H2AS and H8S failed to inhibit the testosterone production. In fact there was a marked increase in the amount of testosterone produced, the reason for which is not yet understood. The amount of cGMP accumulated in response to rAP-II was not affected by the presence of H2AS or H8S. In unstimulated cells, neither the cyclic nucleotide level nor the amount of steroid produced was affected by the histones. Based on the [^{125}I]hCG binding data it is possible to conclude that histone H2AS inhibits the binding of hCG to its receptors on Leydig cells and thereby causes the inhibition of hCG-stimulated cAMP formation and steroidogenesis.

INTRODUCTION

A variety of factors, some of which have been well-characterized and some still to be characterized, have been proposed to be produced or present within the gonads, where they are supposed to function as autocrine/paracrine regulatory substances (see Refs [1] and [2] for review). Two proteins known to be present almost ubiquitously can also be included in this group of substances. From testicular interstitial fluid, Melsert *et al.* [3], have purified a steroidogenesis stimulating factor which appears to be albumin and it was shown that both albumin and this factor could markedly stimulate steroidogenesis by rat Leydig cells [3]. Recently, Aten and Behrman [4, 5] have purified a GnRH-binding inhibitor from cow ovaries and have identified this to be histone H2A. Furthermore, Aten and Behrman have shown that the protein purified from bovine ovaries and commercially available histone H2A could inhibit LH-stimulated cAMP formation by luteal cells and FSH-stimulated cAMP and progesterone production by granulosa cells. In the present investigation, we have examined whether the antigonadotrophic effect of histone can be demonstrated on testicular steroidogenic cells as well. For this, we have used percoll purified mouse Leydig cells and have tested a number of commercially available histone types in order to compare these preparations for their antigonadotrophic potencies. Furthermore, since mouse Leydig cells are known to respond to atrial natriuretic peptide in terms of an increased cGMP and testosterone production [6–8], it was of interest to examine if histones could exert an inhibitory effect on atrial-peptide stimulated cyclic nucleotide formation and the steroid production by mouse Leydig cells.

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MATERIALS AND METHODS

The hormones, hCG (13,500 IU/mg) and rat atriopeptin II (rAP-II), corresponding to ANF-(99–126) peptide according to Cantin and Genest [9], were obtained from Boehringer Mannheim (Mannheim, F.R.G.) and Bissendorf Biochemicals (Hannover, F.R.G.) respectively. Dibutyryl-cAMP (db-cAMP), the histones type H2A, H2AS, H3S, H5S, H6S, H7S and H8S and 3-isobutyl-1-methyl xanthine (IBMX) were procured from Sigma (Deisenhofen, F.R.G.). Minimum essential medium with Earle's salts was obtained from Gibco Europe (Karlsruhe, F.R.G.).

The methods for isolation, purification and incubation of mouse Leydig cells have been described previously [6–8]. Routinely, 50,000–100,000 cells were incubated in 500 μl minimum essential medium containing 25 mM HEPES, pH 7.4 and 1 mg/ml bovine serum albumin. All incubations were carried out in the presence of the cyclic nucleotide phosphodiesterase inhibitor, IBMX (0.25 mM) in order to keep the phosphodiesterase activity maximally inhibited. In experiments, where cyclic nucleotide formation was measured, the incubation was carried out at 37°C for 15 min and where testosterone production was measured, the incubation was continued for 3 h at 37°C, unless otherwise stated.

After incubation of the cells with various test substances as indicated, 100% ice-cold ethanol was added to the incubation mixture, so that the final ethanol concentration was 80%. The mixture was left standing at 4°C for 4–6 h, after which the precipitated proteins were removed by centrifugation at 2000 *g* for 15 min at 4°C. The ethanol extract was evaporated to dryness and redissolved in 0.1% NaN_3. The amounts of cAMP, cGMP and testosterone were measured by specific RIAs as described previously [6–8]. The solutions of histones were prepared in the incubation medium fresh on the day of use, since in preliminary studies, we had noted that some histone solutions were not very stable on storage.

For hCG-binding assay, hCG was labelled using a chloramine *T* method to a specific activity of 36 μCi per μg protein. The binding experiment was carried out essentially as previously described by Willey and Leidenberger [10]. In brief, 100,000 cells were incubated with 60,000 cpm [^{125}I]hCG and indicated amounts of unlabelled hCG or histones in 200 μl incubation medium as mentioned above, for 1 h at 37°C, following which bound hormone was separated from free and bound radioactivity was measured.

The data presented are mean ± SD from triplicate determinations and each experiment has been repeated at least twice.

RESULTS

In the first experiment, results of which are summarized in Fig. 1, we have examined the

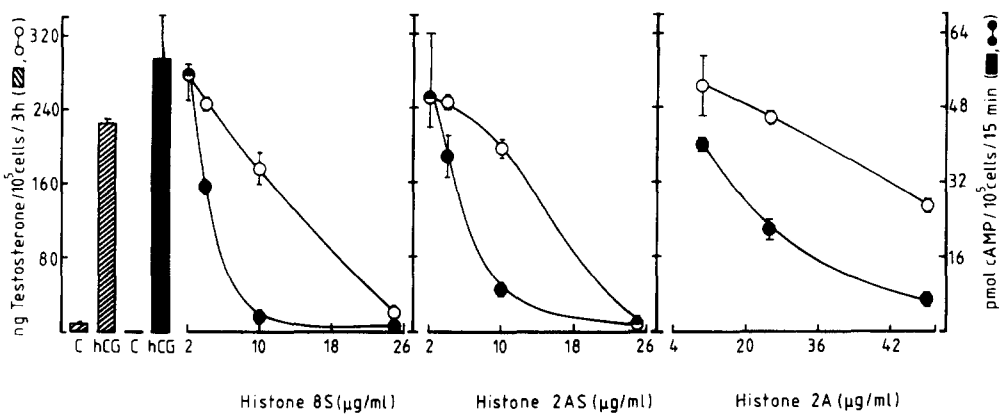

Fig. 1. Effect of histones on hCG-induced testosterone production and cAMP accumulation by isolated mouse Leydig cells. A suspension of Leydig cells in 500 μl minimum essential medium containing 25 mM HEPES, pH 7.4, 0.25 mM IBMX and 1 mg/ml bovine serum albumin was incubated at 37°C without any addition (C) or in the presence of hCG and indicated concentrations of histone H8S, H2AS and H2A. The incubation was stopped by the addition of 2 ml 100% cold ethanol and testosterone and cAMP was measured in the ethanol extract by specific radioimmunoassays as mentioned in the Materials and Methods section. In the experiment, where cAMP was measured the incubation was carried out for 15 min and the amount of hCG added was 10 ng/ml and where testosterone was measured, the incubation-duration was 3 h and 0.5 ng/ml hCG was added. The data presented are mean ± SD from triplicate determinations.

effects of addition of various concentrations of three types of histones, namely H2A, H2AS and H8S, on hCG-stimulated steroidogenesis and cyclic nucleotide formation by mouse Leydig cells. The cells were incubated for 15 min in the presence of 0.25 mM IBMX and hCG (10 ng/ml) which resulted in a 250-fold increase in the level of cAMP measured. It may be mentioned that at 15 min of incubation, negligible quantities of cAMP [11] or cGMP [7] can be measured in the extracellular medium. Therefore, at the chosen time-point of incubation, the measured cyclic nucleotide level can be taken as that reflecting almost solely the intracellular level of the cyclic nucleotide. The results obtained clearly show that each of the histone types examined produced a dose-dependent inhibition of cAMP accumulation in the cells stimulated with 10 ng/ml hCG. In a parallel experiment, the cells were incubated in the presence of 0.5 ng/ml hCG and 0.25 mM IBMX for 3 h with varying concentrations of histones H2A, H2AS and H8S and the amount of testosterone produced was measured. It is evident here as well, that all three types of histones could inhibit testosterone production in a dose-dependent manner. Comparatively, the amounts of histones required to inhibit cAMP production were approx. 3-fold less than that required for inhibiting testosterone production to a similar extent. Similar dose-related inhibitory effects were observed also when other types of histones, e.g. H5S, H6S and H7S were used (data not presented). From the results obtained, we have calculated the amounts of different types of histone required to produce 50% inhibition of hCG-stimulated cAMP formation (ED_{50} cAMP) and testosterone production (ED_{50} testosterone) as shown in the Table 1. It is evident that all types of histones listed exerted a strong inhibitory effect on hCG-stimulated steroid production and cAMP formation. However, invariably in all cases, the amount of histones required to inhibit hormone-stimulated cAMP formation to an extent of 50% was approx. 3–5-fold less than the amount required to inhibit testosterone production to a similar extent.

Table 1. The amounts of different types of histones required to half maximally inhibit the cAMP formation and testosterone production by mouse Leydig cells

	Histones (μg/ml)					
	H2A	H2AS	H5S	H6S	H7S	H8S
ED_{50} cAMP	17.0	5.0	7.0	13.5	7.0	4.0
ED_{50} test	58.0	16.5	43.0	24.0	25.0	14.0

The mouse Leydig cells were incubated with 10 ng/ml hCG (for cAMP determinations) or with 0.5 ng/ml hCG (for testosterone determinations) in the presence of varying concentrations of different types of histones and for each type of histones, inhibition curves were constructed. The amount of each type of histone required to reduce the hCG-stimulated cAMP formation to half (ED_{50} cAMP) and that required to reduce hCG-stimulated testosterone production to half (ED_{50} test) were calculated. The amounts of cAMP accumulated and testosterone produced by the cells in the presence of hCG without the addition of histones were 59 pmol/10^5 cells/15 min and 225 ng/10^5 cells/3 h (mean from quadruplicate determinations), respectively.

Table 2. Effect of addition of histone H2AS and H8S on rAP-II-stimulated cGMP accumulation by mouse Leydig cells

Additions		cGMP accumulated (pmol/10^5 cells/15 min)
rAP-II	Histone	
—	—	0.12 ± 0.01
20 nM	—	58.80 ± 9.50
20 nM	2 μg/ml H2AS	67.10 ± 11.20
20 nM	4 μg/ml H2AS	71.20 ± 6.20
20 nM	10 μg/ml H2AS	60.80 ± 1.90
20 nM	25 μg/ml H2AS	60.3 ± 3.90
20 nM	2 μg/ml H8S	56.40 ± 17.10
20 nM	4 μg/ml H8S	70.40 ± 22.50
20 nM	10 μg/ml H8S	71.25 ± 14.50
20 nM	25 μg/ml H8S	49.60 ± 5.60

The mouse Leydig cells were incubated with rAP-II for 15 min in the presence of varying concentrations of H2AS or H8S and the amount of cGMP accumulated was measured as described in the Materials and Methods section. The data presented are mean $\pm$ SD from triplicate determinations.

In addition to the types of histones listed in Table 1, we have also used H3S type, which, however, failed to inhibit the hCG-stimulated steroidogenesis and produced only a moderate inhibition on cAMP accumulation. In the absence and presence of 50 μg/ml H3S, the cells incubated with 0.5 ng/ml hCG produced 225 ± 3.7 and 223 ± 16.1 ng testosterone/10^5 cells/3 h, respectively. The amounts of cAMP accumulated when the Leydig cells were incubated with hCG (10 ng/ml) in the absence or presence of 50 μg/ml H3S were 38.9 ± 1.9 and 21.4 ± 1.6 pmol cAMP/10^5 cells/15 min, respectively.

When the cells were stimulated with forskolin, the amount of cAMP formed was increased 40-fold over the basal level (the amounts of cAMP accumulated in the absence or presence of 4 μM forskolin were 0.12 ± 0.03 and 4.95 ± 0.03 pmol/10^5 cells/15 min respectively). Addition of histone H8S upto a concentration of 25 μg/ml had no effect on forskolin-induced cAMP formation. The cells incubated in the presence of 4 μM forskolin and 25 μg/ml H8S accumulated 4.65 ± 0.57 pmol cAMP/10^5 cells/15 min. It may be noted that at this concentration H8S could almost totally inhibit the hCG-induced cAMP formation.

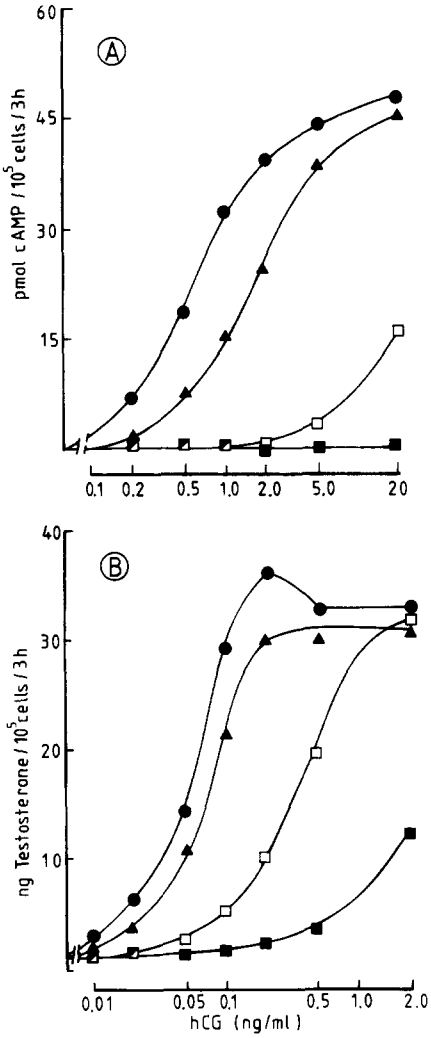

Fig. 2. The effect of histone H2AS on the cAMP (A) and testosterone (B) dose–response curves for hCG. The cells were incubated as described under Fig. 1 for 3 h with varying concentrations of hCG in the absence of any addition (●) or in the presence of the following concentrations of histone H2AS: 5 μg/ml (▲), 12.5 μg/ml (□) and 25 μg/ml (■). The amounts of cAMP accumulated (A) and testosterone produced (B) by the cells were measured. From triplicate determinations the mean ± SD values were determined but for the sake of clarity the bars representing SD have been omitted. In all cases the values for SD were within 10% of the mean values.

In case of unstimulated cells, neither cAMP nor testosterone level was affected by the addition of histone. The cells incubated without any addition or with 25 μg/ml histone H8S produced 0.21 ± 0.05 and 0.15 ± 0.01 pmol of cAMP/10^5 cells/15 min respectively. The amounts of testosterone produced by cells incubated without or with 25 μg/ml H8S were 3.8 ± 0.2 and 4.28 ± 0.5 ng/10^5 cells/3h respectively.

The results presented in Fig. 2 show the dose–response curves of hCG in the presence of various concentrations of histone H2AS. The cells were incubated in the presence of varying amounts of hCG in the absence of the histone or in the presence of 5 μg/ml, 12.5 μg/ml or 25 μg/ml histone H2AS and the amounts of cAMP accumulated (Fig. 2A) and testosterone produced (Fig. 2B) were measured. It is evident that with increasing concentrations of histone added, the dose–response curve of hCG was shifted increasingly to the right. In the presence of 5 μg/ml histone H2AS, the amounts of cAMP accumulated (Fig. 2A) by the cells in response to submaximally stimulating doses of hCG were markedly inhibited. The extent of inhibition, however, decreased gradually with the addition of increasing amounts of hCG until the inhibition was completely reversed in the presence of 20 ng/ml hCG. In the presence of 12.5 μg/ml H2AS, the inhibitory effect was more pronounced and could only be partially reversed by 20 ng/ml hCG. When the histone was added at 25 μg/ml concentration, the inhibition was nearly complete with all doses of hCG. Figure 2B shows a similar experiment where hCG-dose–response curves were constructed with testosterone production as the endpoint. Here as well, it is clear that the histone H2AS at concentrations of 5 and 12.5 μg/ml could inhibit testosterone production when the cells were stimulated with low concentrations of hCG. The extent of inhibition declined with increasing concentrations of hCG added and a

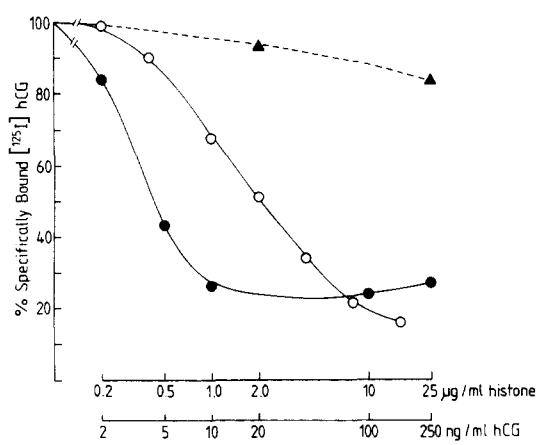

Fig. 3. Inhibition of binding of ^{125}I-hCG to its receptors on Leydig cells by histone H2AS. A suspension of 100,000 cells was incubated with [^{125}I]hCG in the absence of any addition or in the presence of varying concentrations of histone H2AS (●), H3S (▲) or unlabelled hCG (○), as detailed in the Materials and Methods section and the amount of specifically bound [^{125}I]hCG was determined. Each data point represent mean of duplicate determinations. This experiment was repeated three times and the results obtained were qualitatively similar.

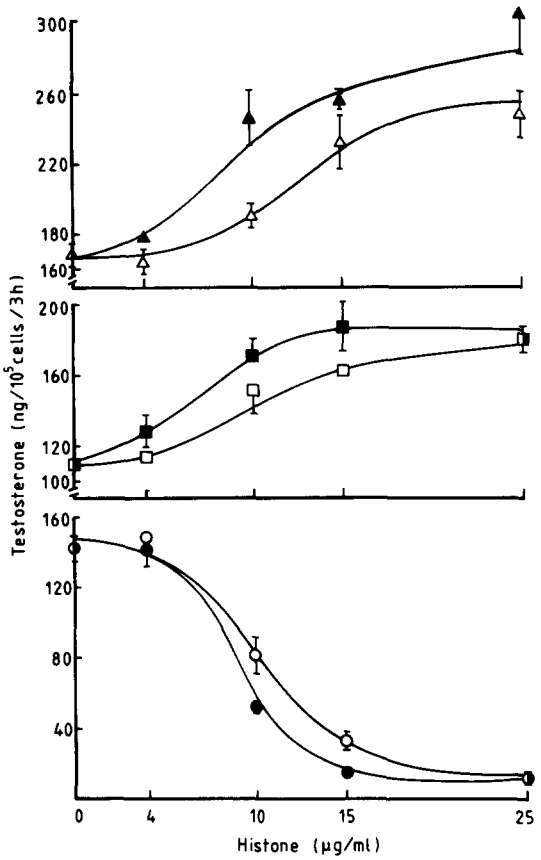

Fig. 4. The effect of histones on db-cAMP, rAP-II and hCG-stimulated testosterone production by isolated mouse Leydig cells. The cells were incubated as detailed under Fig. 1 for 3 h with 1 mM db-cAMP (▲, △) or 20 nM rAP-II (■, □) or 0.5 ng/ml hCG (●, ○), in the presence of varying concentrations of histone H2AS (open symbols) or histone H8S (solid symbols). At the end of the incubation, the amount of testosterone produced was measured. The data presented are mean ± SD from triplicate determinations.

complete reversal of inhibition was achieved with 0.5–2.0 ng/ml hCG. When H2AS was added at 25 μg/ml concentration, the inhibition was more drastic and only a partially reversal was obtained with 2 ng/ml hCG. In a separate experiment (data not shown), where a ten times greater hCG concentration, i.e. 20 ng/ml was added, we observed a near complete reversal of inhibition produced by 25 μg/ml histone H2AS. Thus it appears that the inhibitory effect of histone on Leydig cells can be competitively reversed by increasing the concentrations of hCG added.

We have next addressed the question whether the inhibitory effects of histones might be exerted by interfering with the binding of hCG to its receptors on Leydig cells. To examine this possibility, 100,000 Leydig cells were incubated with [^{125}I]hCG for 1 h in the absence of any addition or in the presence of varying concentrations of histones, H2AS and H3S and unlabelled hCG. The non-specific binding was determined by incubating the cells with ^{125}I-hCG in the presence of 100 IU/ml of unlabelled hCG. The results shown in Fig. 3 show clearly that both unlabelled hCG and histone H2AS could inhibit binding of [^{125}I]hCG in a dose-dependent manner. Approximately 0.5 μg/ml histone H2AS could effect 50% inhibition of [^{125}I]hCG binding to the cells. Interestingly, histone H3S which had negligible inhibitory potency failed to inhibit binding of the radioactive ligand significantly.

We have next examined the effect of addition of varying concentrations of H2AS and H8S on rAP-II-stimulated testosterone production (Fig. 4). For comparison, the data also include the amounts of testosterone produced by the cells stimulated with either dbcAMP (1 mM) and 0.5 ng/ml hCG in the presence of various amounts of H8S. It is evident that, though hCG-stimulated steroidogenesis was strongly inhibited by H8S, there was no inhibitory effect of H8S either on rAP-II-stimulated or on db-cAMP-stimulated testosterone production. In fact, in the presence of 25 μg/ml H2S or H8S, there was a marked enhancement of rAP-II-stimulated testosterone production. Similar observation was made with respect to db-cAMP-stimulated steroid production. It may be noted that at 25 μg/ml concentrations both H2AS and H8S histones almost completely inhibited the hCG-stimulated testosterone production.

DISCUSSION

The results obtained in this study confirmed the observation made by Aten and Behrman [4, 5] that histones can interfere with the actions of gonadotrophin on gonadal steroidogenic cells. These authors have evaluated the effect of histone type H2A on LH-stimulated cAMP formation by rat luteal cells and FSH-stimulated cAMP formation by rat granulosa cells and have shown that concentrations of approx. 70 and 30 μg/ml of H2A were required to bring about a half-maximal inhibition of LH-stimulated and FSH-stimulated cAMP formation, respectively. In addition, H2A was also shown to inhibit FSH-stimulated progesterone production by rat granulosa cells. In the present study, we have compared the inhibitory effects of various histone types and have shown that all histones

examined could inhibit the stimulatory effects of hCG on mouse Leydig cells and that histone types H2AS and H8S are the most potent inhibitors of hCG-stimulated cAMP formation and testosterone production by these cells. It was observed that approximately three times less H2AS and H8S were required to inhibit the hCG-stimulated cAMP formation compared to what was required to inhibit testosterone production to a similar extent. In the case of granulosa cells, however, the inhibitory concentrations of H2A required for blocking FSH-stimulated cAMP and progesterone production were reported to be nearly identical [5]. The fact that histones failed to inhibit db-cAMP-stimulated testosterone production would indicate that the inhibitory effect of histones is exerted at a point proximal to hCG-stimulated cAMP formation. This is also supported by the observation that hCG-stimulated cAMP formation could be inhibited by lower concentrations of histones than those required to inhibit hCG-stimulated testosterone production. It is possible to suggest that histones interfere with gonadotrophin-receptor mediated adenylate cyclase activation, which is in agreement with the proposal put forward by Aten and Behrman [5]. Since histone H8S did not affect forskolin-stimulated cAMP formation by the Leydig cells, it appears that adenylate cyclase activity *per se* is not affected by histones. Only at high concentrations ($>100\ \mu$g/ml) histones, H2AS and H8S could inhibit forskolin-stimulated cAMP formation (data not presented), which presumably was due to an unspecific toxic effect on the cells. It is concluded, therefore, that histones interfere with the gonadotrophin-receptor mediated activation of adenylate cyclase activity, by preventing the binding of hCG to its receptors. It is also possible to argue that the inhibitory histones bind to the gonadotrophin itself and therefore do not allow the hCG to bind to its receptors. This question can not yet be answered directly from the data obtained in the present study. However, we have carried out a radioimmunoassay of hCG in the absence or presence of $25\ \mu$g/ml histone H2AS and observed that the histone did not interfere in the radioimmunoassay (data not shown). It may be argued that if histones could bind to hCG, one would have seen interference in the binding of hCG to the antibody. This would obviously cause interference in the radioimmunoassay, but such interference was not observed. Therefore, it appears unlikely that the inhibitory histones bind to gonadotrophin directly. It may be of some consequence for studies where bio-/immunopotencies of LH/hCG in biological fluid samples are compared, since the presence of histones in these fluid samples may affect the bio-potency but not the immuno-potency of the gonadotrophins. This will be of particular relevance for such samples where low levels of gonadotrophins are present, since the inhibitory effect of histone H2AS on steroid production could be reversed by higher concentrations of hCG.

Furthermore, it was of interest to note that when the cells were stimulated with either db-cAMP or with rAP-II, there was a marked stimulation of steroidogenesis in the presence of histones. The concentration which was found to be maximally inhibitory on hCG-stimulated steroid production was maximally stimulatory when db-cAMP or rAP-II was used as the stimulatory ligand. The level of cGMP in rAP-II stimulated cells remained unaffected by the addition of histones. There was however, no effect of histones on the basal steroidogenesis or on the level of cGMP in unstimulated cells.

Thus, under the short-term incubation conditions employed in this report, we have shown that several types of histones could inhibit the gonadotrophin-stimulated testosterone production by inhibiting binding of hCG to its receptors on Leydig cells. This inhibitory effect was specific to gonadotrophin, since atrial peptide-stimulated steroidogenesis and cGMP accumulation was not inhibited by the histones. The observed inhibition brought about by histones was not a result of an activation of the cyclic nucleotide phosphodiesterase activity, since all incubations were carried out in the presence of the phosphodiesterase inhibitor, IBMX. The inhibitory effect could be competitively reversed by the presence of increasing amounts of hCG, which excludes the possibility of nonspecific cytotoxic effects of histones, at least in the concentration ranges used in this study. This is also supported by the fact that no inhibition was observed when db-cAMP or rAP-II was used as the stimulatory ligand. The physiological significance of the observations reported here are not at present clearly understood. However, in studies where the presence of antigonadotrophic substances or factors that interfere with gonadotrophin binding to its receptors, in testicular or ovarian materials is investigated, it will be important to bear in mind

that histones could constitute one class of such antigonadotrophic substances.

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