BIOLOGY OF REPRODUCTION 43, 1058–1063 (1990)

Effect of Histone H2A on Progesterone Production by Bovine Luteal Cells[1]

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ABSTRACT

Histone H2A competitively inhibits binding of GnRH to high affinity rat ovarian receptor sites and blocks gonadotropin-stimulated steroid and cAMP accumulation during culture of rat granulosal or luteal cells. The objective of our study was to examine the progesterone suppressive effects of histone H2A on bovine luteal cells. In the first study, luteal cells were treated at Time = 0 h with a partially purified preparation of bovine ovarian histone H2A (3 ng GnRH equivalents, 800 μg protein), equivalent amounts of GnRH (3 ng), or BSA (800 μg) and incubated for a total of 4 h. At Time = 2 h, cells were treated with 5 ng bovine LH (bLH) or with medium. Histone H2A completely blocked both basal and LH-induced accumulation of progesterone compared with untreated cultures or cultures treated with bLH. Neither BSA nor GnRH suppressed LH-induced progesterone accumulation. In the second study, histone H2A was added to cultures at Time = 0 h and bovine luteal cells were cultured for 8 h. After 2 h of treatment, histone H2A (3 ng GnRH equivalents) was removed from selected cultures and replaced with fresh medium. Four hours later cultures were treated with 5 ng bLH or medium. LH treatment of cultures from which histone H2A had been removed resulted in an increase in accumulation of progesterone compared with control cultures treated throughout the treatment period with histone H2A. The third study examined the effect of 9–181 pg GnRH equivalents (1.7–34 μg protein) of a highly purified preparation of bovine ovarian histone H2A on basal and LH-induced progesterone production during 2 or 3 h of culture. ED_{50} values for progesterone suppression were 140 pg GnRH equivalents (34 μg protein) for LH-treated cultures and 200 pg equivalents (40 μg protein) for cultures not treated with LH. The fourth study examined the suppressive effect of 25–200 μg of a commercial preparation of calf thymus histone H2A on progesterone production. Calf thymus histone H2A did not suppress basal production of progesterone during 6 h of culture compared with untreated control cultures. However, calf thymus H2A suppressed LH-induced progesterone accumulation in a dose-responsive fashion (ED_{50} = 195 μg protein). We concluded that ovarian histone H2A may have a role in regulation of progesterone production in cattle. Also, since bovine luteal cells were not responsive to GnRH and may not contain high affinity GnRH receptors, an ovarian GnRH receptor is probably not required for the progesterone suppressive effects of histone H2A.

INTRODUCTION

Ovaries from humans, cows, sheep and rats contain a potent GnRH-binding inhibitor with antigonadotropic activity (GnRH-BI) [1–4]. In cattle, GnRH-BI is in gonadal and numerous nongonadal tissues, but its highest concentration is in granulosal cells [5]. GnRH-BI recently has been purified from bovine ovaries, partially sequenced, and identified as histone H2A [6, 7]. Bovine ovarian histone H2A competitively inhibits binding of GnRH agonists to high affinity rat ovarian GnRH receptor sites. Because histone H2A binds specifically to rat ovarian GnRH receptor sites, the antigonadotropic action of histone H2A may be mediated through the ovarian GnRH receptor. In comparison with GnRH, histone H2A is a markedly more potent suppressor of steroid and cAMP production by rat granulosal and luteal cells [8, 9]. Although the mechanism of action of histone H2A is unknown, the aforementioned results indirectly support an antigonadotropic role for histone H2A in rats.

Antigonadotropic effects of histone H2A have only been reported for rats [8, 9], which have an ovarian GnRH response system [10, 11]. Our objective was to determine whether histone H2A suppresses progesterone production in cattle, a species without an ovarian GnRH response system [12, 13]. A positive outcome from our study would support previous results in rats that bovine ovarian histone H2A may have antigonadotropic properties [8, 9], and would strongly imply that the mechanism of action of histone H2A is not mediated through a GnRH receptor. In this report, we will show that bovine ovarian histone H2A and a commercial preparation of calf thymus histone H2A both suppress LH-induced progesterone accumulation during incubation of bovine luteal cells. We have concluded that ovarian histone H2A may have a role in regulation of pro-

Accepted August 14, 1990.
Received April 9, 1990.
[1]This work was supported by NIH Grant HD-06968-01 to J.J.I., USDA Grant 87-CRCR-1-2539 to R.A.M., and NIH Grant HD-15403 to H.R.B.
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gesterone production in cattle, and that an ovarian GnRH receptor is probably not required for histone H2A action.

MATERIALS AND METHODS

Assays

During purification of histone H2A from bovine ovaries, a highly specific radioreceptor assay (RRA) using rat ovarian membranes and a GnRH agonist (D-ALA^6, DES-GLY^{10}-GnRH ethylamide, GnRH-A) was used to identify histone H2A activity [2, 3, 5, 8, 9]. Amounts of bovine ovarian histone H2A are expressed as pg GnRH equivalents or as μg of total protein. One picogram GnRH equivalent indicates that bovine ovarian histone H2A displaced specific binding of 1 pg of GnRH agonist in a rat ovarian membrane GnRH RRA. Protein concentrations were determined by the method of Lowry et al. [14]. RIAs were used to determine concentrations of progesterone in media [15, 16].

Purification of Ovarian Histone H2A

Detailed procedures for isolation of histone H2A from bovine ovaries have been published [2, 3, 5, 8, 9]. In general, a combination of low-pressure chromatography and reversed-phase HPLC were used to prepare a crude preparation of histone H2A. For more purified histone H2A, the previous purification steps were coupled with cation-exchange and gel-permeation chromatography. Both the crude and highly purified preparations of histone H2A suppress cAMP, estradiol, and/or progesterone production during culture of granulosal or luteal cells in rats [1, 8, 9]. Histone H2A activity was identified by the rat ovarian GnRH RRA described above. In the first two studies, a single, large dose of the crude preparation of histone H2A, shown to suppress cAMP and steroid production in rats [1], was tested for its capacity to suppress accumulation of progesterone and for reversibility of its progesterone suppressive activity using cultures of bovine luteal cells. The third study tested the progesterone suppressive effect of various doses of a more highly purified preparation of bovine ovarian histone H2A on bovine luteal cells.

Preparation of a Nonovarian Source of Histones

The possibility that the progesterone-suppressive effect of histone H2A was unique to ovarian preparations of histone H2A was examined in the fourth study. Since histone H2A is in the nucleus of all cells, we tested whether a nonovarian source of histone H2A produced results similar to those for ovarian histone H2A. Calf thymus histone H2A (Sigma Chemical Co., St. Louis, MO; No. H-6881, Lot #88F-8125) was dissolved in saline overnight at room temperature and added without further purification to bovine luteal cells.

Luteal Cell Cultures

Corpora lutea (CL) were obtained from Holstein cows, under epidural anesthesia (2% lidocaine), at mid cycle via an incision in the anterior vagina; CL were placed in ice-cold Medium 199 (GIBCO, Grand Island, NY). Luteal tissue was dispersed with collagenase according to previously published procedures [17, 18], and 1×10^6 viable cells/ml/well were incubated in Medium 199 for the first three experiments or in Dulbecco's Modified Eagle's Medium buffered with HEPES (3.6 g/L) and $NaHCO_3$ (2.2 g/L) and supplemented with penicillin (100 000 U/L), streptomycin (100 mg/L), and fresh glutamine (2 mM, all reagents from Sigma) for the last study.

Luteal cells were incubated 20–30 min before treatments were added (Time = 0 h). bLH was added 1–6 h after treatments began. At appropriate times before incubations ended, 100–200-μl samples of cells and medium were removed and replaced with the same volume of fresh medium. Spent medium with cells was stored at −20°C for assay of progesterone. Cell viability was evaluated with Trypan blue dye on four separate occasions following incubations of luteal cells with the highest dose of histone H2A. Viability of luteal cells was greater than 85%.

First study: progesterone-suppressive effects of a partially purified preparation of bovine ovarian histone H2A. At Time = 0 h, aliquots (100 μl) of medium were removed from each luteal cell culture well and replaced with medium with or without histone H2A (3 ng GnRH equivalents, 800 μg protein), BSA (800 μg protein), or GnRH (3 ng). At Time = 2 h, 5 ng NIH B9 bLH or medium was added to cultures. Incubations ended at Time = 4 h. Concentrations of progesterone in medium were determined at Time = 0, 2, and 4 h of culture.

Second study: reversibility of progesterone-suppressive effects of a partially purified preparation of histone H2A. Histone H2A (3 ng GnRH-BI equivalents, 800 μg protein) or medium was added to bovine luteal cells at Time = 0 h. At Time = 2 h, cells and medium were centrifuged (200 × *g*, 10 min) and cells were washed three times with fresh medium. After the last centrifugation, supernatants were replaced with medium alone or with medium containing 3 ng equivalents of histone H2A. At Time = 6 h, 10 μl of medium with or without 5 ng NIH B9 bLH was added to each culture well. At Time = 8 h, all incubations ended. Progesterone concentrations were determined at Time = 0, 2, 6, and 8 h of culture.

Third study: progesterone-suppressive effects of a highly purified preparation of ovarian bovine histone H2A. Ten different doses (9–181 pg GnRH equivalents, 1.7–34 μg protein) of highly purified bovine ovarian histone H2A were added to cultures of bovine luteal cells at Time = 0 h. Selected cultures received 5 ng bLH or medium at Time = 1 h. Concentrations of progesterone were determined at the end of incubations, which was Time = 2 h for the basal group and Time = 3 h for the LH-treated group.

Fourth study: progesterone-suppressive effects of a commercial preparation of histones. At Time = 0 h, bovine luteal cells were treated with medium containing 25–200 μg calf thymus histone H2A or with medium alone. Saline or 5 ng bLH (NIH bLH-4) was added at Time = 2 h. Incubations ended at Time = 6 h. Concentration of progesterone was determined at Time = 0 and 2 h for the basal group, and at Time = 0, 2, and 6 h for the LH-treated group.

Statistical Analysis

The first study was designed to determine whether histone H2A inhibited progesterone accumulation in medium, whereas the second study was designed to determine whether the inhibitory action of histone H2A on progesterone accumulation in medium was reversible. Statistical differences between treatment groups in the first two studies were determined by use of a completely randomized design analysis of variance [19]. Differences between means were determined by the Bonferoni *t*-test [19]. The third study was designed to examine the dose-response effect of a highly purified preparation of bovine ovarian histone H2A on progesterone accumulation in medium, whereas the fourth study was designed to examine the dose-response effect of a commercial preparation of histone H2A. Regression analysis was used in the third and fourth studies to analyze each dose-response curve. For each of the four studies, there were four or five replicates for a single CL per time point for each treatment. Each of the four studies was repeated two or three times with luteal cells from different cattle. Significance was determined by Fisher's test for combined evidence to calculate an overall p value [19] for each study. Fisher's test of combined evidence is used to combine results from several experiments with similar hypothesis. Although the combined p value is reported, the results for one of the replicates from each of the four studies are shown since results for each replicate were similar.

RESULTS

Bovine ovarian histone H2A blocked ($p < 0.01$) basal and LH-induced progesterone accumulation during culture of bovine luteal cells (Fig. 1). LH-induced accumulation of progesterone was unaffected by BSA or GnRH.

The progesterone-suppressive effects of bovine ovarian histone H2A (3 ng GnRH equivalents, 800 μg protein) on basal and LH-induced progesterone accumulation were both partially reversible (Fig. 2). After removal of histone H2A at Time = 2 h, recovery of basal and LH-induced progesterone accumulation (refer to two intermediate lines in Fig. 2) was greater ($p < 0.025$) than that for cultures continuously incubated with histone H2A (refer to the two bottom lines in Fig. 2), but less ($p < 0.025$) than that for cells treated with medium (refer to the two top lines in Fig. 2).

Highly purified histone H2A inhibited LH-induced ($r = -0.67$, $p < 0.01$) and basal ($r = -0.72$, $p < 0.01$) accu-

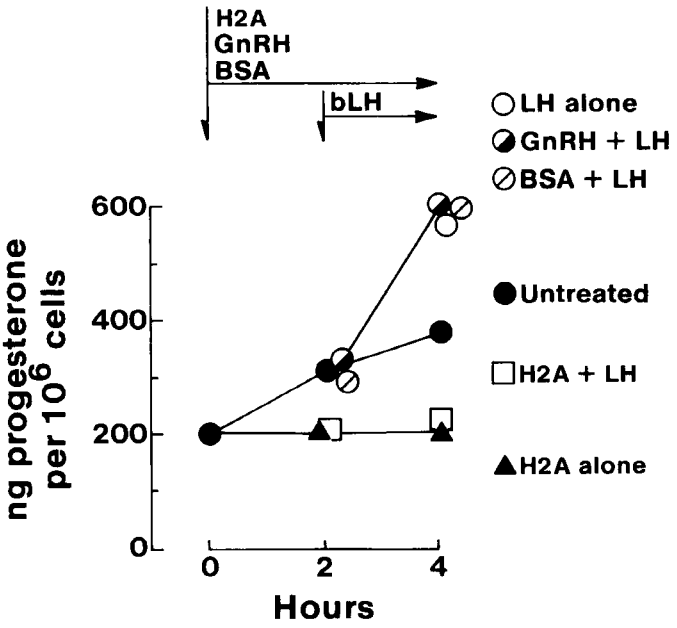

FIG. 1. The effect of bovine ovarian histone H2A (H2A) on basal and LH-induced accumulation of progesterone during incubation of bovine luteal cells. Bovine luteal cells (10^6/well) were incubated for 4 h. Histone H2A (3 ng GnRH-like equivalents, 800 μg protein), GnRH (3 ng), BSA (800 μg), or bLH (5 ng) were added to cultures as shown by arrows. Specific treatments are defined in the *figure*. Symbols represent mean values for progesterone for 5 culture wells. SEM was less than 10% of the mean for each mean.

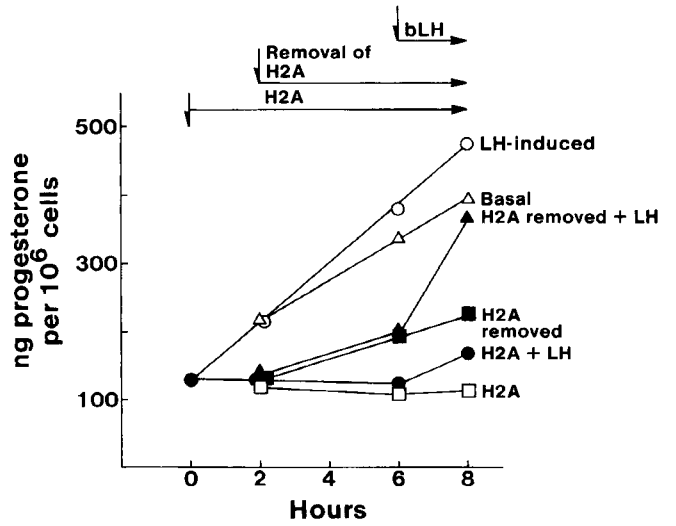

FIG. 2. The reversibility of progesterone-suppressive effects of bovine ovarian histone H2A (H2A) on basal and LH-induced accumulation of progesterone during culture of bovine luteal cells. Bovine luteal cells (10^6/well) were incubated for 8 h. Histone H2A (3 ng GnRH equivalents, 800 μg protein) was added at Time = 0 h (or removed at Time = 2 h), and 5 ng bLH was added at Time = 6 h, as shown by arrows. In LH-treated groups, bovine luteal cells were cultured with histone H2A for 8 h (H2A + LH), with the histone H2A for 2 h followed by replacement of histone H2A-containing medium with fresh medium (H2A removed + LH), or without histone H2A for 8 h (LH-induced). In controls, cultures were untreated (basal), or treated only with histone H2A for 2 h (H2A removed) or for 8 h (H2A). Each symbol represents mean values for progesterone concentrations in 5 replicate culture wells. SEM was less than 10% of the mean for each mean.

mulation of progesterone in a dose-dependent fashion (Fig. 3). ED_{50} values for the progesterone-suppressive effects of bovine histone H2A were 140 pg GnRH equivalents for LH-treated cultures and 200 pg equivalents for untreated cultures. Total protein in 140–200 pg GnRH equivalents was less than 40 μg. Amount of protein for the highest dose of the highly purified ovarian histone H2A preparation (Fig. 3) was 24-fold less than that used in the first two studies (Figs. 1, 2).

In contrast to results with bovine histone H2A, a commercial preparation of calf thymus histone H2A did not suppress basal accumulation of progesterone (Fig. 4a). However, calf thymus histone H2A did suppress ($p < 0.025$) LH-induced progesterone accumulation in a dose-dependent fashion (Fig. 4b). The ED_{50} for calf thymus histone H2A was approximately 195 μg compared with an ED_{50} of approximately 34 μg of total protein (140 pg GnRH equivalents) for the highly purified preparation of bovine ovarian histone H2A (Fig. 3).

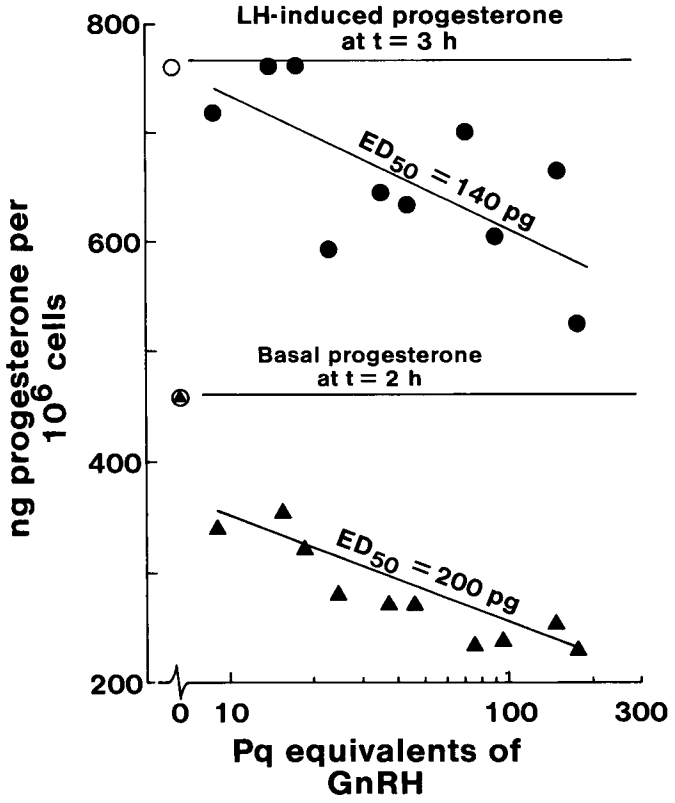

FIG. 3. The dose-response effect of a highly purified preparation of bovine ovarian histone H2A (H2A) on LH-induced and basal accumulation of progesterone during culture of bovine luteal cells. Luteal cells (10^6) were incubated for 3 h (top panel, LH-induced) or for 2 h (bottom panel, basal) and treated with ten different doses of histone H2A at Time = 0 h. bLH (5 ng) was added at Time = 1 h to the LH-induced cultures. Doses of histone H2A were from 9 (1.7 μg protein) to 181 pg (34 μg protein) GnRH equivalents. Estimates of ED_{50}s are shown in the *figure*. Treatments included untreated cultures (open circle with triangle, lower panel), cultures treated with histone H2A (triangles, lower panel), cultures treated with histone H2A followed by bLH (dark circles, top panel), and cultures treated with bLH alone (open circles, top panel). Progesterone concentrations were determined after 3 h culture for the LH-treated group and after 2 h culture for the basal treatment group. Symbols represent mean value for 4–5 replicate determinations of concentrations of progesterone. SEM was less than 10% of the mean for each mean.

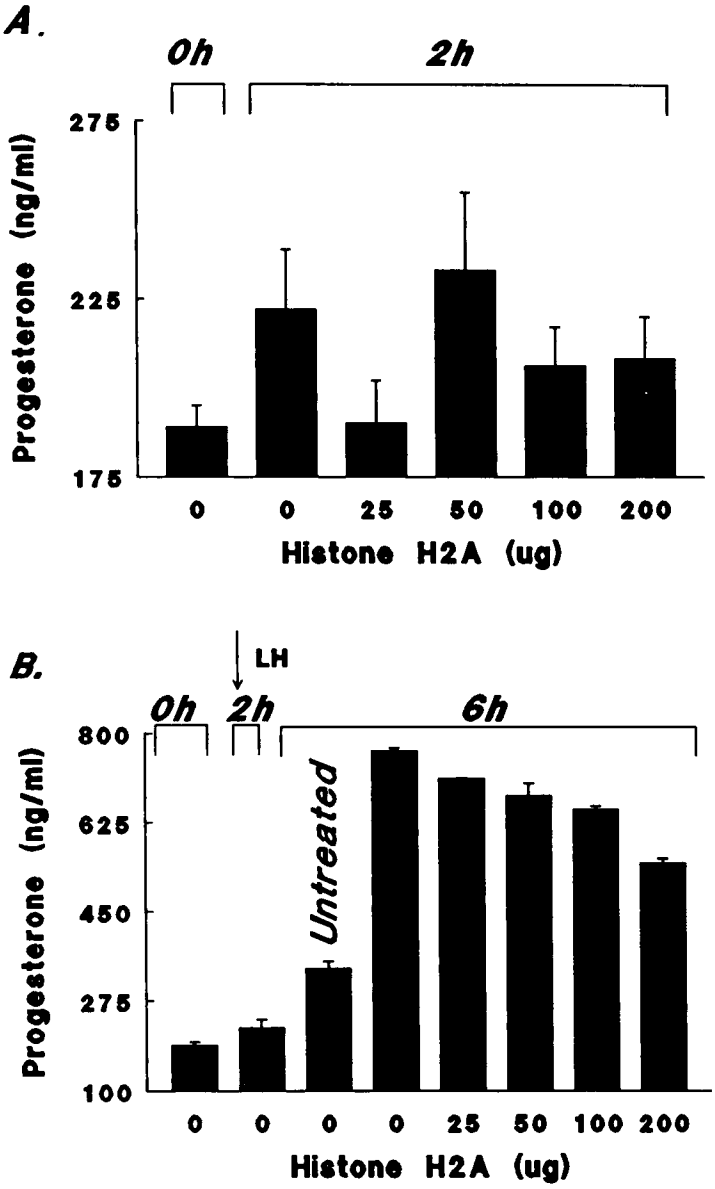

FIG. 4. Effects of calf thymus histone H2A on basal and LH-induced progesterone accumulation during culture of bovine luteal cells. Bovine luteal cells (10^6) were incubated for 2 h (basal) or 6 h (LH-induced) with and without 25–200 μg of histones. Histones were added at Time = 0 h in both groups, whereas 5 ng bLH was added at Time = 2 h to the LH-treated group. Concentrations of progesterone in medium were determined at Time = 0 h and Time = 2 h for the basal group and at Time = 0, 2, and 6 h for the LH-treated group. Bars represent mean and SEM for 4 replicate determinations of progesterone.

DISCUSSION

The most significant finding of our research was that bovine ovarian histone H2A suppressed in reversible fashion

both basal and LH-induced accumulation of progesterone during short-term culture of bovine luteal cells. Similar results were recently reported for rats [8, 9]. Our results, coupled with those for rats, support a potential antigonadotropic role for histone H2A in the bovine ovary.

In contrast to results of others in rats [10, 11], GnRH was without effect on LH-induced progesterone accumulation during culture of bovine luteal cells. This finding supports the report from another laboratory that GnRH does not suppress progesterone production in cattle [13]. Surprisingly, although GnRH antagonists block the progesterone-suppressive effect of GnRH on rat ovarian cells, they do not inhibit the progesterone-suppressive effect of histone H2A on rat ovarian cells [8, 9]. Thus, the mechanism of action of histone H2A is probably not mediated through the ovarian GnRH receptor in rats.

Whether the progesterone suppressive effect of histone H2A is mediated through specific receptor sites has been addressed. For example, in rats, histone H2A does not inhibit the interaction of epidermal growth factor with its ovarian receptor [8]. Also, spermine and spermidine, highly charged molecules similar to histone H2A, do not have progesterone-suppressive effects on rat ovarian cells [8]. In our study, BSA, added in amounts equal to the total amount of protein in the bovine ovarian histone H2A preparation, had no effect on LH-induced progesterone accumulation. Also, GnRH added in amounts equivalent to the total amount of GnRH equivalents in the histone H2A preparation did not suppress progesterone accumulation during culture of bovine luteal cells. The possibility that histone H2A has antagonistic properties on the LH receptor response system in luteal cells in cattle or other species has not been studied.

The suppression of progesterone accumulation during culture of bovine luteal cells (Figs. 1, 2) could have been the result of cytoxic effects of the large amounts of proteins in the ovarian preparation of histone H2A. This possibility seems unlikely, however, since the progesterone-suppressive effect of histone H2A on both basal and LH-induced progesterone accumulation was partially reversible. Nevertheless, a crude ovarian preparation of histone H2A could contain factors other than histone H2A that suppress progesterone accumulation. Although this possibility was not completely ruled out, a highly purified preparation of bovine ovarian histone H2A and a commercial nonovarian source of histone H2A both suppressed LH-induced progesterone accumulation in a dose-responsive fashion. Similar results have been reported for rats [8, 9]. Differences in purity of commercial calf histone H2A compared with the highly purified preparation of bovine ovarian histone H2A may explain why commercial histones did not alter basal progesterone (Fig. 4), and why ED_{50} values differed for the LH-treated groups (Fig. 3 vs. Fig. 4). In support, the commercial preparation contains several types of histones (manufacturer's specifications), whereas the highly purified bovine ovarian GnRH-BI/histone H2A preparation contains primarily histone H2A.1 and H2A.2 [8].

In conclusion, our results support the possibility that bovine ovarian histone H2A may have antigonadotropic properties in cattle, especially because of the progesterone-suppressive effect of histone H2A. Also, because bovine luteal cells do not have measurable amounts of GnRH binding sites as do those of rats [12], and because bovine luteal cells do not respond to GnRH treatment [13], our results clearly showed that an ovarian GnRH receptor is probably not required for the progesterone-suppressive effects of histone H2A.

The mechanism of action of ovarian histone H2A is unknown. Indeed, whether the progesterone-suppressive effect of histone H2A has a specific physiological role in ovarian function remains to be determined, especially since microgram amounts of the highly purified histone H2A must be added to bovine luteal cell cultures (Fig. 3) or to rat granulosal and luteal cultures [8, 9], before progesterone accumulation is suppressed. Since histones are in high abundance in the nucleus (histone mass = 0.2% of cell mass or 10% of total proteins), and in low amounts on the cell surface and in blood and milk [20–22], it is possible that microgram amounts of histone H2A are released during cell death. Therefore, it is not unreasonable to hypothesize that release of large amounts of histones during cell death would inhibit gonadotropin support of live cells and, in turn, accelerate luteolysis or atresia [8, 9].

ACKNOWLEDGMENTS

We thank Dr. Roy L. Fogwell for providing bovine tissue, Alan Ealy and Leslie Jackson for technical assistance, Trudy Hughes for critique of this manuscript, and Larry Chapin for statistical analyses.

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