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Gonadotrophin-releasing activity of histones H2A and H2B

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Abstract. We report that histones H2A and H2B possess gonadotrophin-releasing activity in vitro and assess the signal transduction pathways involved in these effects. Perifused and incubated rat anterior pituitary (AP) cells were used, and luteinizing hormone (LH) and follicle stimulating hormone (FSH) were measured by RIA. Perifusion of cells with histone H2A (30 μM) or histone H2B (30 μM), markedly stimulated LH release but failed to elicit any FSH response. Cells incubated with 6 or 30 μM histone H2A showed a dose- and time-dependent stimulatory effect on both LH and FSH release which was blocked by 1 μM peptide MB35, an 86–120 amino acid fragment of histone H2A. Incubation of pituitary cells with gonadotrophin-releasing hormone (GnRH) and histones H2A or H2B showed a stimulatory effect on LH and FSH release which was similar to the sum of the separate effects. Trifluoperazine, as well as ethylene glycol bis(b-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), alone or in the presence of the calcium ionophore A23187, significantly reduced the response of AP cells to histones. Various cyclic adenosine monophosphate (cAMP) enhancers had no effect on histone-stimulated release of gonadotrophins in incubated AP cells. Our results confirm previous evidence that histones may act as hypophysiotrophic signals. Calcium- and diacylglycerol-associated pathways, but not cAMP, appear to participate in these effects.

Key words. Histones; pituitary cells; FSH; LH; MB35; calcium; diacylglycerol.

Histones are well-characterized basic proteins known to be an essential component of the nucleosome. In recent years, a growing body of evidence has accumulated suggesting that in addition to their structural role in chromatin, histones may have hormone-like activities when present in extracellular fluids. For instance, it has been reported that histones H3 and H4 have insulin-like effects on isolated rat adipocytes [1] and that bone marrow regeneration is associated with a marked increase in the serum levels of a 14-amino acid osteogenic peptide identical to the C-terminus of histone H4 [2]. Histones H1, H2A, H2B and H3 have been reported to inhibit adenylate cyclase in canine renal cortical membrane preparations [3]. At pituitary level, homeostatic thymus hormone (HTH), an H2A-H2B histone dimer [4], was able to inhibit thyrotropin and growth hormone (GH) secretion in young rats [5]. This preparation was also active on the adrenal axis in vivo [6]. Histone H2A has been found to stimulate prolactin (PRL) release from perifused rat pituitary cells [7] which showed a reduced PRL response to nucleoproteins with the age of the pituitary donors [8]. Additionally, the thymic peptide MB35, which represents the fragment 86–120 of histone H2A, was reported to stimulate the release of PRL and GH from rat pituitary cells in culture [9].

Although to our knowledge no effects of histones on gonadotrophin secretion have been documented, a gonadotrophin-releasing hormone (GnRH) binding inhibitor from bovine ovaries has been purified and identified as histone H2A [10]. Furthermore, H2A was also reported to inhibit the binding of GnRH to its pituitary membrane receptor [11]. This finding raises the possibility that H2A may influence gonadotrophin secretion.

As a continuation of previous studies on the hypophysiotrophic activities of nucleoproteins, we undertook to

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determine whether histone H2A and histone H2B, two closely related chromosomal peptides, may act as gonadotrophin secretagogues and, if so, whether their effects are due to a ligand-specific, second-messenger mediated action or, alternatively, to nonspecific membrane phenomena related to the high positive charge of histones. The present report describes our findings.

Materials and methods

Test substances. Calf thymus histone H2A, H2B, A23187 ionophore, EGTA, poly-L-lysine (average MW = 8.8 kDa) and rat GnRH were purchased from Sigma Chemical Company, St. Louis, MO, USA. Trifluoperazine was purchased from RBI, Natick, MA, USA. Peptide MB35 was a gift of Dr. Allan L. Goldstein, Department of Biochemistry, the George Washington University Medical Center, Washington DC, USA.

Animals. Young (4 months) female Sprague-Dawley rats, kindly provided by Bagó Pharmaceuticals, City Bell, Argentina, were used as donors. Animals were housed in a temperature-controlled room (22 ± 2 °C) on a 14:10 h light/dark cycle. Food and water were available ad libitum.

Preparation of rat median eminence extracts. Median eminences (ME) were dissected by cutting a squared area around the pituitary stalk, and placing it into a homogenizer containing 1 ml/ME of 0.05 M HCl + 1% w/v ascorbic acid. After thorough homogenization, the extract was frozen and thawed three times and then submitted to three 20-s sonication bursts, leaving 10-s intervals between bursts. Finally, the homogenate was centrifuged at 10,000 rpm for 15 min, and the clear supernatant was saved and considered as undiluted ME extract (protein content = 210 ± 20 μg/ml). ME extracts did not cause any interference with the radioimmunoassays (RIAs) performed in this study.

Cell dispersion. Ten anterior pituitaries (AP) were cut with a razor blade into 8 to 10 pieces each and placed together in a Petri dish where they were washed twice with Earle's Balanced Salt Solution (EBSS) containing 1 g/l glucose, 1 g/l $NaCO_3H$, 0.5% bovine serum albumin, 30 μg/ml ascorbic acid and 50 IU/ml aprotinin (perifusion medium, PM). The pieces were transferred to a plastic tube containing 10 ml of PM with 30 mg of collagenase type IV and 1 mg DNase type I. After 1 h incubation at 37 °C under constant shaking, the cell suspension was repeatedly flushed with a Pasteur pipette to complete the dispersion process. The suspension was centrifuged at 500*g* for 20 min at 4 °C. The cell pellet was gently resuspended in 4 ml of PM, an aliquot was mixed with an equal volume of 0.4% Trypan Blue in saline, and the mix was used for the assessment of cell viability, which ranged from 90 to 98%. A second aliquot was homogenized and used to determine cell DNA content by a microfluorometric method using bisbenzimidazole (Hoechst H33258) as a fluorescent dye [12].

Cell perifusion. An average of $(4.4 \pm 1.0) \times 10^6$ freshly dispersed pituitary cells ($n = 12$) obtained as described above, were packed with Biogel P-2 into short columns (1 cm diameter; 4 cm length) kept in a water bath at 37 °C and continuously perifused with PM. The perifusion system used allowed for the simultaneous perifusion of two cell columns in parallel, which permitted an estimation of intercolumn variability within individual experiments. The substances to be tested (stimuli) were dissolved in PM and, at appropriate times, a pulse of 2.5 ml per stimulus was pumped through the perifusion circuit (flow rate = 0.7–0.8 ml/min) at the end of which 1.5-ml fractions were collected with two synchronized fraction collectors. A 1/30 ME extract, pooled from several young animals, was routinely used at the beginning and at the end of each experiment as a system performance control. The quantity of LH and FSH released by each stimulus was assessed by RIA with the materials obtained through the National Hormone and Pituitary Program (NHPP), National Institute of Diabetes, Digestive and Kidney Diseases; National Institute of Child Health and Development; United States Department of Agriculture. Hormone secretion was expressed in terms of LH RP-3 and FSH RP-2, as nanograms of hormone/μg cell DNA. Histones are known to have a negative interference on RIAs [13], a phenomenon that was confirmed by us for the case of the gonadotrophin RIAs used here. The degree of negative interference for different histones was H1 > H3 > H2A ≈ H2B (H4 not tested). Conversely, H2A and H2B were more soluble in PM than H1 and H3.

Cell incubation. Two hundred microlitres of freshly dispersed pituitary cells (5×10^5 cells) obtained as described above but from six to seven pituitaries was placed into single Eppendorf tubes containing PM. The tubes were placed in a metabolic incubator and preincubated at 37 °C for 20 min under continuous shaking. At the end of the preincubation period, tubes were centrifuged at 1000*g* for 2 min. Pellets were gently resuspended in PM containing the appropriate concentration of the different stimuli (each stimulus was tested in triplicate). Cells were further incubated for the indicated times and centrifuged at 1000*g* for 2 min. Gonadotrophin levels were measured in the supernatants and cell DNA in the pellets.

Statistics. Assessment of the level of significant differences between stimulated and basal values of LH or FSH released by the pituitary cells was carried out by one-way analysis of variance (ANOVA), while time course experiments were assessed by two-way ANOVA.

When significant, the ANOVAs were followed by Duncan's multiple range test to assess the significance of differences between means. A P value lower than 0.05 was considered to represent a significant difference.

Results

Dynamic studies with perifused cells. In perifusion experiments both histone H2A and H2B induced sharp LH secretory peaks, while peptide MB35 failed to elicit any significant hormonal response at the dose tested. Neither peptide MB35 nor histones H2A or H2B generated any significant secretory peak of FSH (fig. 1). The LH and FSH secretory responses to pulses of ME extract run through the columns at the beginning and at the end of perifusions were comparable, indicating a preserved function of cells throughout the experiments.

Time course experiments. Incubation experiments were carried out in order to expose cells for longer times than in perifusion studies, to different stimuli. Histone H2A stimulated LH and FSH release in a time- and dose-dependent manner (fig. 2). The same pattern of LH or FSH responses was obtained with 1, 6 and 30 μM histone H2B (data not shown). When the cells were incubated with 1 μM peptide MB35 for up to 40 min, no significant release of LH or FSH was observed, but after 3 h of incubation with MB35, a stimulatory effect was detected (fig. 2). Despite its lack of effect on gonadotrophin release in short-term incubation, 1 μM peptide MB35 was able to block completely the release of both LH and FSH induced by 6 and 30 μM histone H2A.

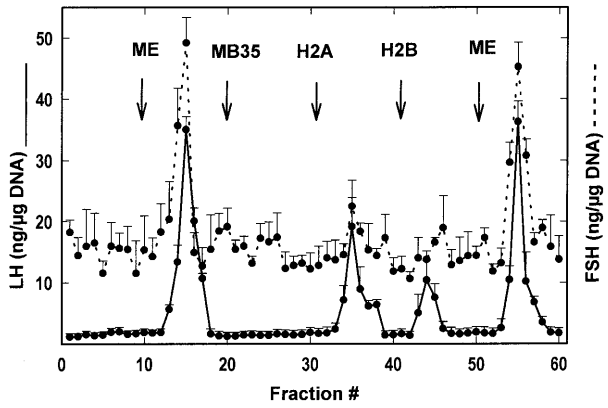

Figure 1. Luteinizing hormone and FSH release profiles in rat AP cells perifused with histone H2A (30 μM), histone H2B (30 μM), peptide MB-35 (1 μM) and ME extract (1/30 v/v). Arrows indicate the point at which the corresponding stimuli were applied. Data points and bars represent the mean and SEM, respectively, of 12 perifusion experiments.

Interaction of histones with GnRH. When cells were incubated with 6 μM histone H2A, 6 μM histone H2B or 8 μM poly-L-lysine in the presence or not of 10^{-2} μM GnRH, both histones showed a stimulatory effect on LH and FSH release which was similar to the sum of the separate effects (table 1). Poly-L-lysine was unable to exert any stimulatory effect on either LH or FSH release, nor did it affect the release of gonadotrophins induced by GnRH. In perifusion experiments, 1 μM histone H1 and H3 did not appear to affect gonadotrophin release (data not shown). However, because of the low solubility of these histones and the significant negative interference that they exert on our RIA, a lack of effect on gonadotrophin release cannot be assured from our experiments.

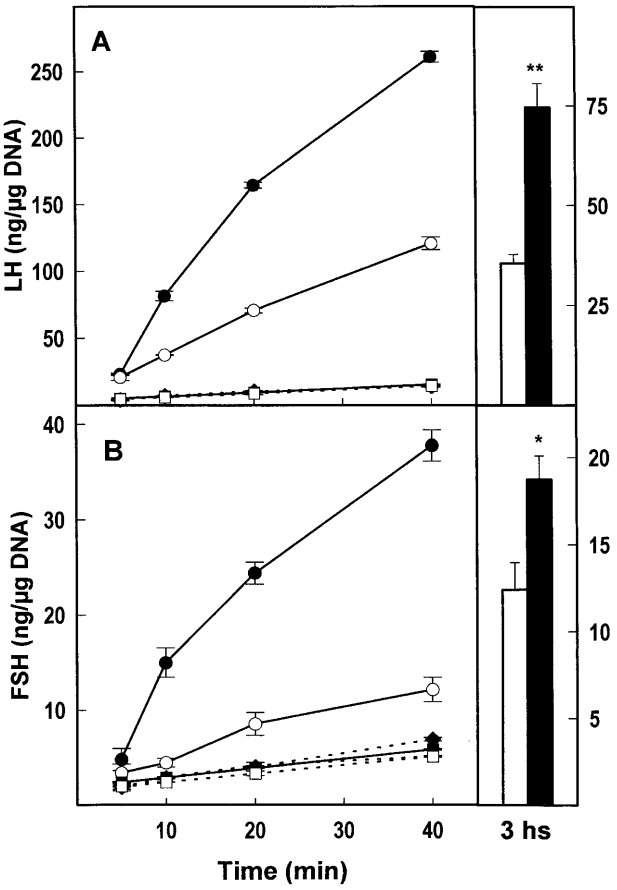

Figure 2. Time course for LH (A) and FSH (B) release by AP cells incubated with 30 μM histone H2A (●——●), 6 μM histone H2A (○——○), 1 μM peptide MB-35 (◆----◆), 30 μM histone H2A plus 1 μM peptide MB-35 (■ · · · · · · ■), 6 μM histone H2A plus 1 μM peptide MB-35 (□ · · · · · · □), and medium alone (----). Data points represent the mean of six sets of incubation experiments. Error bars are indicated only when they are larger than symbols. Insets: Hormone release after incubation of cells for 3 h with medium alone (open column) or 1 μM peptide MB-35 (solid column). * $P < 0.05$; ** $P < 0.01$, as compared with medium alone.

Table 1. Specificity of histones and interaction with GnRH.

Stimuli	LH (ng/μg DNA)	FSH (ng/μg DNA)
Medium	4.02 ± 0.35 (6)	2.42 ± 0.13 (6)
GnRH 10^{-2} μM	70.22 ± 2.87 (6)*	16.25 ± 1.43 (6)*
H2A 6 μM	120.00 ± 2.15 (6)*	34.15 ± 2.21 (6)*
H2B 6 μM	115.36 ± 2.73 (6)*	26.87 ± 1.15 (6)*
Poly-L-lysine 8 μM	3.98 ± 0.12 (6)	2.51 ± 0.25 (6)
H2A 6 μM + GnRH 10^{-2} μM	191.62 ± 4.16 (6)*	46.23 ± 2.15 (6)*
H2B 6 μM + GnRH 10^{-2} μM	145.12 ± 2.11 (6)*	40.01 ± 1.97 (6)*
Poly-L-lysine 8 μM + GnRH 10^{-2} μM	72.18 ± 2.74 (6)*	18.14 ± 1.16 (6)*

Amount of LH or FSH released by AP cells incubated for 40 min with either medium, 6 μM histone H2A, 6 μM histone H2B or 8 μM poly-L-lysine in the presence or absence of 10^{-2} μM GnRH. Data represent the mean ± SEM of six sets of incubation experiments. * $P < 0.01$ as compared with the respective basal level.

Signal transduction pathways involved in the secretagogue activity of histones. The incubation of AP cells with the calcium chelator EGTA completely blocked the stimulatory effect of histones H2A and H2B on LH and FSH release (fig. 3). Incubation of AP cells in the presence of the calcium ionophore A23187 had no effect on gonadotrophin secretion.

The protein kinase C inhibitor trifluoperazine (TFP) was also able to block the gonadotrophin response of AP cells incubated with histones H2A and H2B (fig. 3). Neither basal nor histone-stimulated gonadotrophin release was significantly affected by the presence of the ionophore A23187 in the secretion medium.

The adenylate cyclase activator forskolin, but not its inactive derivative 1,9-dideoxyforskolin, increased gonadotrophin release in nonstimulated AP cells but had no effect on hormone release in histone-stimulated AP cells (fig. 4). Caffeine and NaF, two intracellular cAMP enhancers which act at proximal and distal sites, respectively, of the cAMP pathway, were without effect on histone-stimulated gonadotrophin release (fig. 4).

Discussion

The finding that gonadotrophic cells are able to respond to histones H2A and H2B is in line with previous studies showing that histones and nucleohistones can act directly on perifused pituitary cells to stimulate the release of adrenocorticotrophic hormone [14] and PRL [7]. In perifusion, the clearance rate is equal to the flow rate of the system. This rate is the same for all secreted products, and there is no influence of cell-secreted products [15]. Therefore, it is unlikely that the LH-releasing activity of histones H2A and H2B in our pituitary cell preparations may have been exerted through a paracrine influence on gonadotrophs by factors released from nongonadotrophic cells in response to these nuclear proteins. The lack of FSH response to histones in perifusion but not in incubation experiments may be due to a reaction time of FSH which is too slow to respond to the rapid histone pulses applied during perifusion.

Our finding that highly positively charged peptides such as poly-L-lysine, with a molecular size similar to that of H2A and H2B, fail to stimulate either LH or FSH release suggests that the secretagogue action of histones H2A and H2B on gonadotrophins is not due to nonspecific electrostatic charge effects. This is in agreement with the previously reported observation that the basic peptides spermine and spermidine mimic some actions of the histone H2A only at concentrations 100–1000 times higher than those used for H2A [10]. Our incubation studies additionally suggest that the effect of H2A and H2B on gonadotrophin secretion may be a receptor-mediated event. This is based on two observations: (i) both histones were able to stimulate the release of LH and FSH in a dose-dependent manner, and (ii) the effectiveness of the synthetic peptide MB35 to block the effects of H2A on gonadotrophin release, which suggests that MB35 binds with high affinity to a putative histone H2A receptor but is less effective than H2A to elicit its biological effect. Indeed, we were able to show a gonadotrophin-releasing activity of MB35 only after 3 h incubation. At shorter times or in perifusion experiments, MB35, unlike H2A, its parent molecule, did not affect basal LH release. It has been previously reported that histone H2A is capable of inhibiting the binding of GnRH to rat AP cell membranes [11]. However, the same study showed that in FSH-stimulated rat granulosa cells, which do not possess GnRH receptors, H2A was able to specifically inhibit cAMP production and progesterone release. Moreover, a chemically modified GnRH molecule which blocks the effects of GnRH, did not block those of histone H2A in LH-stimulated luteal cells [16]. Therefore, histone H2A should act, at least in part, via a GnRH receptor-independent mechanism. It should be pointed out that although our data suggest that H2A and H2B release gonadotrophins by a receptor-mediated mechanism, they do not prove so, and

therefore the possibility of a receptor-independent action of histones remains open. Likewise, because of the technical difficulties indicated in the 'Results' section, we cannot rule out the possibility that other histones may also stimulate gonadotrophin release.

The stimulation of gonadotrophin release by GnRH is known to be mediated by increased intracellular calcium as well as by increased intracellular levels of diacylglycerol (DAG) and inositol triphosphate (IP_3). The latter induces a rhythmic release of calcium from an IP_3-sensitive store which is coupled to a GnRH-stimulated gonadotrophin burst of exocytosis [17–19]. Our finding that TFP, an inhibitor of protein kinase C, blocked the gonadotrophin response of pituitary cells to histones H2A and H2B suggests that the DAG pathway is involved in the gonadotrophin-releasing action of H2A and H2B. The additive effect of H2A and H2B with GnRH, at submaximal doses, is in line with this hypothesis, which is also supported by the report that incremented levels of DAG exerted an additive effect on LH release stimulated by submaximal doses of GnRH [18].

The fact that the adenylate cyclase activator forskolin failed to influence histone-(submaximally) stimulated gonadotrophin release from AP cells suggests that, in addition to their stimulatory actions on hormone release, histones may concomitantly block the cAMP signal transduction pathway. This hypothesis is further supported by the fact that the inhibition of phosphodiesterase with caffeine or the activation of protein G_S with NaF did not enhance LH or FSH release by H2A and H2B.

It has been reported that histone H2A possesses antigonadotrophic effects when acting on gonadotropin-stimulated ovary cells, but the mechanisms of action appear to be different in LH-stimulated luteal cells

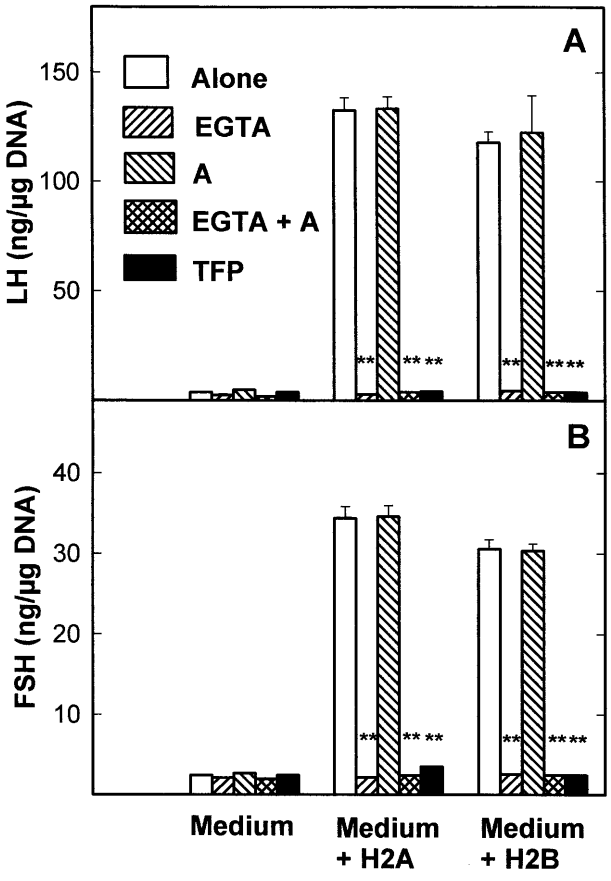

Figure 3. Amount of LH (*A*) and FSH (*B*) released by AP cells incubated for 40 min with either medium, 6 µM histone H2A or 6 µM histone H2B in the presence or absence of 5 mM EGTA, 5 µM A23187 ionophore, 5 mM EGTA + 5 µM A23187 ionophore or 15 mM TFP, as indicated in the figure. Data points represent the average of six sets of incubation experiments. The significance of the effect of the added substances on the secretagogue action of histones is indicated by * $P < 0.05$ or ** $P < 0.01$.

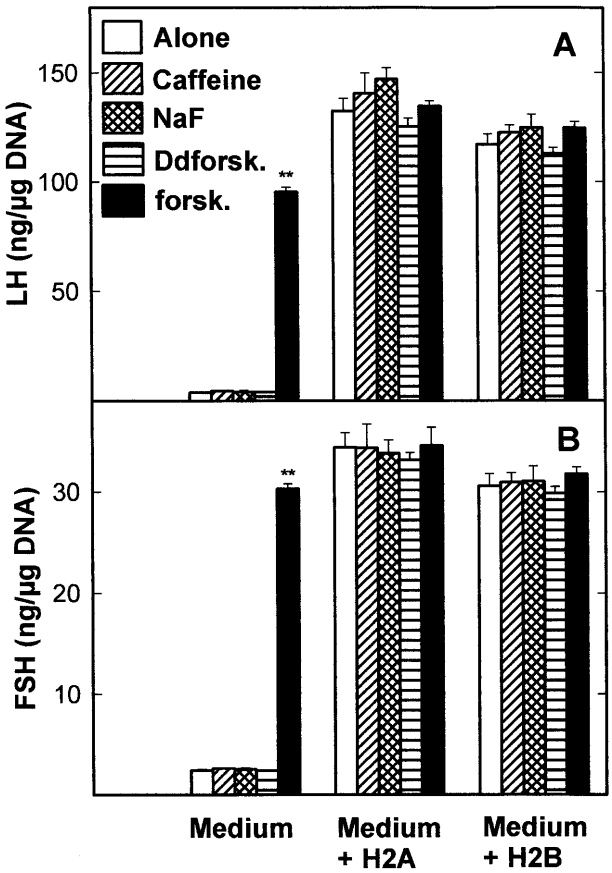

Figure 4. Amount of LH (*A*) and FSH (*B*) released by AP cells incubated for 40 min with either medium alone, 6 µM histone H2A or 6 µM histone H2B in the presence or absence of 10 mM caffeine, 10 mM NaF, 30 µM 1,9-dideoxyforskolin or 30 µM forskolin, as indicated in the figure. Other details are as in figure 3.

from those in FSH-stimulated granulosa cells. In the former, both GnRH and histone H2A reversibly inhibited cAMP production, although histone H2A was more effective and faster than GnRH and may involve both a noncompetitive inhibition of LH union to its receptor and inhibition of G protein activation, whereas the histone inhibition of both cAMP and progesterone production in FSH-stimulated granulosa cells may be due to the inhibition of adenylate cyclase [11, 16]. Our results with forskolin are also in line with those that report that histone H2A inhibited selectively, reversibly and in a dose-dependent manner cAMP and testosterone production by human chorionic gonadotrophin (hCG)-stimulated Leydig cells, but did not show any effect in nonstimulated cells or stimulated cells with other bioactive peptides such as atrial natriuretic peptide [20].

Our results with EGTA and the ionophore A23187 indicate that the gonadotrophin-releasing activity of histones H2A and H2B is dependent on the presence of appropriate levels of extra and intracellular calcium rather than on calcium transport mechanisms. This is in line with the finding that removal of the extracellular/intracellular calcium gradient by calcium chelators completely abolished the activation of nitric oxide synthase by histones in intact neuronlike neuroblastoma N1E-115 cells [21].

Taken together, the data on the intracellular pathways activated by histones H2A/H2B and GnRH, as well as the additive effects of these molecules on gonadotrophin release, suggest that histones and GnRH may act on similar signal transduction pathways via separate receptors. Whether the action of histones involves other second messengers such as cyclic guanosine monophosphate (cGMP) remains to be investigated.

Although it is not clear at present whether nuclear proteins have a physiological role as extracellular messengers, there is evidence indicating that chromatin fragments can specifically bind to the plasma membrane of leukocytes and other cell types [22, 23]. Furthermore, it has been reported that the mono- and oligonucleosomes released by spleen and thymic T cells undergoing programmed death in short-term tissue culture have mitogenic and polyclonal effects on normal B lymphocytes [24]. This results in a generalized enhancement of immunoglobulin synthesis and anti-DNA antibody responses in vitro. More recently, it has been reported that nucleosomes or histones can inhibit natural killer (NK) cell activity in vitro, and that in cancer patients with abnormally high circulating levels of nucleosomal DNA (i.e. mono- and oligonucleosomes), NK activity is depressed [25].

Nucleosomal release is a hallmark of programmed cell death [26]. Therefore, it is possible that during physiological or pathological processes involving massive programmed cell death, the nucleoproteins released by the dying cells may convey important 'apoptotic signals' to integrative systems of the body such as the neuroendocrine and immune networks. We are currently conducting systematic experiments in order to test this hypothesis in the neuroendocrine system.

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