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Hypophysotropic activity of histone H3 in vitro

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Abstract

To assess the effect of histone H3 on pituitary hormone secretion, rat anterior pituitary (AP) cells were used and growth hormone, prolactin, thyrotropin, luteinizing hormone and follicle stimulating hormone measured by radioimmunoassay. Incubation of cells with H3 (1, 6, and 30 μM) stimulated the release of all five hormones in a dose-dependent manner. This effect was blocked by preincubation of H3 with an anti-H3 antibody. Incubation of AP cells with 6 μM H3 in the presence of specific AP hormone secretagogues (GRP-6, thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormone (GnRH)) showed additive effects on hormone secretion. Pharmacological experiments suggested that calcium- and diacylglycerol- (DAG) associated pathways, but not cAMP, participate in the hypophysiotropic activity of H3. Our results confirm previous evidence that histones may act as hypophysiotropic signals.

Keywords: Histones; Hypophysotropic activity; Pituitary cells; Second messengers; Apoptotic signals

1. Introduction

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 possess 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 [28] 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 [34]. A gonadotropin-releasing hormone (GnRH) binding inhibitor from bovine ovaries has been purified and identified as histone H2A [1]. At pituitary level, histone H2A was found to decrease the basal secretion of luteinizing hormone (LH) by bovine pituitary cells in vitro [33]. Furthermore, H2A was also reported to inhibit the binding of GnRH to its pituitary membrane receptor [27]. Homeostatic thymus hormone (HTH), an H2A–H2B histone dimer [31], was able to inhibit thyrotropin (TSH) and growth hormone (GH) secretion in rats [17]. This preparation was also active on the adrenal axis in vivo [18]. Histone H2A has been found to stimulate prolactin (PRL) release from perifused rat pituitary cells [7]. Additionally, the thymic peptide MB35, representing the fragment 86–120 of histone H2A, was reported to stimulate the release of PRL and GH from rat pituitary cells in culture [3]. More recent studies showed that separately, histone H2A and histone H2B have PRL-, TSH-, GH-, LH-, and FSH-releasing activity on the pituitary in vitro and suggested that such activity may be mediated by specific cell receptors [8–11]. As a continuation of a systematic characterization of the hypophysiotropic activities of nucleoproteins, we undertook to determine whether histone H3 may also modulate anterior pituitary (AP) hormone secretion and, if so, whether its 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.

2. Materials and methods

2.1. Test substances

Calf thymus histone H3, A23187 ionophore, EGTA, caffeine, poly-L-lysine (poly-lys) MW = 8.8 kDa, rat GnRH, rat thyrotropin-releasing hormone (TRH) and growth hormone-releasing hormone (GH-RH) were purchased from Sigma Chemical Company (St. Louis, MO). Trifluoperazine (TFP) was purchased from RBI (Natick,

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MA). GH-releasing peptide-6 (GHRP-6) was a kind gift from Carlos Dieguez Department of Physiology, Faculty of Medicine, University of Santiago de Compostela, Spain. Specific TNT-2 anti-histone H3 monoclonal antibody [29], was a kind gift from Alan Epstein, Department of Pathology, School of Medicine, University of Southern California, USA.

2.2. Animals

Female Sprague–Dawley rats, kindly provided by Bagó Pharmaceuticals, City Bell, Argentina, were used as donors. Animals were housed in a temperature-controlled room ($22 \pm 2\,^{\circ}C$) on a 14/10 h light/dark cycle. Food and water were available ad libitum.

2.3. Cell dispersion

In each incubation experiment, 10 APs were cut with a razor blade into 8–10 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 (incubation medium (IM)). The pieces were transferred to a plastic tube containing 10 ml IM with 30 mg 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 $500 \times g$ for 20 min at 4 °C. The cell pellet was gently resuspended in 4 ml IM, and an aliquot mixed with an equal volume of 0.4% Trypan Blue in saline, and the mix 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].

2.4. Cell incubation

Two hundred microliters of freshly dispersed pituitary cells obtained as described earlier were placed into single Eppendorf tubes containing IM. 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 $1000 \times g$ for 2 min. Pellets were gently resuspended in PM containing the appropriate concentration of the different stimuli (each stimulus was tested in quadruplicate). Cells were further incubated for the indicated times and centrifuged at $1000 \times g$ for 2 min. Hormone levels were measured in the supernatants by radioimmunoassay (RIA) with the materials provided by Dr. A.F. Parlow, Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA, USA. Hormone secretion was expressed in terms of nanograms of hormone per microgram cell DNA.

2.5. Experimental design

In the experiments described in each of the three figures of this paper, six sets of incubations were performed. In each incubation, individual data points were the average result of quadruplicate incubation tubes. The average result of each of these quadruplicates was considered as a single data point for that particular incubation set. Since six sets of incubation experiments were carried out for each type of experimental assessment (i.e. Figs. 1–3), the number of replicates for the different experiments of this study was considered to be $n = 6$. (For further details, see the corresponding figure legends.)

2.6. Statistics

Assessment of the level of significance of differences between stimulated and basal values of the different hormones released by the pituitary cells was carried out by one-way ANOVA, while time course experiments were assessed by two-way ANOVA. When significant, the ANOVAs were followed by the 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.

3. Results

3.1. Hypophysiotropic activities of histone H3 and their immunological blockade

When AP cells were incubated with 1, 6, and 30 μM histone H3 during 40 min, a significant ($P < 0.001$) dose-dependent release of GH, PRL, LH, FSH, and TSH was observed (Fig. 1). The same pattern was obtained with 10- and 20-min incubations (data not shown). Preincubation of histone H3 with 1/50 dilution of TNT-2 antibody during 3 h at 37 °C, completely abolished the secretagogue action of H3 at the three concentrations tested. The same dilution of the antibody alone did not affect the basal secretion of GH, PRL, LH, FSH, and TSH (Fig. 1).

3.2. Interaction of histone H3 with different hypophysiotropic peptides

Incubation of AP cells with 6 μM histone H3 alone or in the presence of either 0.1 μM GHRH or 1.0 μM GHRP-6 revealed different interactions between H3 and these GH secretagogues. While H3 plus GHRP-6 induced an additive effect on GH secretion, the resulting effect of the co-incubation of the histone with GHRH was synergistic (Fig. 2).

Both 6 μM histone H3 and 0.1 μM TRH induced a significant release of both PRL and TSH. Incubation of AP

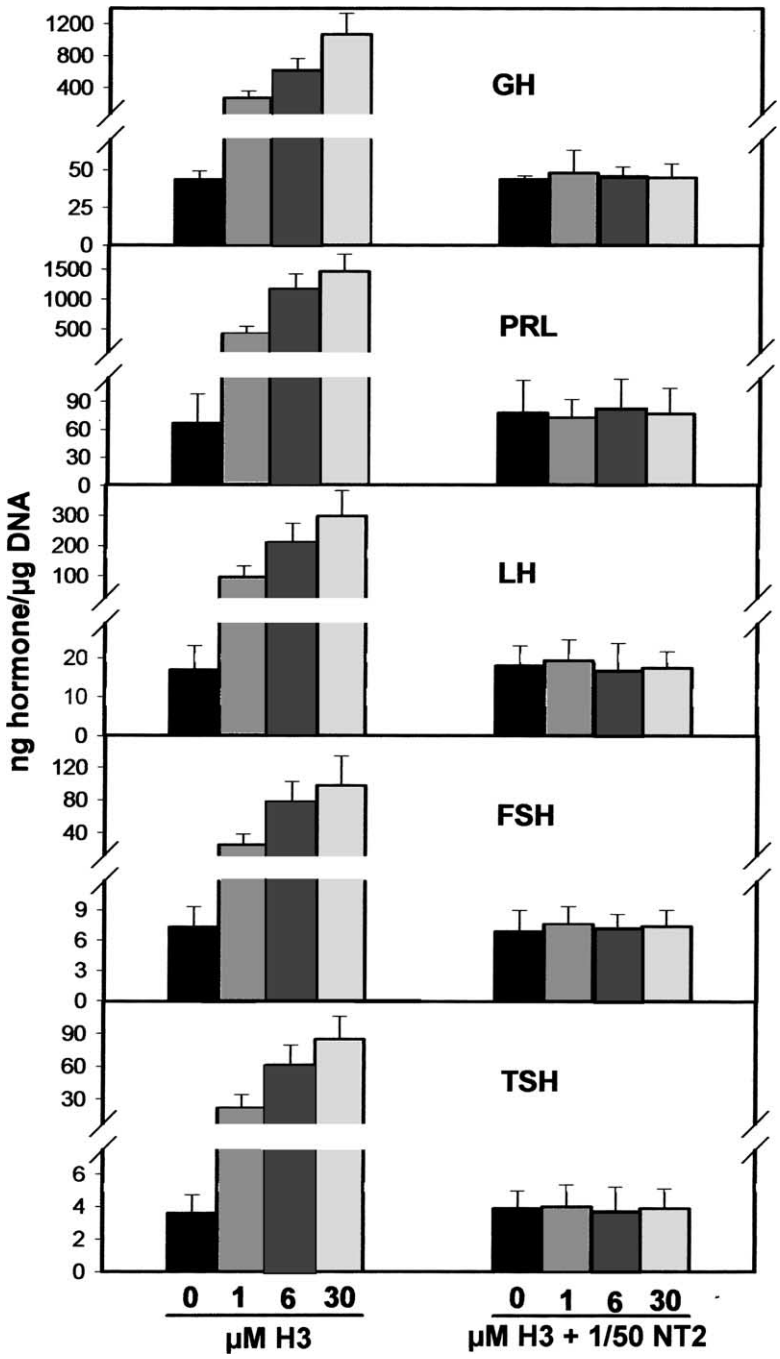

Fig. 1. Multiple pituitary hormone-releasing activity of H3. Mass of GH, PRL, LH, FSH, and TSH released by rat pituitary cells incubated for 40 min with the either: 0, 1, 6, and 30 μM histone H3 alone or previously incubated with a 1/50 dilution of antibody NT2. Data bars represent the mean ± S.E.M. of six sets of incubation experiments.

cells with histone H3 plus TRH induced an additive release of both PRL and TSH. Also, incubation of AP cells with the histone plus GnRH induced an additive release of both gonadotropins (Fig. 2).

Poly-lys (8 μM) had neither a secretagogue effect on AP cells, nor was it able to modify the magnitude of the stimulatory action of GHRH, GHRP-6, TRH and GnRH on their target hormones.

3.3. Signal transduction pathways involved in the secretagogue activity of histone H3

The calcium chelator EGTA alone (5 mM) or associated with ionophore A23187 (5 μM) completely blocked the stimulatory effect of histone H3 on GH, PRL, LH, FSH, and TSH release. Neither basal nor histone H3-stimulated hormone release was significantly affected by the presence

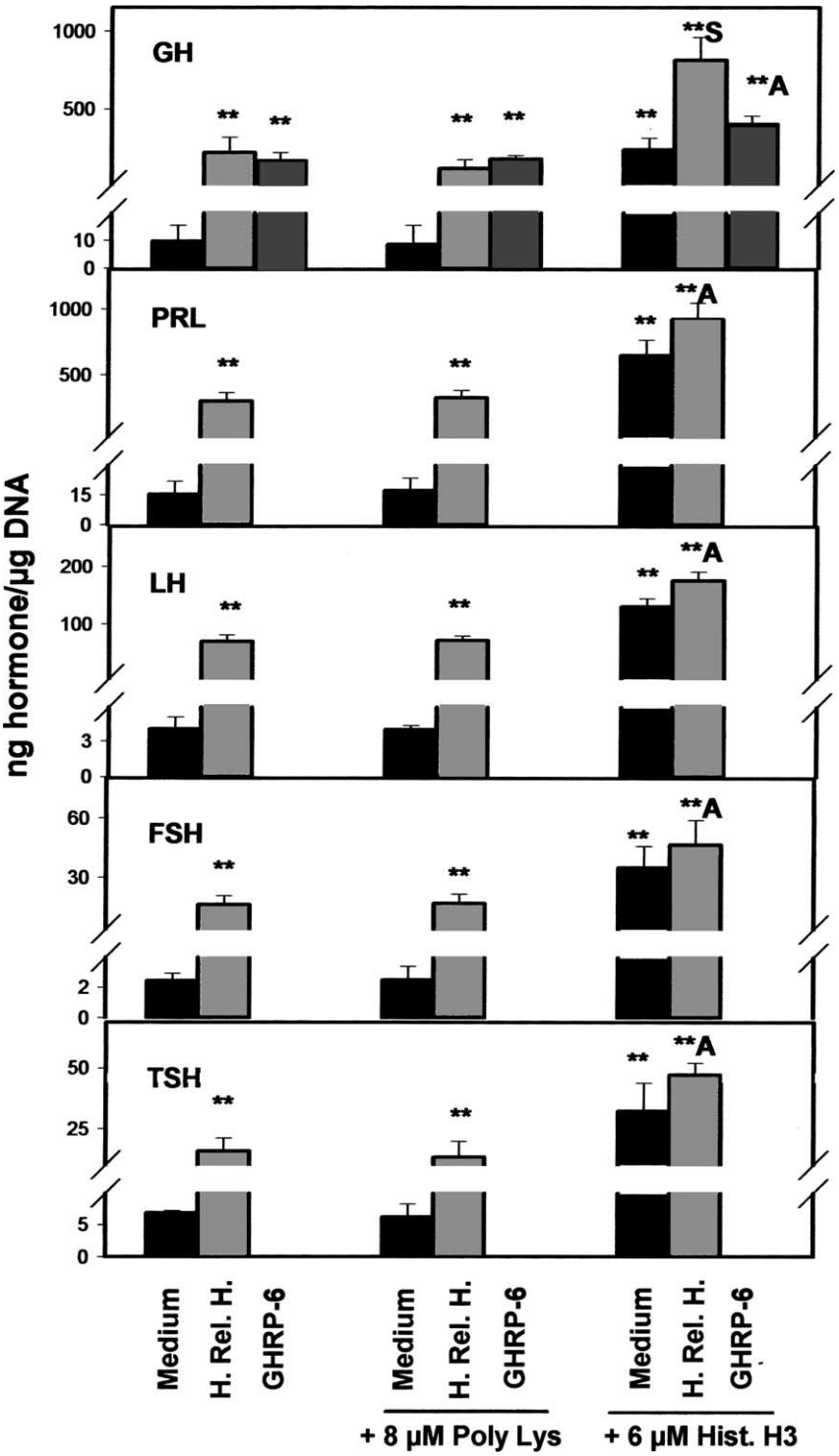

Fig. 2. Interactions of H3 with other pituitary hormone secretagogues. Mass of GH, PRL, LH, FSH, and TSH released by rat pituitary cells incubated for 40 min with the either: medium, 8 μM poly-lys or 6 μM histone H3 alone or plus hormone-releasing hormone (H. Rel. H.) or 1 μM GHRP-6. H. Rel. H. = 0.1 μM GHRH for GH, 0.1 μM TRH for PRL and TSH, and 0.01 μM GnRH for LH and FSH. Data bars represent the mean ± S.E.M. of six sets of incubation experiments. $^{**}P < 0.01$ when compared the effect with the medium alone. A: additive effect and S: synergistic effect when compared the stimuli alone with stimuli plus histone.

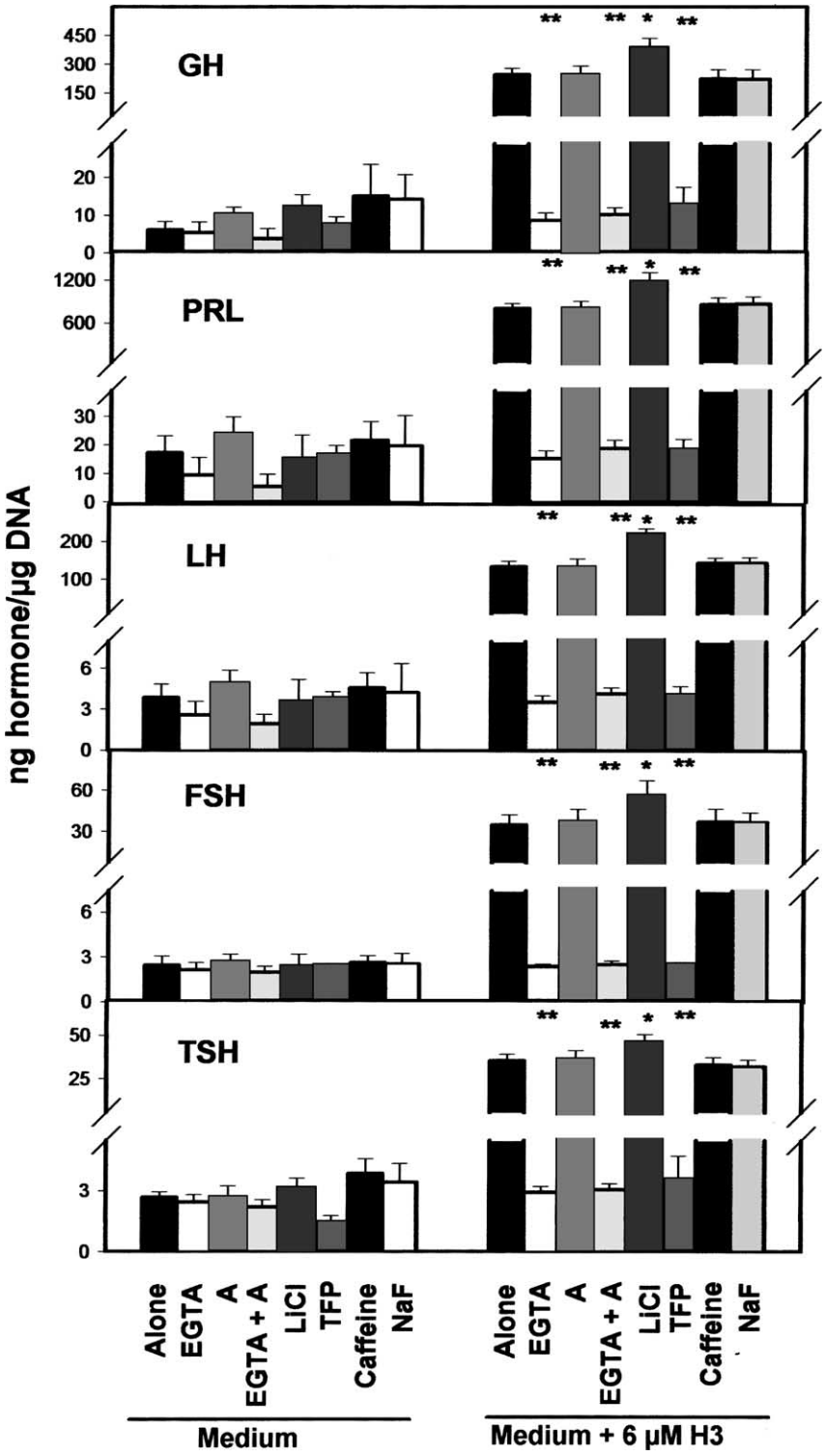

Fig. 3. Signal transduction pathways activated by H3 in pituitary cells. Amount of GH, PRL, LH, FSH, and TSH released by rat pituitary cells incubated for 40 min with the either: medium or 6 μM histone H3. Cells were incubated alone or in combination with: 5 mM EGTA, 5 μM A23187 ionophore, 5 mM EGTA + 5 μM A23187 ionophore, 10 mM LiCl, 10 mM caffeine, 10 mM NaF or 15 mM TFP, as indicated on the figure. Data points and bars represent the mean ± S.E.M. of six sets of incubation experiments. Effect of the substance in relation to the cells stimulated with the histone: $^{*}P < 0.05$, $^{**}P < 0.01$.

of the ionophore A23187 in the secretion medium (Fig. 3).

The protein kinase C (PKC) inhibitor TFP (15 mM) significantly inhibited the stimulatory activity of H3 on the above AP hormones. The inhibition of the inositol triphosphate (IP_3) degradative pathway by LiCl (10 mM) caused a significant enhancement of histone-induced AP hormone release (Fig. 3).

Caffeine (10 mM) and NaF (10 mM), two intracellular cAMP enhancers which act at proximal and distal sites, respectively, of the cAMP pathway, were without effect on histone H3-stimulated GH, PRL, LH, FSH, and TSH release (Fig. 3).

4. Discussion

Our finding that histone H3 may act directly on AP cells stimulating the release of GH, PRL, TSH, and gonadotropins is in line with previous reports on the multiple releasing activity of other histones on different AP hormones. Thus, histones H2A and H2B were reported to act directly on AP cells to stimulate the release of adrenocorticotropic hormone [19], PRL [7,11], GH [9], TSH [8], and gonadotropins [10,33].

Our incubation studies suggest, although do not prove, that the effect of H3 on AP hormone secretion is a receptor-mediated event. This is based on two observations: (a) Histone H3 was able to stimulate the release of GH, PRL, LH, FSH, and TSH in a dose-dependent manner. (b) The effectiveness of monoclonal anti-histone H3 to block the hormone release by the histone at all doses tested.

Our finding that a highly positively charged peptide like poly-lys, which has a similar molecular weight than histone H3, fails to stimulate AP hormone indicates that the secretagogue action of histone H3 is not due to nonspecific electrostatic charge effects. The observation that the basic peptides spermine and spermidine mimic some actions of histones only at concentrations 100–1000 times higher than those used for histone H3 [1] strengthens the hypothesis of a specific action of H3 on AP cells.

The synergistic effect of histone H3 with GHRH, whose action is known to be mediated by cAMP [15], suggests that histone H3 and GHRH act on GH release via separate mechanisms.

Our pharmacological experiments suggest that histone H3 may share at least some steps of the intracellular pathway that mediates the action of GHRP-6 on GH release, which is known to be effected by an increase in intracellular Ca^{2+} and involves phosphoinositides and activation of PKC by diacylglycerol (DAG) [13].

Thyrotropin release induced by TRH is mediated by DAG and cAMP, while TRH stimulates PRL via the phosphoinositide pathway [16]. The additive response observed here between submaximal doses of H3 and TRH suggests that histone H3 may share at least some steps of the intracellular pathway that mediates the action of TRH on AP cells. The stimulation of gonadotropin release by GnRH is known to be mediated by increased DAG and IP_3 levels [6,26,32]. Our data suggest that histone H3 may also act via the DAG–IP_3 pathways (also see below).

Our finding that TFP, an inhibitor of PKC, strongly reduced the response of pituitary cells to histone H3, while LiCl, known to inhibit degradation of IP_3, enhanced it, suggests that the DAG–IP_3 pathways are significantly involved in the multiple AP hormone-releasing action of H3. The fact that the inhibition of phosphodiesterase with caffeine or the activation of protein G_S with NaF did not enhance the AP hormone-releasing activity of histone H3 suggests that cAMP may not be involved in the secretagogue activity of H3.

In addition, our results with EGTA and the ionophore A23187 indicate that the AP hormone-releasing activity of histone H3 is dependent on the presence of appropriate levels of extracellular calcium rather than on calcium transport mechanisms.

Whether the actions of histone H3 involve other second messengers such as cGMP or prostaglandins remains to be investigated.

Although it is not clear at present whether nuclear proteins may 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 [4,5,22–25,30]. Furthermore, it has been reported that the mononucleosomes 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 Ig synthesis and anti-DNA antibody responses in vitro [5]. 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. mononucleosomes and oligonucleosomes), NK activity is depressed [25]. Also supportive of the idea that histones may act as extracellular messengers is the fact that, in the receptive stage of the luteal phase, the human endometrium releases, along with several other proteins, histones H2A, H2B, H3, and H4 [4]. Two days after dexamethasone administration in the drinking water of rats, a significant increase occurs in plasma levels of nucleosomal DNA, which peaks on day 4 [21]. This DNA is mainly of thymic origin where dexamethasone induces massive thymocyte death. It should be mentioned that preliminary studies in which we i.p. injected different purified histones (including H3) in young rats, failed to reveal any changes in serum PRL and GH levels, as measured by RIA (unpublished results). Although it is not clear why purified histones failed to stimulate pituitary hormone release in vivo, two reasons can be suggested: (a) Histones, but not nucleohistones, are rapidly degraded in serum and other biological fluids. (b) In high concentrations, histones exert a negative interference with RIAs which causes a underestimation of actual serum hormone

concentrations and could therefore mask small increases in GH and PRL levels [20].

Clearly, in vivo confirmatory data are necessary to firmly establish a physiological role for extracellular histones as hypophysiotropic signals. Nevertheless, the fact that nucleosomal release is a hallmark of programmed cell death [14], makes it attractive the idea 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.

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