Prolactin Storage in a Clonal Strain of Rat Pituitary Tumor Cells Is Cell-cycle Dependent

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ABSTRACT GH_4C_1 cells (GH cells) are a clonal strain of rat pituitary tumor cells which secrete prolactin. We measured intracellular prolactin at different stages of the cell cycle using flow microfluorometry. Prolactin was stained by an indirect immunocytochemical technique using fluorescein isothiocyanate (FITC)-conjugated antiserum, and DNA was stained simultaneously with propidium iodide. We found that prolactin storage in GH cells was cell-cycle dependent; prolactin storage increased as cells passed from G_1 to S to $G_2 + M$. We have shown previously that insulin and 17β-estradiol act synergistically to increase intracellular prolactin three- to sevenfold and slow the rate of cell growth to ~70% of control cells. In this study we observed that insulin and estradiol increased prolactin storage at each stage of the cell cycle but did not affect the cell-cycle distribution of the population even though cell growth was slowed. We conclude that insulin and estradiol did not increase prolactin storage by affecting the cell-cycle distribution of the population.

GH_4C_1 cells (GH cells) are a clonal strain of rat pituitary tumor cells known to secrete prolactin (1, 2). Immunocytochemical analysis of GH-cell prolactin content by Hoyt and Tashjian (3) showed that, although these cells are a clonal strain, they are heterogeneous with respect to the amount of prolactin stored within them. Use of two fluorescent dyes which have similar excitation wavelengths but optically separable emmission peaks to stain GH cells simultaneously for intracellular prolactin and DNA followed by analysis with a fluorescence-activated cell sorter allows correlations to be made between prolactin storage and the cell cycle. These correlations can be made without manipulations to induce synchronous cell populations. We decided to examine GH cells using flow microfluorometry to determine whether their heterogeneity in prolactin content is due to a cell-cycle dependence of prolactin storage. We found that prolactin storage in GH cells was cell-cycle dependent; prolactin storage increased as cells passed from G_1 to M.

Insulin and 17β-estradiol synergistically increase intracellular prolactin three- to sevenfold and slow the rate of cell growth (4). Since prolactin storage was cell-cycle dependent, the hormone-induced increase in prolactin storage could be due to a change in the cell-cycle distribution of the population. Using flow microfluorometric analysis, we determined that insulin and estradiol did not increase prolactin storage by affecting the cell-cycle distribution of the population.

MATERIALS AND METHODS

We used culture medium and serum from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY). Rat prolactin used for production of rabbit antiserum and as a standard for microcomplement fixation was supplied by Dr. A. F. Parlow of the hormone distribution program of the National Institute of Arthritis, Metabolism, and Digestive Diseases (Bethesda, MD). Bovine pancreatic insulin, 17β-estradiol, bovine pancreatic ribonuclease A, propidium iodide, and acriflavine were obtained from Sigma Chemical Co. (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was obtained from Miles Laboratories, Inc. (Elkhart, IN).

Cell Culture

We grew GH cells as monolayers in Ham's F10 medium supplemented with 2.5% fetal bovine serum and 15% horse serum. Cultures used for cell number and intracellular prolactin determinations were plated in triplicate at an initial density of 10^5 cells/35-mm diameter tissue culture well; cells used for microfluorometric analysis were seeded at an initial density of 1.53×10^5 cells/150-cm^2 tissue culture flask. 2 d after plating, control cells received fresh medium and treated cells received fresh medium containing 300-nM insulin and 1-nM 17β-estradiol. We applied fresh medium and hormones every 2 d for 6 d.

Measurement of Intracellular Prolactin and Cell Number

GH cells used for measurement of intracellular prolactin were collected and cell number was determined using a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL) as previously described (4). We measured intracellular prolactin in cell sonicates by microcomplement fixation (1, 5). Each value is the mean ± SE

of triplicate wells. Differences between control and hormone-treated cells were analyzed statistically by analysis of variance.

Fixing and Staining Cells for Microfluorometric Analysis

Cells used for microfluorometric analysis were removed from tissue culture flasks with 1-mM EDTA in phosphate-buffered saline. We fixed the cells in suspension for 1 h in 70% ethanol and treated them with ribonuclease using the method described by Crissman and Steinkamp (6).

Intracellular prolactin was stained by incubating cells with rabbit antiserum to rat prolactin (30 min, room temperature) followed by incubation with FITC-conjugated goat antiserum against rabbit IgG (20 min, room temperature). The specificity of this staining procedure for prolactin was tested by substituting either serum from non-immunized rabbits or antiserum to rat prolactin which had been preadsorbed with excess rat prolactin for the untreated prolactin antiserum. DNA was then stained with propidium iodide (6) to determine the cell cycle distribution and to group cells into G_1, S, or G_2 + M. The absorption spectra of FITC and propidium iodide are similar but the fluorescence emission peaks are optically separable. In experiments where only DNA was stained, cells were fixed in 10% formalin and stained by the Feulgen-acriflavine method (7). Greater cell recovery was achieved using this procedure and gave results identical to those obtained with propidium iodide.

Microfluorometric Analysis of Stained GH Cells

The fluorescence intensities of FITC and propidium iodide were determined using a Becton Dickinson FACS IV fluorescence activated cell sorter (Becton Dickinson FACS Systems, Sunnyvale, CA) equipped with an argon ion laser (488 nm). Channel number is proportional to fluorescence intensity, and the median channel is the channel above and below which 50% of the cell population falls. The cell-cycle dependence of intracellular prolactin was determined by measuring prolactin-associated FITC fluorescence separately for cells in G_1, S, and G_2 + M. The intensity of propidium iodide fluorescence was used to group cells into each of these cell-cycle compartments. The fraction of cells in each cell-cycle compartment was determined using a modification of the computer-fit program developed by Dean and Jett (8).

RESULTS

FITC Fluorescence Is Specific for Prolactin

Control GH cells stained using prolactin antiserum preadsorbed with excess rat prolactin showed very low FITC fluorescence intensities compared to cells stained under normal conditions using untreated prolactin antiserum (Fig. 1). The median channel number is proportional to fluorescence intensity and was 7 for cells stained with preadsorbed antiserum and 113 for cells stained with untreated antiserum. Similar results were obtained using serum from non-immunized rabbits (data not shown).

Insulin and 17β-Estradiol Slow GH Cell Growth and Increase Intracellular Prolactin

Insulin plus estradiol slowed cell growth to 70% and increased intracellular prolactin to 540% of control values (Table I). The hormone-induced increase in intracellular prolactin was associated with a greater fluorescence intensity of the cell population (Fig. 2); the median channel number increased from 78 to 162 (Table I). Insulin and estradiol increased prolactin storage fivefold but prolactin-associated FITC fluorescence increased only twofold. The binding of prolactin antiserum to intracellular prolactin in ethanol-fixed cells may not be stoichiometric, unlike binding to solubilized prolactin. We could not successfully resolubilize immunoreactive prolac-
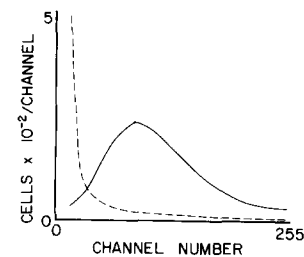

FIGURE 1 Prolactin-associated FITC fluorescence distribution of GH cells. Cells were grown in the absence of hormones and fixed as described for Table I. Channel number is proportional to fluorescence intensity. The solid line represents cells stained using rabbit antiserum to rat prolactin, and the broken line represents cells stained under identical conditions substituting rabbit antiserum to prolactin which had been preadsorbed with excess rat prolactin.
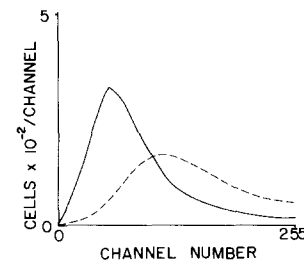

FIGURE 2 The effect of insulin + estradiol on the prolactin-associated FITC fluorescence distribution of GH cells. GH cells were grown, fixed, and stained as described for Table I. The solid line represents control cells and the broken line represents cells treated for 6 d with 300-nM insulin + 1-nM 17β-estradiol.

tin from ethanol-fixed GH cells to measure the hormone after fixation.

Intracellular Prolactin Is Cell-cycle Dependent

Table II shows that intracellular prolactin in both control and hormone-treated populations was cell-cycle dependent; FITC fluorescence due to prolactin increased as cells passed from G_1 to S and was greatest for cells in G_2 + M. The hormone-induced increase in prolactin-associated fluorescence was present in each stage of the cell cycle.

Insulin and Estradiol Do Not Affect the Cell-cycle Distribution of the Population

Since intracellular prolactin is a function of cell cycle and insulin and estradiol slow the growth of GH cells, insulin and estradiol might increase intracellular prolactin by increasing the number of cells in S and G_2 + M, the cell-cycle compartments associated with higher levels of intracellular prolactin. Fig. 3 presents the DNA histograms of control and hormone-treated GH cells; it is representative of four independent experiments. It should be noted that both control and treated populations were in logarithmic growth at the time these histograms were determined (data not shown). Insulin and estradiol did not increase the fraction of cells in S or G_2 + M. Treatment with either estradiol alone or insulin alone had no effect on cell-cycle distribution (not shown) but increased intracellular prolactin to 190% and 170% of controls, respectively (Table I). From these we conclude that insulin and estradiol do not increase prolactin storage in GH cells by increasing the fraction of cells in S or G_2 + M.

TABLE I

Effect of Insulin and Estradiol on Cell Number, Intracellular Prolactin, and Prolactin-associated Fluorescence

	Controls	Insulin + Estradiol	Insulin	Estradiol
Cell number (per well × 10^{-6} ± SE)	1.89 ± 0.05	1.36 ± 0.02	1.56 ± 0.03	1.64 ± 0.05
Intracellular prolactin ($\mu g/10^6$ cells ± SE)	0.166 ± 0.006	0.892 ± 0.018	0.280 ± 0.014	0.311 ± 0.017
FITC median channel number	78	162	119	Not determined

The effect of insulin and estradiol on cell number, intracellular prolactin and prolactin-associated FITC fluorescence. GH cells were treated with 300-nM insulin and 1-nM 17β-estradiol for six days as described in Materials and Methods. Values for intracellular prolactin and cell number are the mean ± SE of triplicate wells. Intracellular prolactin levels and cell number were significantly different from controls for all treatment groups ($P < 0.005$). Cells used for microfluorometric analysis were grown and stained as described in Materials and Methods. Prolactin-associated fluorescence is reported as the median channel number of the cell population.

TABLE II

Cell-cycle Dependence of Prolactin-associated Fluorescence in the Presence and Absence of Insulin + Estradiol

	FITC median channel number	
	Controls	Insulin + Estradiol
G_1	64	133
S	89	190
G_2 + M	100	203

Cell-cycle dependence of prolactin-associated FITC fluorescence in the presence and absence of insulin + estradiol. Cells were grown, fixed, and stained as described for Table I. FITC fluorescence was determined separately for cells in G_1, S, and G_2 + M phases and is reported as the median channel number. These values are from one of two independent experiments which gave similar results.
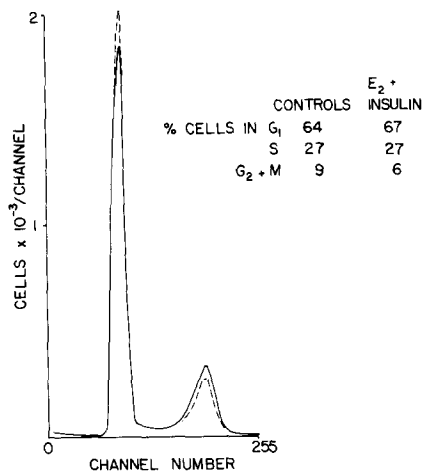

FIGURE 3 Cell-cycle distribution of GH cells grown in the presence and absence of insulin + estradiol. GH cells were seeded and grown as described for Table I. Cells were fixed in formalin and stained with acriflavine as described in Materials and Methods. The solid line represents control cells and the broken line hormone-treated cells. The coefficient of variation for the G_1 peak was 5.6% for control cells and 5.4% for hormone-treated cells.

DISCUSSION

We have shown that insulin- and estradiol-induced increases in GH-cell prolactin storage cannot be accounted for by increased prolactin synthesis, decreased secretion, or an increase in the time for newly synthesized prolactin to be processed for secretion into the medium (4). Here we show that prolactin storage is a function of cell cycle in both control and hormone-treated populations, but insulin and estradiol do not increase prolactin storage by affecting the cell cycle distribution of the population. This confirms findings by Clausen and co-workers (9) that estradiol does not influence GH-cell cell-cycle distri-

bution. A previous study by Faivre-Bauman et al. (10) did not find that intracellular prolactin varied during the cell cycle in the closely related GH3 rat pituitary cell line. However, the cells had been synchronized using a 36-h serum deprivation which could have altered their normal function or could have resulted in a selected population. The advantage of flow microfluorometry is that synchronous populations are not required.

Our experiments demonstrate that although insulin and estradiol slow cell growth and reduce the density the cells achieve at plateau (4), there is no hormone-induced difference in the fraction of cells in each cell-cycle compartment. The 3% increase in the fraction of cells in G_1 after hormone treatment seen in the experiment shown in Fig. 3 was not reproducible. In two of three other experiments there was no difference in the cell-cycle distribution between control and hormone-treated cells.

Karyotypic analysis of GH cells shows them to be aneuploid (11, 12), and this could affect the results of our cell-cycle analyses. If the cell populations were sufficiently aneuploid, the G_1 peak would be broadened and this would be indicated by a relatively large coefficient of variation. The coefficient of variation of the G_1 peak was 5.6% for control cells and 5.4% for hormone-treated cells. These values are comparable to those obtained with diploid cell populations (13) and indicate that the GH cells used in our study were not sufficiently aneuploid to affect our results.

Our observation that intracellular prolactin is dependent on cell cycle is important because GH cells are used extensively to study the regulation of prolactin synthesis and secretion. It is possible that agents which affect prolactin regulation in GH cells exert their effects through changes in the cell cycle although insulin and estradiol do not.

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