Regulation by Calcium of Prolactin and Growth Hormone mRNA Sequences in Primary Cultures of Rat Pituitary Cells

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Addition of Ca\(^{2+}\) to primary cultures of female pituitary cells incubated in serum-free medium lacking added Ca\(^{2+}\) yielded no effects on levels of prolactin or growth hormone mRNA, assayed by cytoplasmic dot hybridization. However, incubation of the cells in serum-free medium containing sufficient ethylene glycol bis(\(\beta\)-aminoethyl ether)-\(N,N',N'\),\(N''\)-tetraacetic acid to reduce medium Ca\(^{2+}\) levels below the 10–40 \(\mu M\) present as a trace contaminant yielded a decrease in the levels of both mRNAs. The decrease was dose-dependent at extracellular Ca\(^{2+}\) concentrations below 1.0 \(\mu M\), had an apparent half-maximum at about 0.3 \(\mu M\), and did not appear to plateau with increasing incubation times. Following 2–3-day incubations in low Ca\(^{2+}\), a reduction of prolactin mRNA (23–70-fold) consistently greater than the reduction of growth hormone mRNA (9–15-fold) was observed. Similar effects of reduced extracellular Ca\(^{2+}\) were obtained with primary cultures of male pituitary cells. The specificity of these effects of lowered extracellular Ca\(^{2+}\) was demonstrated by the following observations. The decreases in these mRNAs were substantially reversible by readdition of Ca\(^{2+}\) to the incubation medium. Reduction of extracellular Ca\(^{2+}\) led to no detectable change in cellular ribosomal RNA levels or overall RNA synthesis. In male pituitary cells, the level of another metal-regulated mRNA, that for metallothionein, was not decreased by a reduction of extracellular Ca\(^{2+}\) that caused a 40-fold decrease in levels of prolactin and growth hormone mRNA. Hence, Ca\(^{2+}\) exhibits specificity in its regulation of pituitary prolactin and growth hormone gene expression.

The role of free intracellular Ca\(^{2+}\) in the regulation of a host of cellular processes, including adrenohypophyseal hormone secretion, is currently an area of intense investigation (1). By contrast, there have been few studies of the possible involvement of Ca\(^{2+}\) in the regulation of specific gene expression. In previous studies with a line of rat pituitary (GH\(_3\)) cells, we showed that addition of Ca\(^{2+}\) to cells which had been incubated in a Ca\(^{2+}\)-depleted medium strongly stimulates (7–200-fold) prolactin mRNA levels, while only slightly increasing (by 60%) growth hormone mRNA levels (2). Combinatorial analysis under these conditions of the regulation by epidermal growth factor, thyrotropin-releasing hormone, and/or Ca\(^{2+}\) suggested a possible role for Ca\(^{2+}\) as a mediator of peptide hormone action on prolactin gene expression (3).

At present, we have found that the results of the Ca\(^{2+}\) studies described above arose from the transformed nature of the GH\(_3\) cells and thus might not represent an accurate reflection of regulatory mechanisms occurring in normal rat pituitary cells. To investigate this possibility, we have carried out studies of the regulation by Ca\(^{2+}\) of prolactin and growth hormone mRNA levels in primary cultures of normal rat pituitary cells. We report here that prolactin and growth hormone mRNAs are regulated in a qualitatively similar fashion in normal pituitary cells and the GH\(_3\) cells. However, the effects of Ca\(^{2+}\) occur over a much lower extracellular concentration range in pituitary cells than in the GH\(_3\) cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—A monolayer serum-free medium (SFM*) containing no added Ca\(^{2+}\) or hormones, prepared as described previously (2), was employed for all incubations. Primary cultures of rat pituitary cells were prepared by trypsin digestion essentially as described by Hymer et al. (4). Pituitaries removed from either female retired breeder or male Sprague-Dawley rats were minced in SFM to pieces approximately 1 mm in size. The pieces were washed twice and incubated at 37 °C in SFM plus trypsin and bovine serum albumin (1 mg/ml each; Sigma) for 2 h, during which the pieces were repipetted at 30-min intervals. The resultant dissociated cells were then diluted 10–20-fold with SFM and plated at 0.5 X 10\(^6\)/ml in 35-mm plastic Petri dishes (not tissue culture-treated). The average cell yield was 1 X 10\(^6\) and 2–3 X 10\(^6\) from individual male and female pituitaries, respectively. Cell viability, determined by trypan blue exclusion, was greater than 98%. Cells were incubated at 37 °C in 5% CO\(_2\) and were observed to attach to the plates within 15 min. The medium was changed after 15 min and about 16 h, at which time experimental additions were made.

**Determination of Ca\(^{2+}\) Concentration of Serum-free Medium**—A spectrophotometric assay employing the metallochromic Ca\(^{2+}\) indicator arsenazo III (O-(1,8-dihydroxy-3,6-disulphonaphthalene-2,7-bis(azo))bisbenzenesulfonic acid) (5) was used. Arsenazo III binds free Ca\(^{2+}\) avidly and is most sensitive to Ca\(^{2+}\) in the micromolar range. A standard curve was constructed by adding known amounts of Ca\(^{2+}\) or EGTA to SFM. One-half of each sample was made 1.0 mM in Ca\(^{2+}\)-EGTA of 4.4 \(\mu M\). A value for the \(K_d\) of Ca\(^{2+}\)-EGTA of 0.15 \(\mu M\) was employed to calculate the amount of EGTA required to yield the desired SFM Ca\(^{2+}\) concentration.

**Quantification of Specific RNAs**—Cells were scraped with a rubber policeman into phosphate-buffered salts. Relative levels of prolactin

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or growth hormone mRNA, or 18 S ribosomal RNA were then assayed by cytoplasmic dot hybridization as described (7). Briefly, cytoplasmic samples were heated in the presence of formaldehyde, 2-fold dilutions dotted onto each of three nitrocellulose sheets, and the sheets baked. Each sheet was then hybridized against a 32P-labeled hybrid plasmid (2-4 x 10^6 cpm/μg, 10 μg/ml) containing cDNA for rat prolactin (pPRL-1 (8)), rat growth hormone (pBR322-GH1 (9)), or 18 S ribosomal DNA (pXC-1 (10)), washed, autoradiographed, and scanned. When a wide range of spot intensities was analyzed, a series of autoradiographic exposures was employed to yield conditions where the intensity of each spot was in the linear range of the film. All values of mRNA levels have been divided by 18 S ribosomal RNA content, which was little affected by variations in extracellular Ca^2+ (see "Results").

**Total RNA Synthesis**—Following incubation in 2.0 ml of SFM containing 20 μCi of [3H]uridine for 30 min, cells were scraped into phosphate-buffered salts, washed once, and lysed by vortexing 30 s in 0.1% sodium dodecyl sulfate, 150 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.5. Trichloroacetic acid-precipitable radioactivity was determined by scintillation counting.

**RESULTS**

**Ca^2+ Regulation of Prolactin and Growth Hormone mRNA Levels in Female Pituitary Cells**—In previous experiments, it was found that addition of Ca^2+ (0.4 mM) to a line of rat pituitary tumor (GH3) cells incubated in a serum-free medium lacking added Ca^2+ yielded a maximal and sizable stimulation of prolactin mRNA levels (2). However, in preliminary experiments, when Ca^2+ was added at concentrations up to 1.0 mM to primary cultures of female rat pituitary cells incubated in the same serum-free medium, no significant stimulation of cytoplasmic levels of either of these mRNAs occurred (data not shown).

Assays with arsenazo III of the Ca^2+ concentration of the serum-free medium employed in these experiments yielded values in the range of 10-40 μM. It seemed possible that normal pituitary cells incubated at these low levels of extracellular Ca^2+ could maintain high intracellular Ca^2+ levels, thus preventing a response to exogenously added Ca^2+. The Ca^2+ concentration of this medium was therefore further reduced by the addition of EGTA. Pituitary cells were incubated for 72 h in the presence of a range of resultant Ca^2+ concentrations, and the effect on levels of prolactin mRNA, growth hormone mRNA, and ribosomal RNA was analyzed by cytoplasmic dot hybridization (Fig. 1). Decreasing extracellular Ca^2+ from 25 to 5 μM yielded no detectable effect on cellular levels of either prolactin or growth hormone mRNA. However, incubation at 0.1 μM Ca^2+ significantly reduced the levels of both mRNAs. This reduction was not due simply to an overall change in the RNA metabolism of these cells, since incubation for 72 h at this Ca^2+ concentration led to no detectable change in ribosomal RNA levels (Fig. 1), nor in overall RNA synthesis (data not shown). The observation that addition of Ca^2+ but not Mg^2+ to the incubation medium reversed the effects of EGTA shows that these effects are due to chelation by EGTA of Ca^2+. Finally, it can be seen in Fig. 1 that female pituitaries incubated with high (5-25 μM) Ca^2+ contain considerably more prolactin mRNA than growth hormone mRNA.

**Dose Dependence of Prolactin and Growth Hormone mRNA on Extracellular Ca^2+**—The results presented in the previous section show that prolactin and growth hormone mRNA levels in pituitary cells remain insensitive to decreasing extracellular Ca^2+ concentrations until quite low concentrations are reached. To define further the effect of reduced extracellular Ca^2+ on cellular levels of these mRNAs, we examined the effect of incubation of pituitary cells at various submicromolar concentrations of extracellular Ca^2+. Incubating cells for 2 days in the presence of decreasing Ca^2+ concentrations in the range of 1.0-0.1 μM yielded a dose-dependent decrease in cellular levels of both prolactin and growth hormone mRNA (Fig. 2). The observation that levels of either mRNA were not significantly different in cells incubated at 1.0 or 0.4 μM implies that the levels of these mRNAs do not become sensitive to extracellular Ca^2+ until submicromolar concentrations are achieved. This was confirmed by the results of other similar experiments, in which we have found no changes in prolactin and growth hormone mRNA levels when extracellular Ca^2+ was varied in the range of 1.0-400 μM (data not shown; see Fig. 4).

The dose-response relationship for prolactin and growth hormone mRNA were similar, with apparent half-maximal effects at about 0.3 μM Ca^2+ (Fig. 2). However, at the lowest

| [Ca^2+] (μM): |
|-------------|
| 25 | 20 | 15 | 5 | 0.1 |

**Fig. 1.** Effect of micromolar extracellular Ca^2+ on prolactin and growth hormone mRNA levels in female pituitary cells. Cells were incubated for 3 days in the presence of sufficient EGTA to reduce extracellular Ca^2+ to the indicated concentrations (see "Experimental Procedures"), plus other additions as indicated. Levels of the indicated RNAs were analyzed by cytoplasmic dot hybridization. *Rous* 1-4 and 5-8 represent 2-fold serial dilutions of cytoplasm from cells treated as shown above and below the dots, respectively. *Rous* 1 and 5 received cytoplasm from 2 x 10^6 cells. In *rrous* 5-8, cytoplasm was applied only to the indicated four columns. Duplicate dots correspond to cells from duplicate cultures. Autoradiographic exposure times (~70 °C, one screen) were: prolactin, 5 h; growth hormone, 18 h; 18 S ribosomal RNA, 5 h.
Calcium Regulation of Prolactin and Growth Hormone mRNA Levels. Female pituitary cells were incubated for 2 days in the presence of the indicated Ca\(^{2+}\) concentrations and relative mRNA levels assayed, as in Fig. 1. The insets show the dots following a 3-day autoradiographic exposure. Total RNA synthesis was analyzed in separate cultures (bottom panel). Each point represents the mean and range of results with duplicate cultures.

Ca\(^{2+}\) concentration tested (0.1 \(\mu\)M), prolactin and growth hormone mRNA levels were reduced to 1.4 and 6.7%, respectively, of the levels observed in cells incubated at 1.0 \(\mu\)M Ca\(^{2+}\). Thus, prolactin mRNA levels appear to be more sensitive to very low extracellular Ca\(^{2+}\) than are growth hormone mRNA levels (see also Fig. 3 and Table I). As observed previously, total RNA synthesis was constant over the range of Ca\(^{2+}\) concentrations tested (Fig. 2, bottom panel).

Kinetics of the Decrease of Prolactin and Growth Hormone mRNA at Low Extracellular Ca\(^{2+}\) Concentrations—Female rat pituitary cells were incubated for various times in the presence of either low (0.1 \(\mu\)M) or high (25 \(\mu\)M) concentrations of Ca\(^{2+}\). The effects on prolactin and growth hormone mRNA levels and on overall RNA synthesis are shown in Fig. 3. Incubation for 6 days in high Ca\(^{2+}\) yielded no detectable effect on prolactin mRNA levels and a gradual decrease in growth hormone mRNA levels to about 50% of their initial value. Incubation for 1 day in low Ca\(^{2+}\) yielded no effect on prolactin mRNA levels and a small (30%) decrease in growth hormone mRNA levels. Between 1 and 3 days, in the presence of low Ca\(^{2+}\), levels of both mRNAs declined with approximately first-order kinetics of the decrease of prolactin and growth hormone mRNA induced by incubation of cells with EGTA.

Female pituitary cells were incubated as indicated, and the relative levels of prolactin and growth hormone mRNA and of 18S ribosomal RNA levels were assayed, as for Fig. 1. Each point represents the mean and range of results with duplicate cultures. Total RNA synthesis was analyzed in separate single cultures. d, days.

| Additions | Prolactin mRNA % of control | Growth hormone mRNA | Total RNA synthesis | 18S rRNA |
|-----------|-----------------------------|---------------------|---------------------|---------|
| None (6 d) | 100 ± 4 | 100 ± 61 | 100 | 100 ± 4 |
| 20 \(\mu\)M EGTA (6 d) | 4 ± 1 | 11 ± 3 | 84 | 104 ± 4 |
| 20 \(\mu\)M EGTA (3 d), then 0.4 mM Ca\(^{2+}\) (3 d) | 31 ± 3 | 35 ± 4 | 96 | 116 ± 13 |
kinetics, with half-times for prolactin mRNA and growth hormone mRNA of approximately 12 and 19 h, respectively. By 6 days of incubation with low Ca²⁺, prolactin mRNA and growth hormone mRNA levels had declined by approximately 80- and 35-fold, respectively, relative to the levels in cells incubated with high Ca²⁺. During the 6-day incubation period, overall RNA synthesis by the cells declined gradually to about 50% of its initial value, with no apparent difference at any time point between the cultures incubated in the presence of high or low Ca²⁺ (Fig. 3, bottom panel).

Reversibility of the Effect of Reduced Extracellular Ca²⁺—It seemed possible that the decrease in prolactin and growth hormone mRNA levels in pituitary cells incubated in the presence of low extracellular Ca²⁺ was due to irreversible inactivation of the pituitary cells which produce these mRNAs. To investigate this possibility, pituitary cells were incubated in the presence or absence of EGTA (20 μM) for 3 days, after which some of the EGTA-treated cultures received Ca²⁺ (0.4 mM). It is seen in Table I that incubation of pituitary cells with EGTA for 6 days caused prolactin and growth hormone mRNA levels to decrease to 4 and 11%, respectively, of the levels in control cultures incubated without EGTA. Addition of Ca²⁺ after 3 days to the EGTA-treated cultures led, by 6 days, to increases of both mRNAs to about 30-35% of the levels in the control cultures. Thus, the effects of incubation in low Ca²⁺ on the levels of both mRNAs are substantially reversed by readdition of Ca²⁺ to the incubation medium. As observed previously, none of these incubation conditions led to significant changes in either total RNA synthesis or ribosomal RNA content (Table I).

In Male Pituitary Cells Ca²⁺ Regulates the mRNAs for Prolactin and Growth Hormone, but Not Metallothionein mRNA.—To investigate whether the effects described above are sex-dependent, the effect of Ca²⁺ on mRNA levels in male pituitary cells was investigated (Fig. 4). It is seen in Fig. 4A that Ca²⁺ exerted effects quite similar to those in female cells. No effect was observed as extracellular Ca²⁺ was reduced from 10 to 0.4-0.6 μM. As the Ca²⁺ concentration was further reduced, cellular levels of prolactin and growth hormone mRNA declined sharply.

The observation (Figs. 1-3 and Table I) that lowering extracellular Ca²⁺ had no effect on pituitary cell total RNA synthesis or ribosomal RNA content showed that the accompanying effects on prolactin and growth hormone mRNA were not due simply to a change in the overall RNA metabolism of the cells. However, lowering extracellular Ca²⁺ caused similar declines in both prolactin and growth hormone mRNA. Hence, to examine further the specificity of the effect on the levels of these two mRNAs, the influence of Ca²⁺ on expression of another metal-regulated gene was investigated. Metallothionein gene expression in many cell types is stimulated by heavy metals (11). Reprobing one of the nitrocellulose sheets employed for the analysis shown in Fig. 4A with a rat metallothionein cDNA probe yielded the results shown in Fig. 4B. When extracellular Ca²⁺ concentrations were lowered, metallothionein mRNA levels did not decrease. Rather, levels of this mRNA were stimulated by decreasing extracellular Ca²⁺ in the range from 10 to 0.6 μM. When extracellular Ca²⁺ was reduced from 0.6 to 0.15 μM, there was a 40-fold increase in prolactin and growth hormone mRNA levels, while metallothionein mRNA levels were not significantly affected.

**DISCUSSION**

We have found that incubation of rat pituitary cells in a serum-free medium containing submicromolar Ca²⁺ concentra-

![Fig. 4. Effect of various extracellular Ca²⁺ concentrations on levels of the mRNAs for prolactin, growth hormone, and metallothionein in male pituitary cells.](image-url)
The use of pulse-chase techniques. However, the decay kinetics of an mRNA following removal of an inducer can be employed to estimate the half-life of the mRNA in the absence of the inducer. Thus, the mRNA decay kinetics observed in pituitary cells incubated in medium containing low (0.1 µM) Ca²⁺ (Fig. 3) suggest that the half-life of the mRNAs for prolactin and growth hormone in pituitary cells incubated in the presence of low Ca²⁺ are approximately 12 and 19 h, respectively.

The present studies show that Ca²⁺ regulates expression of the prolactin and growth hormone genes at a pretranslational level, but do not yield direct information concerning the regulation by Ca²⁺ of the transcription of these genes. However, we have shown previously that incubation of GH3 cells with Ca²⁺ yields increases in all detectable nuclear prolactin mRNA precursors.

In addition, previous investigations have demonstrated a role for Ca²⁺ in the secretion of prolactin (17-19) and growth hormone (17, 20, 21). The cellular mechanisms involved in the regulation of Ca²⁺ in both the expression of these genes and the secretion of their protein products remain to be elucidated. It is, however, intriguing to speculate that these apparently disparate cellular processes may be functionally related.

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