

The Role of Calcium in the Transcriptional and Posttranscriptional Regulation of the Gonadotropin-Releasing Hormone Gene in GT1-7 Cells*

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ABSTRACT

The role of calcium in the regulation of GnRH gene expression and the mechanism for its effects were examined in the present study. Using the immortalized hypothalamic GT1-7 cell line, which synthesizes and secretes GnRH, we demonstrated by ribonuclease protection assay and Northern blot analysis that these cells respond to treatment with the calcium ionophores ionomycin and A23187 with an inhibition of transcription of the GnRH gene and decreases in GnRH messenger RNA (mRNA) levels. Ionomycin treatment caused the GnRH mRNA half-life to decrease from 25 to 9 h, concomitant with

a decrease in mRNA poly(A) tail length, suggesting that ionomycin causes a decrease in GnRH mRNA stability. The ionomycin inhibitory effect on GnRH cytoplasmic mRNA levels was significantly inhibited in the presence of cycloheximide or the RNA synthesis inhibitor 5,6-dichloro-1 β -ribofuranosylbenzimidazole, indicating that novel protein/RNA synthesis is obligatory for this effect. We conclude that an increase in calcium levels caused by ionomycin inhibits GnRH gene expression at multiple levels, including GnRH gene transcription and mRNA stability in GT1-7 cells. (*Endocrinology* **139**: 2685–2691, 1998)

GnRH IS THE key hormone in the control of reproduction. GnRH gene expression and GnRH release are regulated by numerous neurotransmitters and hormones (1–6; reviewed in Ref. 7), whose actions are mediated by several intracellular second messenger pathways, including protein kinase A, protein kinase C (PKC), and calcium/calmodulin pathways (8–11). Because GnRH neurons in the brain are sparse and widely distributed (12, 13), it is difficult to study the molecular mechanisms underlying gene expression in individual GnRH neurons. Therefore, much of the research on GnRH release and gene expression has been carried out using an immortalized mouse hypothalamic GnRH neuronal cell line, GT1 cells (14). Using these cells, we and others have reported that the phorbol ester, phorbol 12-myristate 13-acetate (PMA), a PKC activator, caused an inhibition of pro-GnRH gene transcription, a decrease in messenger RNA (mRNA) levels, a decrease in translational efficiency of the GnRH mRNA (8, 10, 11, 15–18), and a decrease in GnRH mRNA stability (7).

Calcium is one of the most important signal transduction elements in neurons. It functions as a second messenger that mediates a broad range of cellular responses to influence events such as synaptic transmission, neuronal survival (19), axon outgrowth (20), changes in synaptic strength (20), and activation of gene expression (21). In the present study, we examined in detail the effects of elevating intracellular calcium on GnRH gene expression and whether GnRH mRNA

turnover also plays a role in the regulation of GnRH mRNA levels.

Materials and Methods

Cell culture and treatment

GT1-7 cells were cultured in DMEM (Life Technologies, Gaithersburg, MD) with 10% HyClone FBS and 5% antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) and maintained at 37 C with 5% CO₂, as described previously (22). Cells were subcultured into six-well dishes 4–5 days before experiments and allowed to grow to 70–80% confluence. For all experiments, cell medium was removed and replaced with serum-free DMEM 1 h before the drug was added. Studies were performed in triplicate cultures and repeated at least three times. Cycloheximide (CHX; Calbiochem, San Diego, CA) was dissolved in sterile water. 5,6-Dichloro-1 β -ribofuranosylbenzimidazole (DRB; Sigma Chemical Co., St. Louis, MO) and A23187 and ionomycin (Calbiochem, San Diego, CA) were dissolved in dimethylsulfoxide (DMSO). Stock solutions of drugs were diluted with medium, not exceeding 0.1% of the total volume of the culture medium (2 ml).

Experimental design

Previous studies (10) demonstrated that the optimal dose of ionomycin in GT1-7 cells for inhibiting GnRH mRNA is 1 μ M; thus, this dose was used in all of the following experiments. In Exp I, GT1-7 cells were treated in the continuous presence of ionomycin or vehicle for 0, 0.5, 1, 2, 4, 8, 12, and 24 h. In Exp II, GT1-7 cells were treated with 1 μ M A23187 or vehicle for 0, 2, 4, and 8 h. In Exp III, at –2.5 h, GT1-7 cells were treated with medium containing ionomycin (1 μ M) or vehicle (DMSO). At 0 h, cells were treated with the RNA synthesis inhibitor DRB (100 μ g/ml) (22, 23). Cells were harvested 0, 2, 4, 8, and 12 h after DRB treatment. Cytoplasmic mRNA was isolated and subjected to ribonuclease (RNase) protection assay. The GnRH mRNA half-life was determined using a regression analysis on the change in GnRH mRNA levels for both control and ionomycin-treated groups. In Exp IV, GT1-7 cells were treated with either DMSO or 1 μ M ionomycin for 8 h. In Exp V, GT1-7 cells were treated with vehicle (DMSO), the protein synthesis inhibitor CHX (20 μ M), or the RNA synthesis inhibitor DRB (100 μ g/ml) for 15 min, followed by ionomycin (1 μ M) or vehicle, and harvested 8 h after treatment. For Exp I, II, IV, and V, experiments were repeated two to four

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times using triplicate cultures, with similar results each time; the results presented in the corresponding figures are a representative case. For Exp III, data from three different experiments were pooled; the control and ionomycin groups were compared within the same experiment.

Cytoplasmic and nuclear RNA isolation

Cytoplasmic and nuclear RNA were isolated as previously described (22, 24). Cells were homogenized in 500 μ l lysis buffer [10 mM Trizma base (pH 7.5), 1.5 mM MgCl₂, 0.3 M sucrose, 0.5% Nonidet P-40, and 0.25% sodium deoxycholate]. This fraction was layered over 400 μ l cushion buffer [10 mM Trizma base (pH 7.5), 1.5 mM MgCl₂, and 0.4 M sucrose] and centrifuged at 800 \times g. The cytoplasmic fraction was subsequently treated with 100 μ g/ml proteinase K, extracted with phenol-chloroform-isoamyl alcohol (25:24:1) followed by chloroform-isoamyl alcohol (24:1), and precipitated with 1.5 vol isopropanol at -20° C for at least 1 h. After precipitation, cytoplasmic RNA was centrifuged at 4 C and washed with 70% ethanol. The pellet was resuspended in 30 μ l 1 \times TE (10 mM Tris, 1 mM EDTA, pH 7.4) and quantified by absorbance at 260 nm, and 1 μ g total RNA from each sample was dried down and resuspended with 20 μ l hybridization buffer (4 M guanidine monothiocyanate and 0.1 M EDTA, pH 7.5). The remaining cytoplasmic RNA was frozen at -80° C. The nuclear pellets were resuspended in 300 μ l high salt buffer [10 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 5 mM MgCl₂, and 0.1 mM CaCl₂], treated with deoxyribonuclease I (20 U) and then proteinase K, extracted as described above, and precipitated. After precipitation, the nuclear nucleic acid was resuspended with 100 μ l 1 \times TE, and treated with 60 U deoxyribonuclease I, followed by another extraction and precipitation. Then, the nuclear samples were washed, dried down, and resuspended in 20 μ l hybridization buffer.

RNase protection assay

GnRH cytoplasmic or nuclear RNA transcripts were quantified by the RNase A/T1 protection assay method as previously described (22, 24). The DNA template for measuring cytoplasmic GnRH mRNA was a 443-bp mouse complementary DNA (cDNA) clone spanning the *Eco*01091 and *Xba*I restriction sites and subcloned into a Bluescript SK⁺ vector, and the template for measuring cyclophilin mRNA was a 111-bp cDNA clone spanning the *Pst*I and *Xmn*I restriction sites and subcloned into a Bluescript SK⁺ vector (22, 25). The DNA template for measuring nuclear primary transcript was a 383-bp intron A/exon 2/intron B portion of the mouse GnRH gene, spanning the *Spe*I and *Hind*III restriction sites and subcloned into a Bluescript SK⁺ vector (22, 25). [α -³²P]UTP-labeled antisense RNA riboprobe was transcribed using an *in vitro* transcription kit (Promega Corp., Madison, WI). Sense standard RNAs were made following a similar protocol with low specific activity and were quantified by absorbance at 260 nm. Aliquots of standard RNA were stored at -80° C until use. Yeast transfer RNA was added to the tubes with the standard RNA to make their final RNA concentrations comparable to those of the samples. One nanogram of riboprobe in a 5- μ l final volume was added to each tube. Samples were denatured at 56 C for 5 min and incubated at 30 C for 12–16 h. Samples were then digested with RNase in 300 μ l RNase buffer [10 mM Tris (pH 7.5), 5 mM EDTA (pH 8.0), 0.3 M NaCl, 40 μ g/ml RNase A, and 2 μ g/ml RNase T1] at 30 C for 1 h followed by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. Samples were then centrifuged, washed, dried, and resuspended in 5 μ l diethylpyrocarbonate-H₂O with 1.5 μ l 1.5 \times Ficoll loading dye and electrophoresed on 5% acrylamide gels. The amount of mouse GnRH RNA fragments protected was quantified by exposing the gels against a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) and was calculated by comparing the amount of radioactivity in each sample (relative intensity unit by PhosphorImager) to the sense RNA generated by regression analysis.

Electrophoresis and Northern hybridization (Exp IV)

Changes in GnRH polyadenylate [poly(A)] tail size were determined by Northern blot analysis. Cytoplasmic RNA was isolated. In Fig. 4A, 3 μ g total RNA were subjected to Northern blot. For preparing "tail-less" mRNA, the mRNA poly(A) tail from GT1-7 cells was digested: samples were annealed with oligo(deoxythymidine) (Life Technologies), followed by RNase H (Life Technologies) digestion, phenol-chloroform-

isoamyl alcohol (25:24:1) extraction, and ethanol precipitation. In Fig. 4B, cytoplasmic RNA was isolated, and the amount of GnRH mRNA was determined by RNase protection assay. Then, the amount of RNA containing 100 pg GnRH mRNA was calculated for each sample and used for Northern blot analysis. Yeast total RNA was used to bring all samples to an equal amount (5 μ g). The RNA was dried and resuspended with 4.5 μ l diethylpyrocarbonate-H₂O, 2 μ l 10 \times formaldehyde buffer [50% glycerol, 1 mM EDTA (pH 8.0), 0.25% bromophenol blue, and 0.25% xylene cyanol FF], 3.5 μ l formaldehyde, and 10 μ l deionized formamide. An unlabeled RNA ladder (0.24–9.5 kDa; Life Technologies) was used as a molecular marker. Ethidium bromide (1 μ l of a 10 mg/ml stock) was added to the marker before electrophoresis. The samples were then denatured and loaded onto a 2% agarose gel containing formaldehyde. The gel was run at 3 V/cm for 8–10 h. After electrophoresis, a photograph of the gel next to a ruler was taken to determine the relative location of the RNA ladder bands to the ruler. Hybridization and determination of the peak density of the hybridized bands were performed as previously described (22). Briefly, the RNA was transferred and fixed to a Nytran filter (Schleicher and Schuell, Keene, NH). After prehybridization and hybridization with the mouse GnRH cDNA clone, the filter was washed and exposed to x-ray film (Kodak XAR 5, Eastman Kodak, Rochester, NY). The film was then scanned on a Nikon Scantouch 1200 scanner (Melville, NY), and the peak value of each band was determined using the NIH Image program. The average RNA size of each peak value was calculated by comparison to the size of the molecular markers. The differences in peak density between treatments was estimated using ANOVA followed by Fisher's protected least significant difference *post-hoc* analysis. Significance was set at $P < 0.05$.

Results

Exp I: time course of the action of the calcium ionophore ionomycin on GnRH primary transcript and cytoplasmic mRNA levels (Fig. 1)

To determine the time-course effects of ionomycin on GnRH gene expression, GT1-7 cells were treated in the continuous presence of ionomycin or vehicle for 0, 0.5, 1, 2, 4, 8, 12, and 24 h. Cells were harvested, and cytoplasmic and nuclear RNAs were isolated and subjected to RNase protection assay. As shown in Fig. 1A, there were significant effects of ionomycin treatment ($P < 0.0001$) and time ($P < 0.0001$) on GnRH mRNA levels. *Post-hoc* analysis indicated that this suppression was first observed at 1 h of treatment ($P < 0.05$), and levels continued to decrease and were significantly suppressed through the 24-h time course. Figure 1B shows that the levels of GnRH primary transcript were also significantly suppressed by ionomycin treatment ($P < 0.0001$) beginning 1 h after ionomycin treatment ($P < 0.05$), with a maximal decrease after 8–24 h of treatment ($P < 0.001$).

Exp II: effect of the calcium ionophore A23187 on GnRH gene expression (Fig. 2)

In addition to ionomycin, A23187, another type of calcium ionophore, was used to verify the effect of calcium on GnRH gene expression. GT1-7 cells were treated with 1 μ M A23187 or vehicle for 0, 2, 4, and 8 h. As shown in Fig. 2A, A23187 caused a significant decrease in GnRH mRNA levels ($P < 0.0001$) starting at 2 h of treatment ($P < 0.001$), and the effect was sustained over the 8-h treatment period. Cyclophilin mRNA levels were not significantly affected by A23187 treatment (Fig. 2B). Figure 2C shows that primary transcript levels were also significantly decreased ($P < 0.0001$) beginning at 2 h of treatment ($P < 0.001$) and remained suppressed over the 8-h treatment period.

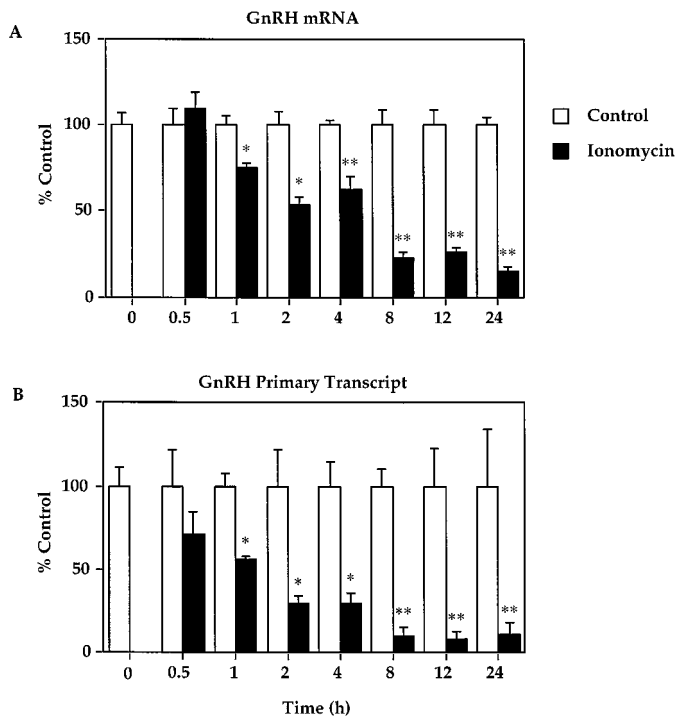


FIG. 1. Time course of the action of the calcium ionophore ionomycin on GnRH primary transcript and cytoplasmic mRNA levels. GT1-7 cells were treated with DMSO vehicle (control, *white bar*) or 1 μ M ionomycin (*black bar*) continuously. Cells were harvested 0, 0.5, 1, 2, 4, 8, 12, and 24 h after the initial treatment, and the levels of GnRH primary transcript and mRNA were determined by RNase protection assay. Values are expressed as a percentage of the control value (vehicle-treated group at the same time point). Each value represents the mean \pm SEM ($n = 3$). A, Mean GnRH mRNA levels in the cytoplasm. B, Mean GnRH primary transcript levels in the nucleus. *, $P < 0.05$; **, $P < 0.001$ (*vs.* corresponding control).

Exp III: effect of ionomycin on GnRH mRNA half-life (Fig. 3)

The GnRH mRNA half-life has been reported to range from 22–31 h (8, 22), suggesting that even if transcription of the GnRH gene were completely blocked by ionomycin treatment, it would take more than 20 h to observe a 50% decrease in cytoplasmic GnRH mRNA levels. The rapid decrease in GnRH mRNA levels observed in Exp I thus suggested that an additional mechanism, such as a decrease in GnRH mRNA stability, is responsible for the decrease in GnRH mRNA levels after ionomycin treatment. Thus, the half-life of GnRH mRNA was determined after treatment with ionomycin or vehicle. As shown in Fig. 3, in control GT1-7 cells treated with vehicle (DMSO) for 2.5 h, the GnRH mRNA half-life was 25 h. When treated with ionomycin for 2.5 h before DRB treatment, the half-life of the GnRH mRNA decreased to 9 h, indicating that there was a decrease in GnRH mRNA stability upon ionomycin treatment.

Exp IV: involvement of calcium in GnRH poly(A) tail shortening (Fig. 4)

To determine whether the GnRH mRNA poly(A) tail size is shortened by ionomycin, suggesting a decrease in mRNA stability (26–28), Northern blot analysis was used to deter-

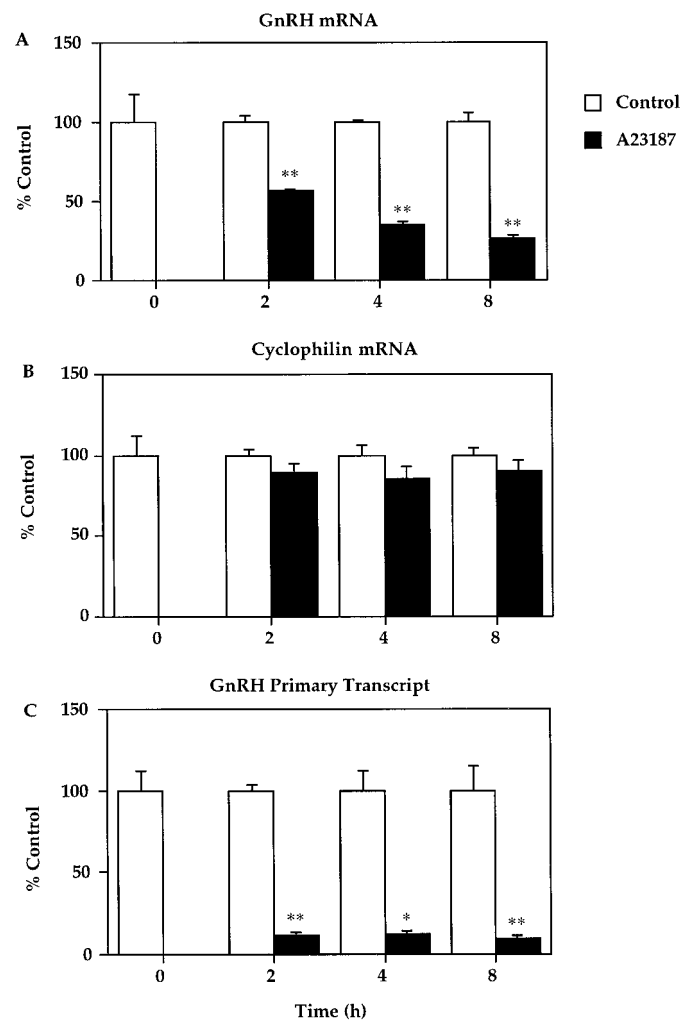


FIG. 2. Time course of the action of the calcium ionophore A23187 on GnRH primary transcript and cytoplasmic mRNA levels. GT1-7 cells were treated with DMSO vehicle (control, *white bar*) or 1 μ M A23187 (*black bar*) continuously. Cells were harvested 0, 2, 4, and 8 h after the initial treatment, and the levels of GnRH primary transcript and mRNA were determined by RNase protection assay. Values are expressed as a percentage of the control value (vehicle-treated group at the same time point). Each value represents the mean \pm SEM ($n = 3$). A, Mean GnRH mRNA levels in the cytoplasm. B, Mean cyclophilin mRNA levels in the cytoplasm. C, Mean GnRH primary transcript levels in the nucleus. *, $P < 0.05$; **, $P < 0.001$ (*vs.* corresponding control).

mine the change in GnRH mRNA poly(A) tail length after ionomycin or vehicle treatment for 8 h. GT1-7 cells were treated with either DMSO or 1 μ M ionomycin for 8 h. A representative Northern blot is shown in Fig. 4A. The GnRH mRNA species in the control samples without RNase treatment are present in a relatively broad band in our high resolution RNA gel, probably because under basal conditions, GnRH mRNAs are in different states of degradation of the poly(A) tail, with the most recently synthesized mRNAs having a longer tail, and older mRNAs having shorter poly(A) tails. After 8 h of ionomycin treatment, GnRH mRNA bands became conspicuously narrowed, with a loss of the high mol wt mRNA species, suggesting a decrease in poly(A) tail length. To confirm that the decrease in GnRH

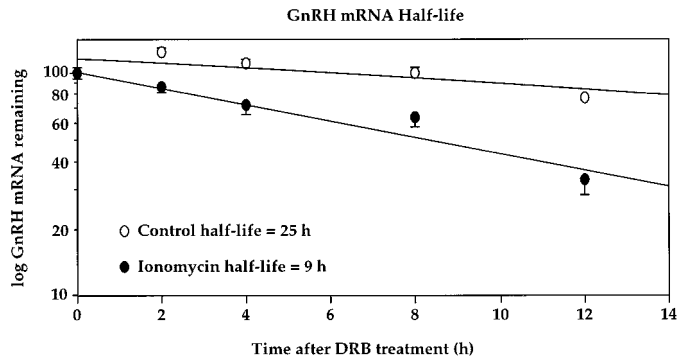


FIG. 3. Half-life of GnRH mRNA, as determined by RNase protection assay. GT1-7 cells were treated with DMSO vehicle (control) or $1 \mu\text{M}$ ionomycin for 2.5 h before DRB treatment. Cells were harvested 2, 4, 8, and 12 h after DRB treatment. Cytoplasmic GnRH mRNA was determined by RNase protection assay. Values were expressed as a percentage of the 0 h control or ionomycin-pretreated group value and plotted on a log scale. In control (DMSO-treated) GT1-7 cells, the GnRH mRNA half-life was calculated by regression analysis to be 25 h. In ionomycin-treated GT1-7 cells, the GnRH mRNA half-life was reduced to 9 h.

mRNA size is due to the shortening of that poly(A) tail length, $3 \mu\text{g}$ total RNA from control and ionomycin-treated GT1-7 cells were subjected to RNase H treatment and the resulting "tail-less" mRNA was subjected to Northern blot. As shown in Fig. 4A, the size of RNase H-treated GnRH mRNA from both control and ionomycin-treated samples stayed the same, indicating that the decrease in GnRH mRNA size is a result of poly(A) tail shortening. It is possible that the decrease in GnRH mRNA levels after ionomycin treatment could affect the resolution of the GnRH mRNA size on the Northern gel; thus, the amount of cytoplasmic mRNA was determined by RNase protection assay, and the total RNA containing 100 pg GnRH mRNA of each sample was used for Northern blot analysis. As shown in Fig. 4B, despite the equal amounts of GnRH mRNA in both control and ionomycin-treated samples, the size of GnRH mRNA was still shortened in ionomycin-treated samples, confirming the observation in Fig. 4A.

Exp V: requirement for new protein and RNA synthesis in the effect of ionomycin on GnRH mRNA levels (Fig. 5)

To determine whether new RNA and protein synthesis was required for the decrease in GnRH mRNA induced by ionomycin, GT1-7 cells were treated with vehicle (DMSO), the protein synthesis inhibitor CHX ($20 \mu\text{M}$), or the RNA synthesis inhibitor DRB ($100 \mu\text{g/ml}$) for 15 min, followed by treatment with ionomycin ($1 \mu\text{M}$) or vehicle, and were harvested 8 h after treatment.

A representative autoradiogram showing GnRH and cyclophilin mRNA levels in individual GT1-7 cell cultures is shown in Fig. 5A. ANOVA indicated a significant effect of treatment on GnRH mRNA levels ($P < 0.0001$). As shown in Fig. 5B, although neither CHX nor DRB treatment resulted in a significant alteration of the GnRH mRNA level, ionomycin caused a significant decrease ($P < 0.0001$ vs. control), and this effect was abolished when DRB or CHX was preadministered 15 min before ionomycin ($P < 0.0001$, ionomycin plus

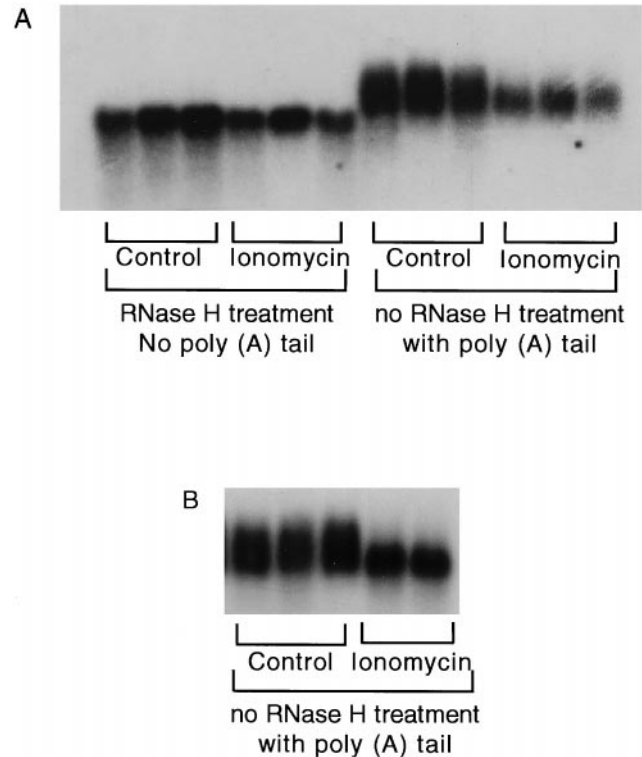


FIG. 4. Autoradiogram of GnRH mRNA electrophoresed through a denaturing agarose gel. A, GnRH mRNA that was digested with RNase H is shown in the six left lanes, and undigested mRNA containing the poly(A) tail is shown in the six right lanes. The treatment condition is indicated below each lane. The RNase H-treated GnRH mRNA migrated in a discrete, uniformly sized band for both control and ionomycin-treated samples. mRNA containing the poly(A) tail migrated in a broader band. The size of the GnRH mRNA containing poly(A) tail was reduced by ionomycin treatment for 8 h. B, Equal amounts of GnRH mRNA were subjected to Northern blot analysis. GT1-7 cells were treated with DMSO or $1 \mu\text{M}$ ionomycin for 8 h. The total RNA containing 100 pg GnRH mRNA, as determined by RNase protection assay, was subjected to Northern blot. The GnRH mRNA poly(A) tail size was reduced in ionomycin-treated samples.

DRB vs. ionomycin; $P < 0.01$, ionomycin plus CHX vs. ionomycin). These results indicate that the ionomycin-induced reduction in the GnRH mRNA level required new protein and/or RNA synthesis. Cyclophilin mRNA levels were not significantly affected by ionomycin treatment.

A representative autoradiogram showing GnRH primary transcript levels in individual GT1-7 cell cultures is shown in Fig. 5C. There was a significant effect of treatment on GnRH primary transcript levels ($P < 0.0001$). As shown in Fig. 5D, both DRB and ionomycin caused a significant decrease in GnRH primary transcript levels ($P < 0.001$ and $P < 0.0001$ vs. control, respectively). Pretreatment with DRB followed by ionomycin caused a significant decrease compared with the effect of DRB alone ($P < 0.05$ vs. DRB) or that of ionomycin alone ($P < 0.05$ vs. ionomycin). CHX treatment itself also caused a significant decrease in GnRH primary transcript levels ($P < 0.001$ vs. control). Pretreatment with CHX followed by ionomycin did not result in a further decrease ($P = 0.10$) in GnRH primary levels compared with the effect of treatment with CHX or ionomycin alone.

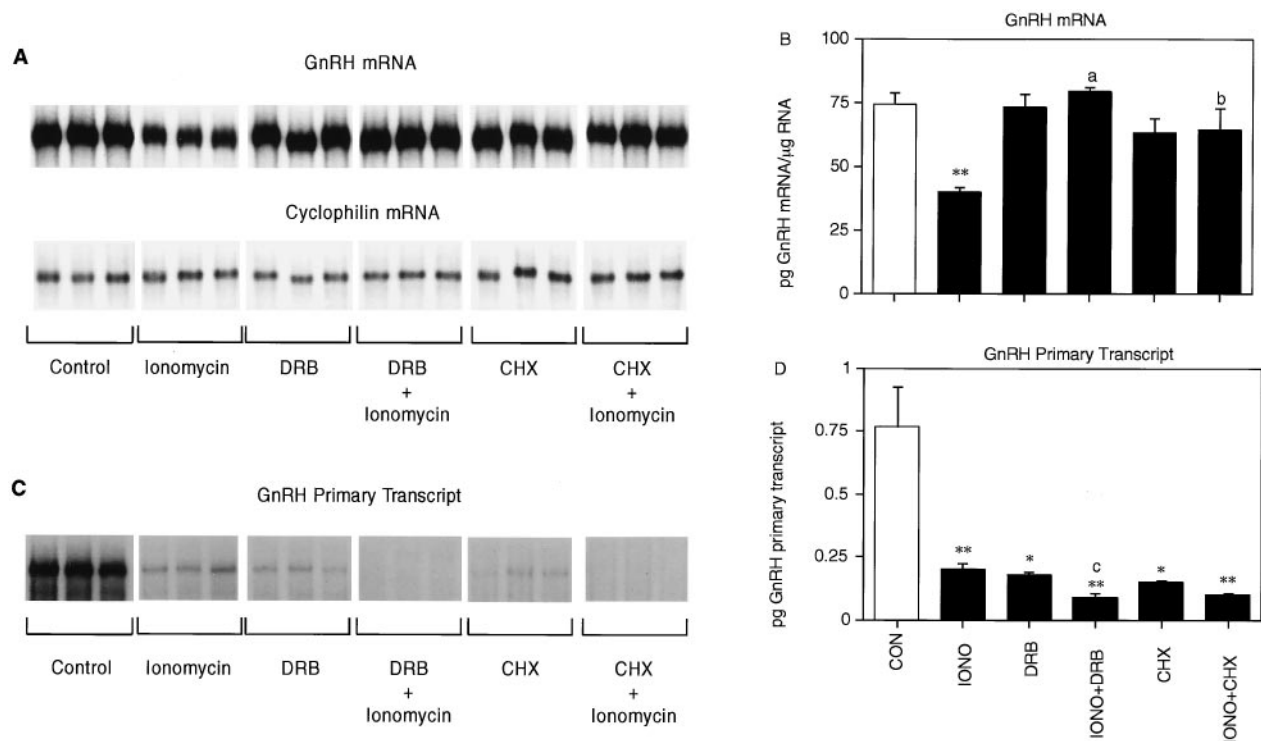


FIG. 5. Effect of the RNA synthesis inhibitor DRB and the protein synthesis inhibitor CHX on the ionomycin (IONO)-induced inhibition of GnRH mRNA and primary transcript levels. GT1-7 cells were treated with DMSO vehicle [control (CON)], treated with 1 μ M ionomycin, pretreated with 100 μ g/ml DRB for 15 min followed by the addition of vehicle (DRB), pretreated with 100 μ g/ml DRB for 15 min followed by the addition of 1 μ M ionomycin (IONO+DRB), pretreated with 20 μ g/ml CHX for 15 min followed by the addition of vehicle (CHX), or pretreated with 20 μ g/ml CHX for 15 min followed by the addition of 1 μ M ionomycin (IONO+CHX). Cells were harvested 8 h after vehicle or ionomycin treatment. A, A representative autoradiogram of GnRH and cyclophilin mRNA levels in individual GT1-7 cultures is shown. One microgram of total RNA was loaded in each lane. B, Ionomycin caused a significant decrease in GnRH mRNA levels; this decrease was abolished by pretreatment with DRB or CHX. DRB or CHX alone had no effect on GnRH mRNA levels. C, A representative autoradiogram of GnRH primary transcript levels with individual GT1-7 cell cultures is shown. The RNA extracted from the nuclear fraction of one six-well plate was loaded in each lane. D, Ionomycin, DRB, and CHX caused similar decreases in GnRH primary transcript levels. *, $P < 0.001$ vs. control; **, $P < 0.0001$ vs. control; a, $P < 0.0001$ vs. ionomycin alone; b, $P < 0.01$ vs. ionomycin alone; c, $P < 0.05$ vs. DRB or ionomycin alone. There was no significant difference between ionomycin plus DRB and ionomycin plus CHX.

Discussion

The level of a specific mRNA present in a cell is largely determined by the rate of transcription of the gene encoding this RNA and the rate of degradation of this mRNA in the cytoplasm. Calcium affects GnRH gene expression in GT1-7 cells at both transcriptional and posttranscriptional levels. In the present study, we observed a significant decrease in the GnRH primary transcript levels, an indicator of GnRH gene transcriptional activity (25), beginning 1 h after the onset of ionomycin treatment. This result is consistent with a previous observation that GnRH primary transcript levels were decreased after 1.5 h of ionomycin treatment (10). In the present study, GnRH mRNA levels also decreased after 1 h of ionomycin treatment. Similar to ionomycin, we observed that another calcium ionophore, A23187, significantly inhibited GnRH mRNA levels. The observation made by our and another laboratory that the half-life of the GnRH mRNA is greater than 20 h (17, 22) suggests that even if transcription of the GnRH gene were completely blocked by ionomycin, it would take more than 20 h to observe a 50% decrease in the cytoplasmic GnRH mRNA level. However, we observed that GnRH mRNA levels are decreased 50% after only 2 h of ionomycin treatment. Therefore, the rapid decrease in the

GnRH mRNA level is not only a reflection of the decline in GnRH gene transcription, but must also result from a major activation of a GnRH mRNA turnover system.

A recent study by Belsham and colleagues (29) reported that treatment with similar doses of ionomycin or A23187 did not affect GnRH gene transcription and mRNA levels in GT1-7 cells, but their presence was necessary to allow for an inhibitory effect of *N*-methyl-D-aspartate and nitric oxide on GnRH mRNA to be observed. The reason for the discrepancy is not clear; possibly it relates to variations in the GT1-7 cell lineage or the experimental culture conditions. In our studies, cells are in serum-free conditions for only 1 h before treatment, whereas Belsham *et al.* (29) incubated their cells in serum-free conditions for 16 h before drug treatment. Both studies, however, agree on the involvement of elevated calcium in the inhibition of GnRH gene transcription and the decrease in GnRH mRNA levels.

To determine whether GnRH mRNA stability is altered by an elevation in calcium levels, we measured the half-life of GnRH mRNA in the presence or absence of ionomycin. In control (DMSO-treated) GT1-7 cells, the GnRH mRNA half-life was 25 h, similar to that reported in previous studies (17, 22). When GT1-7 cells were pretreated with ionomycin for

2.5 h, the half-life decreased to 9 h. This observation suggested that ionomycin decreased GnRH mRNA stability.

Poly(A) tail length has been reported to be an important indicator of mRNA stability in many systems (reviewed in Ref. 30). Numerous studies have provided evidence that a longer poly(A) tail confers stability upon the mRNA, whereas poly(A) tail shortening precedes and may result in degradation of the mRNA (26–28, 31, 32). In the present study, we found that the average size of the GnRH mRNA was significantly reduced in GT1-7 cells by treatment with ionomycin, but the size of RNase H-treated mRNA from both control and ionomycin-treated GT1-7 cells stayed the same. This most likely represents a mRNA poly(A) tail shortening and probably contributes to the decrease in GnRH mRNA stability and the subsequent decrease in GnRH mRNA level after ionomycin treatment.

Calcium has been implicated in the transcriptional activation of a variety of genes. For example, White and colleagues have shown that calcium activates transcription of the PRL gene, possibly through a calcium/calmodulin-dependent protein kinase mechanism (33). Nguyen and colleagues have demonstrated calcium stimulation of proenkephalin gene expression via calcium/calmodulin-dependent protein kinase through the same *cis*-element responsible for cAMP activation of the proenkephalin gene (34). Studies by Greenberg's laboratory have shown that calcium activation of *c-fos* gene expression occurs by calcium/calmodulin-dependent protein kinase phosphorylation of transcription factors cAMP response element binding protein and serum response factor (35, 36). Several studies have also demonstrated that calcium activates cytokine gene expression by calcineurin-mediated dephosphorylation of the transcription factor nuclear factor-AT (37–39).

Recently, calcium-mediated transcriptional inhibition has been documented. For example, calcium ionophore A23187 has been shown to inhibit heat shock protein-70 expression by altering the phosphorylation state of the heat shock transcription factor (40). Ionomycin has been shown to inhibit transcription factor STAT3 phosphorylation and its DNA binding (41).

In the present study, we observed that ionomycin caused a significant decrease in GnRH gene transcription, as indicated by a decrease in GnRH primary transcript levels and a significant decrease in GnRH mRNA levels, due to a combination of an inhibition of transcription and a destabilization of existing GnRH mRNAs. These effects of calcium could be mediated by posttranslational modification of preexisting factors or by inducing the synthesis of new protein factors that subsequently affect GnRH gene expression. We investigated these possibilities by determining whether new RNA or protein synthesis is required in the ionomycin-induced decrease in GnRH primary transcript and mRNA levels. As expected, treatment with DRB reduced primary transcript levels, but not to zero, most likely because after 8 h the DRB has been metabolized partially, and transcription is starting to rebound. We believe that this is why ionomycin can further suppress primary transcript levels after DRB treatment. Interestingly, CHX alone caused a major decrease in primary transcript levels, suggesting that some labile protein factor(s) is involved in maintaining basal levels of GnRH transcrip-

tion. Changes in the protein binding pattern in the GnRH promoter region by DNA footprint and mobility gel shift assays are necessary to clarify this issue. In contrast to GnRH gene transcription, the ionomycin-induced decrease in GnRH mRNA levels was prevented by DRB and CHX. Like calcium, the PKC activator PMA also causes a decrease in GnRH primary transcript and mRNA levels as well as a decrease in GnRH mRNA stability (18, 22). The effect of PMA on GnRH mRNA stability is both RNA and protein synthesis dependent (18). These findings indicated that the posttranscriptionally mediated down-regulation of the GnRH mRNA level involved a mechanism that requires the new synthesis of cellular factors. It is possible that treatment with ionomycin or PMA induces the expression of a gene(s) whose product(s) could regulate the turnover of GnRH mRNA. Thus, pretreatment of cells with a RNA synthesis inhibitor (DRB) or a protein synthesis inhibitor (CHX) prevents the activation of this factor(s) by PMA or ionomycin and prevents the decrease in GnRH mRNA. Future identification and characterization of these cellular factors will be important to further our understanding of the regulation of GnRH gene expression at the posttranscriptional level.

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