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Post-Transcriptional Regulation of the Gonadotropin-Releasing Hormone Gene in GT1–7 Cells

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Abstract

GT1–7 cells respond to treatment with the phorbol ester, phorbol 12-myristate 13-acetate (PMA), with an inhibition of transcription of the proGnRH gene and decreases in GnRH mRNA levels. However, the timing of this decrease in GnRH mRNA levels suggests that a decrease in GnRH mRNA stability may be involved in addition to an inhibition of transcription of the proGnRH gene. To address this possibility, we treated GT1–7 cells with 100 nM PMA for 4 h and then monitored GnRH mRNA levels over time after blockade of GnRH gene transcription with DRB. PMA treatment caused GnRH mRNA half-life to decrease from 30 to 11 h. Then, to verify this observation, we examined changes in GnRH mRNA poly (A) tail length, which may be a reflection of mRNA turnover, following treatment of GT1–7 cells with PMA or vehicle for 0, 4, 8 or 24 h. The poly (A) tail was removed from half of the GT1 cytoplasmic RNA sample by digestion with RNase H and the difference in GnRH mRNA size with and without RNase H treatment was determined by Northern hybridization. PMA treatment (4 and 8 h) resulted in a significant decrease in the length of the GnRH mRNA poly (A) tail, consistent with a decrease in GnRH mRNA stability. This finding suggests that GnRH mRNA turnover is inducible by substances such as PMA. Our study indicates that a change in mRNA stability is one of a multiplicity of levels at which GnRH gene expression is regulated.

Gonadotropin-releasing hormone (GnRH) mRNA levels are regulated by numerous neurotransmitters, hormones and growth factors (1–6). We have previously reported that GnRH mRNA levels in rat brain can be altered by the glutamate analog N-methyl-D,L-aspartate treatment or during the estrous cycle without concomitant changes in GnRH primary transcript (1, 2), an index of GnRH gene transcription (7). We hypothesized that this increase in GnRH mRNA levels, occurring independently of transcription, is due to a post-transcriptional change such as an enhancement of mRNA stability, i.e. a decrease in degradation of the existing GnRH mRNA pool. However, because of the small number and widespread distribution of GnRH neurons in the mammalian brain (8) we approached this issue of mRNA stability using the immortalized mouse hypothalamic GnRH-secreting cell line, the GT1 cells (9). These cells have facilitated an understanding of how neurotransmitters and other factors affect GnRH secretion and gene expression at a cellular and/or molecular level.

Much of the research on GnRH release and gene expression in the GT1 cell lines has focused on the protein kinase C (PKC)/calcium second messenger systems which mediate the effects of neurotransmitters (10–13). The phorbol ester, phorbol 12-myristate 13-acetate (PMA), a PKC activator, has been reported to inhibit transcription of the proGnRH gene, decrease mRNA levels and decrease translational efficiency of the GnRH mRNA (10–15). While the decrease in GnRH gene transcription by phorbol esters occurs quickly, measurements of GnRH mRNA half-life suggest that it is a stable mRNA ($t_{1/2} > 20$ h; 15) and it would take many hours for such transcriptional changes to be manifested as changes in mRNA levels. It therefore appears that an additional mechanism, along with decreased transcription, is responsible for the decrease in GnRH mRNA levels observed following PMA treatment. Thus, in the present study, we examined whether GnRH mRNA stability is also decreased by PMA treatment in GT1–7 cells.

Results

RNase protection assay (Experiment I)

GnRH primary transcript levels measured using the A2B clone decreased significantly compared to DMSO controls following 3 h PMA treatment ($P < 0.0001$; Fig. 1A). Levels were still significantly depressed at 10 h PMA treatment ($P < 0.001$), but returned to control levels at 21 h PMA treatment. For GnRH mRNA levels in the cytoplasm, a significant decrease was observed beginning 10 h after the initiation of PMA treatment ($P < 0.0001$),

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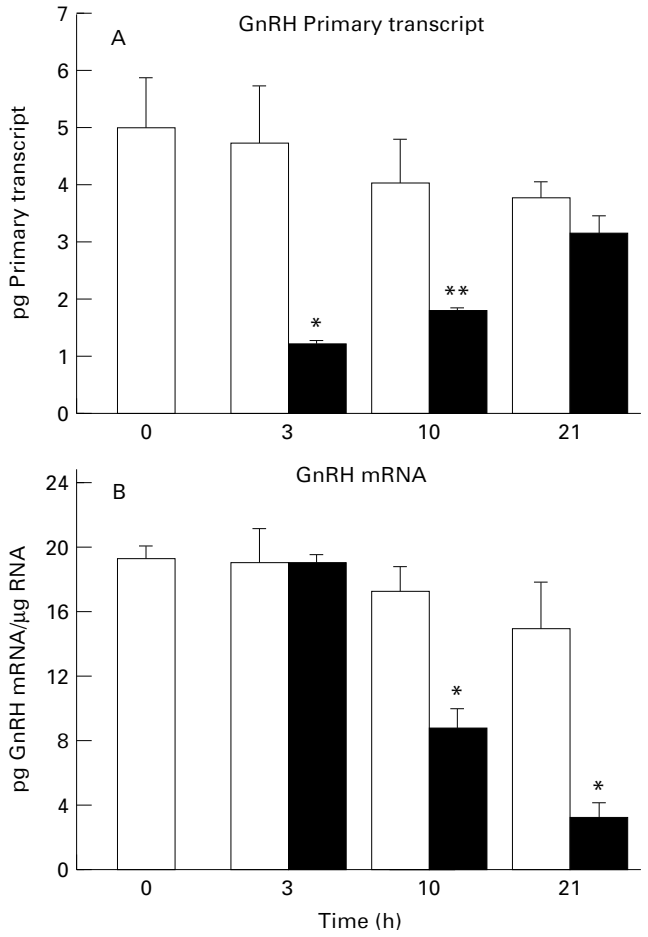

FIG. 1. Mean levels of GnRH primary transcript (A) in the nucleus and GnRH mRNA levels (B) in the cytoplasm of GT1–7 cells treated with PMA for 0, 3, 10 and 21 h. Primary transcript levels were significantly decreased at 3 and 10 h compared to controls. GnRH mRNA levels were significantly lower at 10 and 21 h compared to DMSO controls. **, $P<0.0001$; *, $P<0.001$. Data in this and other figures are presented as mean ± SEM. ■ Control; □ PMA.

and levels were even further decreased at 21 h of PMA treatment ($P<0.0001$; Fig. 1B).

Determination of GnRH mRNA half-life (Experiment II)

GnRH mRNA levels were observed to decay with a half-life of 30 h in control (DMSO)-treated GT1–7 cells following DRB treatment (Fig. 2A). When cells were treated with PMA for 4 h prior to DRB treatment, the half-life of the GnRH mRNA decreased to 11 h (Fig. 2B). In contrast, cyclophilin mRNA half-life in GT1–7 cells was similar both in the presence (14 h) or absence (13 h) of PMA (data not shown), indicating that the effect of PMA is specific to the GnRH system.

Poly (A) tail analysis (Experiment III)

A representative Northern blot demonstrating migration of GnRH mRNA through the agarose gel is shown in Fig. 3. The mRNA that was digested with RNase H is shown in lanes 1–7, and appears as a discrete band. Messenger RNA that was not digested with RNase H migrated in a broader band, as is seen in lanes 8–14.

Neither treatment with PMA nor time affected the size of the RNase H-treated mRNA, and the average size of this 'tailless' GnRH mRNA for all treatment groups, as determined by comparison to RNA and DNA molecular size markers, was approximately 486 bases, which corresponds to the size of the mouse GnRH without a poly (A) tail (Fig. 4A). For the mRNA which had a poly (A) tail, the mean size of the GnRH mRNA did not differ among the control groups, and had a mean size of 587 bases (Fig. 4B). There was a significant shift in the size distribution of the GnRH mRNA transcript following 4 and 8 h PMA treatment ($P<0.001$; Fig. 4B), to 547 and 524 bases, respectively. The mean size of the GnRH mRNA returned to slightly greater than control levels (606 bases) following 24 h PMA treatment (Fig. 4B).

Discussion

Two major factors largely determine the levels of a specific mRNA present in a cell: first, the rate of transcription of the gene encoding this RNA, and second, the rate of degradation of this mRNA in the cytoplasm (i.e., mRNA stability). In the present study, we observed that GnRH mRNA levels decreased two-fold after treatment for ten hours with the phorbol ester PMA. In order to estimate the contribution of GnRH gene transcription to levels of GnRH mRNA, we measured levels of GnRH primary transcript in the nucleus. We have observed that changes in GnRH primary transcript parallel transcription of the GnRH gene, as measured by transcription run-on assays (7), and thus levels of primary transcript in the nucleus are a good indication of transcriptional activity of the proGnRH gene. In the present study, GnRH primary transcript levels were significantly decreased following 3 h of PMA treatment. These results are consistent with studies estimating transcription of the proGnRH gene following PMA treatment using transfection/GnRH promoter activity in GT1 cells (7, 13, 15). GnRH mRNA levels did not decrease until several hours after the decrease in GnRH gene transcription; therefore it is possible that this latter decrease is simply a reflection of the preceding decline in GnRH gene transcription. However, it has been reported in the present and a previous study (15) that the half-life of the GnRH mRNA is quite long (>20 h), suggesting that even if transcription of the GnRH gene were completely blocked by PMA treatment, it would take more than 20 h to see a two-fold decrease in cytoplasmic GnRH mRNA levels.

We addressed this issue by measuring the half-life of GnRH mRNA in the presence or absence of PMA. This would directly determine whether phorbol esters alter GnRH mRNA stability. In control (DMSO-treatment) GT1–7 cells, we determined GnRH mRNA half-life to be 30 h, similar to that (22 h) reported in a previous study using the RNA synthesis inhibitor actinomycin D (15). In the present study, when GT1–7 cells were pre-treated with PMA for 4 h, the half-life decreased to 11 h. In contrast, in another laboratory (15), co-treatment of GT1 cells with the phorbol ester and actinomycin D did not significantly change GnRH mRNA half-life (to 18 h). This lack of effect on GnRH mRNA half-life is most likely due to the co-administration of the two drugs in the previous study (15), compared to 4 h pre-treatment with PMA in the present study. Treatment with PMA

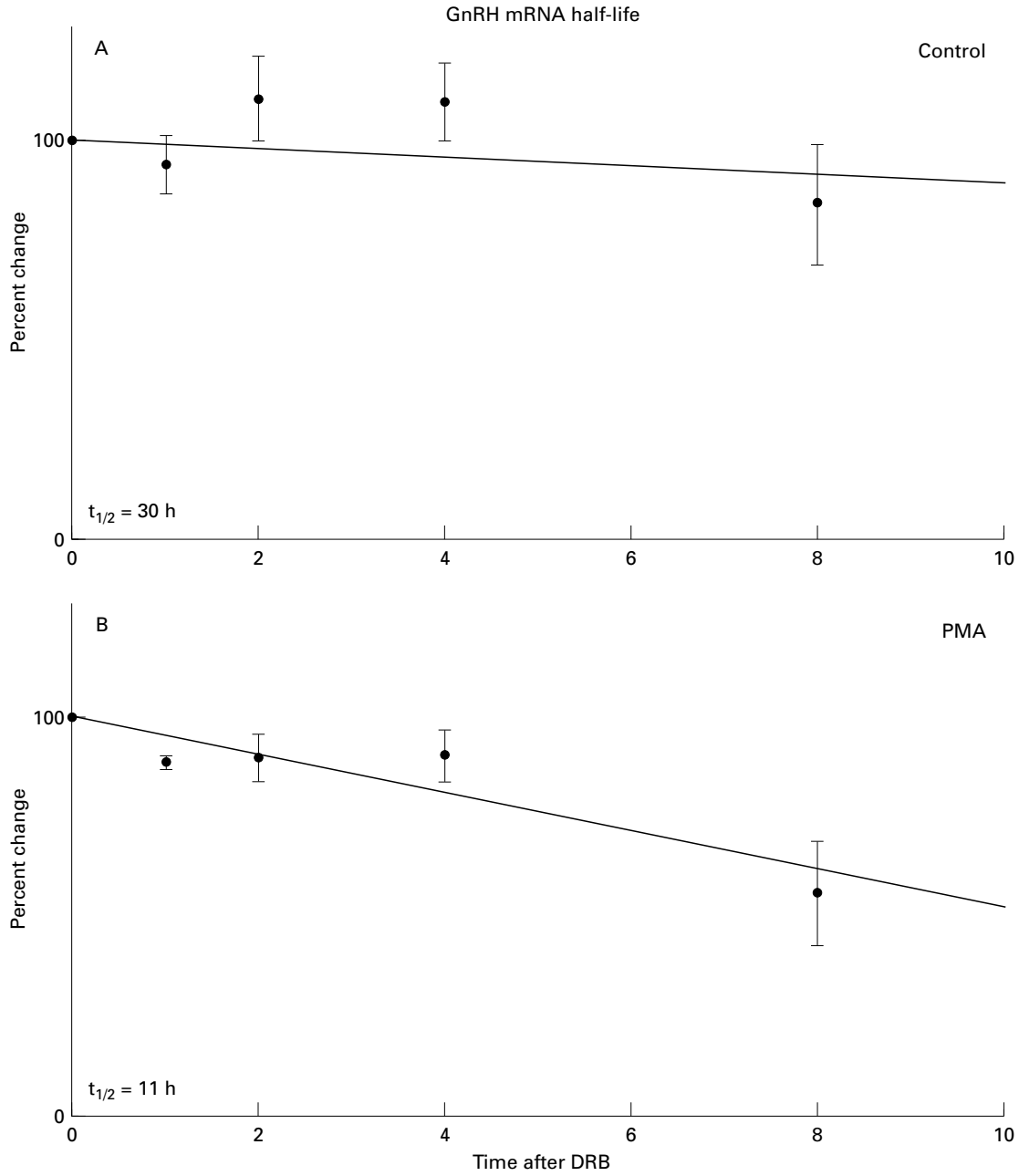

FIG. 2. Half-life of GnRH mRNA as determined by RNase protection assay. In control (DMSO)-treated GT1–7 cells (A), the GnRH mRNA half-life was calculated to be 30 h. In PMA-treated GT1–7 cells (B), the half-life was reduced to 11 h.

appears to cause an activation of cellular factors that are involved in degrading the GnRH mRNA, which under basal conditions in GT1 cells has an extremely long half-life (15, 16). When GT1 cells are treated with PMA and an RNA or protein synthesis blocker simultaneously, these cellular factors cannot be induced, preventing a change in GnRH mRNA turnover rate. Exposure of GT1–7 cells to PMA for 4 h prior to treatment with the RNA or protein synthesis inhibitor is sufficient to activate this 'turnover system' (16) and thus the factors that are responsible for turning over the GnRH mRNA have already been induced and can thus elicit a subsequent decrease in the GnRH mRNA half-life, as in the present study. Our finding supports the hypothesis that PMA has direct effects on GnRH mRNA stability, and it is therefore likely that the rate of turnover of the GnRH mRNA, as well as gene transcription, plays an important role in determining GnRH mRNA levels. Therefore, in Experiment III we assessed changes in GnRH mRNA stability by measuring changes in GnRH mRNA poly (A) tail length in response to treatment with PMA.

Poly (A) tail length has been reported to be an important indicator of mRNA stability in numerous systems (17, 18). For example, deadenylated rabbit globin mRNA injected into *Xenopus* oocytes was found to have a half life of 5–10 h. In contrast, when

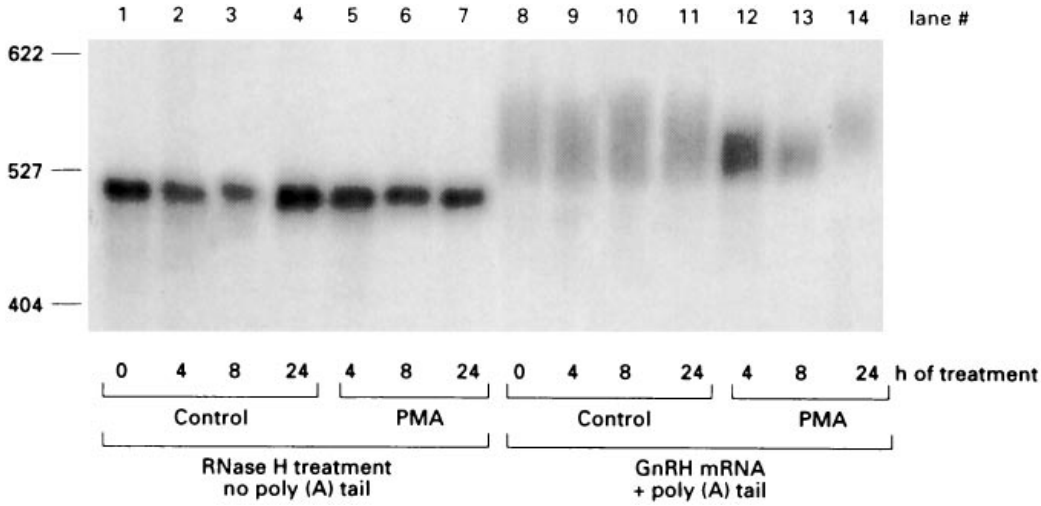

FIG. 3. Autoradiogram of GnRH mRNA electrophoresed through a denaturing agarose gel. Messenger RNA that was digested with RNase H is shown on the left (lanes 1–7), and undigested mRNA containing the poly (A) tail is shown on the right (lanes 8–14). Treatment condition and time are indicated below each lane. The RNase H-treated GnRH mRNA migrated in a discrete, uniform-sized band for all treatments. Messenger RNA containing the poly (A) tail migrated in a broader band. The size of the mRNA containing the poly (A) tail was reduced by PMA treatment for 4–8 h, and returned to control levels at 24 h. The migration of the *Msp*I-digested pBR322 marker is indicated on the far left.

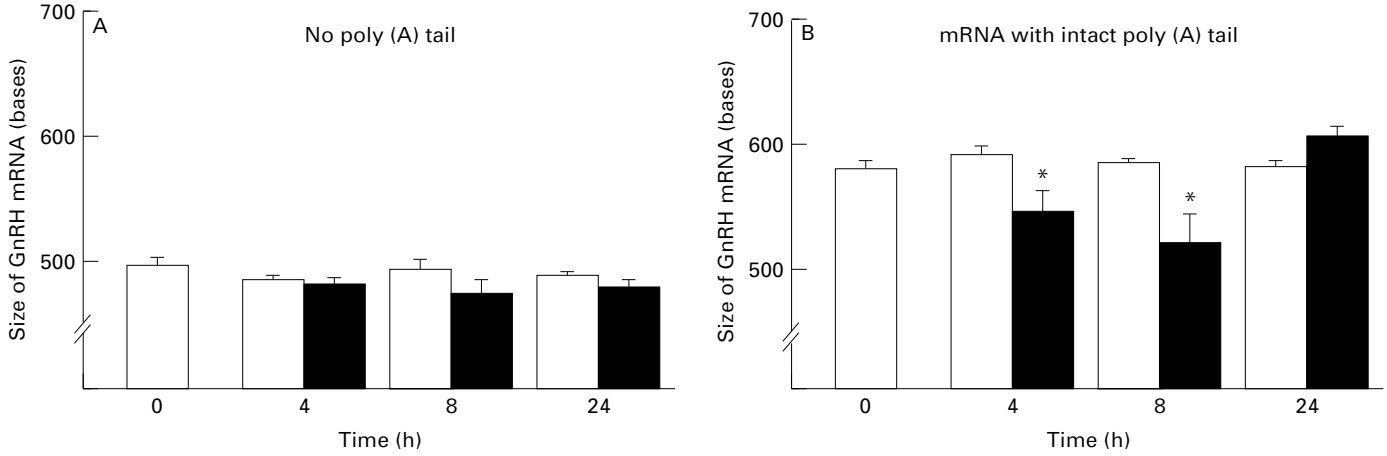

FIG. 4. The size of the GnRH mRNA with and without the poly (A) tail was calculated for each treatment group (n = 6/treatment). A) The mean size of the GnRH mRNA that had been digested with RNase H (no poly (A) tail) for all the groups did not vary. B) The size of the GnRH mRNA that had an intact poly (A) tail did not differ between control groups. A significant reduction in poly (A) tail size occurred following PMA (4 and 8 h) treatment. GnRH mRNA size returned to control levels following 24 h PMA treatment. *, $P < 0.001$ *vs* corresponding control, and *vs* 24 h PMA treatment. ■ Control; □ PMA.

the mRNA containing the poly (A) tail was injected, it was still not degraded at >48 h (19). It was reported in yeast cell-free extracts that polyadenylation of the 3′ end stabilized the mRNA, irrespective of the mRNA species (20). For the c-*fos* system, a shortening of the poly (A) tail from ~200 to a length of 30–60 nucleotides was followed by decay of the mRNA, indicating that the decrease in the poly (A) tail length may in fact be causal to the mRNA degradation (21). These and other studies (22, 23) have provided strong evidence that a longer poly (A) tail probably confers stability upon the mRNA, while in contrast, poly (A) tail shortening precedes and may result in degradation of the mRNA. Furthermore, alterations in poly (A) tail length have been reported in several neuroendocrine and other systems such as vasopressin (24–26), prolactin (27), oxytocin (28, 29), and chorion and globin (30), and in response to changes in the cellular environment such as the steroid hormone milieu (31), lactation (29), phorbol esters (32), osmotic condition (24, 26) or stimulation by neurotransmitters (27). Thus, this appears to be an important and ubiquitous mechanism for the regulation of mRNA levels.

In the present study we found that the average size of the GnRH mRNA poly (A) tail was significantly reduced in GT1–7 cells by treatment with PMA. This most likely represents a decrease in mRNA stability and may contribute to the degradation of GnRH mRNA, with a subsequent decrease in GnRH mRNA levels. The GnRH mRNA species containing the poly (A) tail migrated through the gel in a relatively broad band with an average size of 587 bases as determined by comparison to an

RNA molecular marker. This band is probably wide 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 those older mRNAs having shorter poly (A) tails due to nuclease activity. Other laboratories using Northern hybridization of total RNA have also shown relatively broad GnRH mRNA bands which become conspicuously narrowed following treatment with phorbol ester (10, 11, 15). The particular breadth of the bands in the present study is due to the extensive distance of electrophoresis through a higher percentage (2%) agarose gel in order to get extremely good size resolution. After 4 and 8 h treatment with PMA, the average size of the GnRH mRNA decreased to 547 and 524 bases, an average decrease of 40 and 63 nucleotides, respectively. At 24 h of PMA treatment, the average size of the GnRH mRNA returned to slightly, although not significantly, greater than control levels (606 bases). GnRH gene transcription, as measured by primary transcript levels, was suppressed from 3 to 18 h of PMA treatment (data not shown for 18 h) but returned to baseline levels at 21 h of treatment. Thus, the return of poly (A) tail size to control levels following 24 h of PMA treatment in the present study probably represents newly synthesized GnRH mRNA.

There are clearly numerous levels at which GnRH gene expression and ultimately decapeptide levels are regulated. Transcription of the proGnRH gene, GnRH mRNA levels, as well as GnRH mRNA translational efficiency have all been reported to decrease in response to phorbol ester (7, 10, 11, 13–15), and the results of the present study add mRNA stability to this list. It is not clear whether all or any of these transcriptional and post-transcriptional events occur via the same mechanism or are regulated independently. The time course of events in the GT1–7 cells in response to PMA treatment suggest differential regulation of the above mechanisms. Furthermore, studies conducted *in vivo* indicate that transcriptional and post-transcriptional events are not regulated in parallel. For example, in the rat, treatment with N-methyl-D,L-aspartate increased GnRH mRNA levels without affecting nuclear primary transcript levels (2), an index of GnRH gene transcription, suggesting an increase in GnRH mRNA stability. Similarly, during the estrous cycle, on the day prior to the luteinizing hormone surge, cytoplasmic GnRH mRNA levels increased without a concomitant increase in primary transcript (1, 33). While RNA synthesis inhibitor or pulse-chase studies are extremely difficult to perform *in vivo*, it is possible to measure GnRH mRNA poly (A) tail size in the animal (Gore & Roberts, unpublished observation). We hope to apply the techniques used in the GT1–7 cell line to these types of animal models in order to investigate in a physiological system those mechanisms involved in the regulation of GnRH gene expression.

Materials and methods

Cell culture and treatment

GT1–7 cells were cultured as described previously (14). In Experiments I and II, studies were done in triplicate cultures, and were repeated four times. For Experiments III, single dishes were used per treatment, and experiments were repeated 6 times. Phorbol 12-myristate 13-acetate (PMA; Calbiochem, San Diego, CA; 100 nM) or vehicle (DMSO) was added to dishes for 0, 3, 10 or 21 h (Experiment I) or 0, 4, 8 or 24 h (Experiment III). Cells were harvested, spun down for 5 min at 800 × g, and the cell pellet resuspended in 400 µl lysis buffer (34). This fraction was layered over 350 µl cushion buffer and centrifuged at 800 × g. The cytoplasmic fraction was subsequently treated with proteinase K, extracted with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and then with chloroform : isoamyl alcohol (24 : 1), and precipitated with 2.5 volumes ethanol. The nuclear pellet was treated with DNase I and proteinase K as described previously (2). Subsequent preparation of cytoplasmic and nuclear RNA for RNase protection assays (Experiments I and II) was also performed as described previously (2). For Northern blot experiments (Experiment III), RNA was centrifuged, washed with 70% ethanol, and the pellets resuspended in 30 µl DEPC-H_2O. Half the RNA from a 10 cm dish was incubated with 2 µl oligo $d(T)_{12-18}$ primer (Gibco BRL, Gaithersburg, MD, USA) plus 5 µl of 5 × oligo d(T) buffer (0.5 M KCl, 0.5 mM EDTA) for 3 min at 65 °C, then 30 min at 22 °C. Two µl of RNase H (Gibco BRL) and 2 µl of 10 × RNase H buffer (200 mM Tris-HCl, pH 8.4; 500 mM KCl, 25 mM $MgCl_2$, 1 µg/µl BSA) was added and the RNA was incubated at 37 °C for 30 min. This was phenol : chloroform : isoamyl extracted, chloroform : isoamyl alcohol extracted, and precipitated with sodium acetate and ethanol. The other half of the RNA (untreated) was precipitated for a control.

RNase protection assay (Experiments I and II)

Cytoplasmic and nuclear RNA samples were suspended in 20 µl hybridization buffer (4 M guanidine thiocyanate and 0.1 M EDTA, pH 8) and incubated overnight at 30 °C with 5 µl of probe (1 ng) labelled to high specific activity (1,300,000 cpm/ng) with [α-^{32}P]UTP. Probe was also incubated with increasing concentrations of sense RNA to produce a standard curve. The probe used in Experiment I was a mouse GnRH clone complementary to the intron A-exon 2-intron B (A2B) region of the proGnRH gene, spanning the *Spe*I and *Hind*III restriction sites and subcloned into a Bluescript SK(+) vector. A 383 bp hybrid is observed in the nuclear samples corresponding to the full-length A2B region and represents unprocessed GnRH primary transcript containing both introns. A 144 bp hybrid is identified in the cytoplasmic samples and represents processed GnRH mRNA (exon 2 alone). For Experiment II, a 443 bp mouse GnRH cDNA clone spanning the *Eco*O109I and *Xba*I restriction sites and subcloned into a Bluescript SK(+) vector (14) was used, as well as a cyclophilin 111 bp cDNA clone spanning the *Pst*I and *Xmn*I restriction sites and subcloned into a Bluescript KS(+) vector (2). The remainder of the RNase protection assay was performed exactly as described previously (14). Samples were electrophoresed through a 5% nondenaturing polyacrylamide gel and exposed to a Phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA, USA) overnight for quantitation. The amount of radioactivity in each sample, as determined by the Phosphorimager, was compared to the amount of reference RNA, as calculated by regression analysis, and used to calculate the amount of GnRH RNA in each sample. Differences in GnRH primary transcript or cytoplasmic mRNA levels were determined using analysis of variance followed by Fisher's protected least significant difference (PLSD) *post-hoc* test. Significance was set at $P < 0.05$.

Determination of GnRH mRNA half-life (Experiment II)

GT1–7 cells were cultured as above. At t = -4 h, the medium was changed to DMEM containing PMA (100 nM) or vehicle (DMSO; 35). At t = 0 h, cells were treated with 5,6-dichloro-1-β-ribofuranosylbenzimidazole (DRB; 100 µg/ml final concentration). This reversible RNA polymerase inhibitor is less toxic than actinomycin D and more specific to the RNA polymerase II system (36). The dose of DRB was chosen based on our previous observation that it causes a rapid and almost complete disappearance of GnRH primary transcript in GT1–7 cells within 60 min of administration (16), suggesting a rapid blockade of GnRH gene transcription (7). This result is similar to the inhibition of RNA synthesis reported in primary pituitary cell cultures (37). Cells were harvested at t = 0, 1, 2, 4 or 8 h after DRB treatment.

Experiments were repeated four times with a similar half-life obtained each time; the results presented in Fig. 2 are a representative case. Cytoplasmic mRNA was harvested and assayed by RNase protection assay, as described above. The GnRH mRNA half-life was determined by performing a regression analysis on the change in GnRH mRNA levels for both control and PMA-treated GT1–7 cells. Cyclophilin mRNA levels were measured in the same samples by RNase protection assay and the cyclophilin mRNA half-life was determined in the same manner as the GnRH mRNA half-life.

Electrophoresis and Northern hybridization (Experiment III)

RNA was centrifuged, washed with 70% ethanol, and 3 μg of the pellets resuspended in 4.5 μl H_2O, 2 μl 10× formaldehyde buffer (38), 3.5 μl formaldehyde, and 10 μl deionized formamide. This was denatured and loaded onto a 2% formaldehyde gel which was run at 100 V for 5 h. These conditions were determined to give a high resolution of the size of the GnRH mRNA over a range of poly (A) tail sizes, thus resulting in a broad band. A ^{35}S-labelled marker (*Msp*I-digested pBR322, New England Biolabs, Beverly, MA, USA) or unlabelled RNA ladder (0.24–9.5 Kb, Gibco BRL) to which ethidium bromide (1 μl of 1 mg/ml stock) was added before electrophoresis were used as molecular markers. After electrophoresis a photograph of the gel next to a ruler was taken to determine the location of the RNA ladder bands. RNA was transferred to a Nytran (Schleicher & Schuell, Keene, NH, USA) filter overnight and fixed using a UV crosslinker (Stratagene, LaJolla, CA, USA). The filter was prehybridized and hybridized overnight using a mouse GnRH cDNA clone inserted into the *Eco*RI site of a Bluescript SK(+) vector, that was linearized and labelled to high specific activity (14). The filter was washed at high stringency (0.1% SSC, 65 °C) and exposed to x-ray film (Kodak XAR 5) overnight. The peak density of each band hybridizing to the GnRH probe (n = 6/treatment) was assessed: first, autoradiograms were scanned on a Macintosh scanner. Then, the peak density of each band was determined using the NIH Image program; this peak was compared to the size of the molecular markers. Using these peak values, an average RNA size was calculated from the six Northern blots performed for each of the 14 treatments. The differences in peak density between treatments was estimated using analysis of variance followed by Fisher's PLSD *post-hoc* analysis. Significance was set at $P < 0.05$.

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