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Translational Efficiency of Gonadotropin-Releasing Hormone Messenger Ribonucleic Acid Is Negatively Regulated by Phorbol Ester in GT1–7 Cells*

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ABSTRACT

Our laboratory and others have reported that treatment of GT1–7 cells with the phorbol ester, phorbol 12-myristate 13-acetate (PMA), inhibits transcription of the pro-GnRH gene and decreases messenger RNA (mRNA) levels. We were interested in whether translation of the existing GnRH mRNA decreases in parallel with these other indexes of biosynthesis after PMA treatment. GT1–7 cells were treated with PMA (100 nM) or vehicle for 0, 1, or 4 h. The cytosolic ribosome-associated RNA was isolated and layered on a continuous (10–40%) sucrose gradient, and fractions were analyzed for the distribution of ribosome-associated GnRH mRNA through the gradient by ribonuclease protection assay. The mRNA found in the lighter fractions is associated with fewer ribosomes per RNA, suggesting that these fractions are translated less efficiently, and RNA recovered from heavier fractions has a higher number of ribosomes per mRNA, representing mRNA that is more actively translated. We found that the distribution of the ribosome-associated GnRH mRNA was shifted into lighter fractions (*i.e.* fewer ribosomes per mRNA) after PMA treatment, indicating that a decrease in the translational efficiency of GnRH mRNA occurs after PMA treatment. Thus, PMA exerts inhibitory effects on translation of GnRH mRNA as well as on gene transcription, mRNA stability, and mRNA levels. (*Endocrinology* **136:** 1620–1625, 1995)

THE DECAPEPTIDE GnRH, which is released from neuroterminals in the median eminence, is subject to regulation by numerous excitatory and inhibitory neurotransmitters, growth factors, and hormones. Although significant inroads have been made in the understanding of how these substances control GnRH biosynthesis and release, both independently and through their interactions, the complexity of this network of neurons and glia in the brain makes it difficult to study the regulatory mechanisms controlling GnRH synthesis and secretion at a cellular level *in vivo*. Furthermore, GnRH neurons are scattered and are relatively sparse in the brain (1). Therefore, the development of GT1 cells, an immortalized mouse hypothalamic GnRH-secreting cell line (2), has facilitated the study of the events regulating expression of the pro-GnRH gene.

GnRH release in GT1 cells is affected by a number of neurotransmitters that also exert effects *in vivo*, such as norepinephrine (3), glutamate (4, 5), dopamine (3, 6), and γ-aminobutyric acid (7). These neurotransmitters act through the protein kinase-A (PKA) or protein kinase-C (PKC)/calcium second messenger systems. In fact, it has been shown that neurotransmitters can stimulate cAMP accumulation (6) or calcium influx (4), and their effects are blocked in the absence of calcium (4). It has also been found that the effects of these neurotransmitters can be mimicked by direct application of PKA or PKC activators (8–10). Our laboratory and others (8–10) have reported that phorbol esters such as phorbol 12-myristate 13-acetate (PMA), a PKC activator, stimulate GnRH release from GT1–7 cells while inhibiting transcription of the pro-GnRH gene and suppressing GnRH messenger RNA (mRNA) levels.

We hypothesized that PMA negatively affects the translational efficiency of GnRH mRNA, as transcription and other posttranscriptional processes, such as mRNA levels (8, 10) and stability (11), also decrease after PMA treatment. Because the number of ribosomes associated with the mRNA affects the ability to translate the mRNA into pro-GnRH peptide, in the present study we measured ribosome-associated GnRH mRNA levels in GT1–7 cells treated with or without PMA.

Materials and Methods

Cell culture and treatment

GT1–7 cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and maintained at 37 C with 5% CO_2. Cells were subcultured into individual tissue culture dishes (15 cm) 2–3 days before experiments and grown to approximately 70% confluency. The medium was changed to serum-free medium 1 h before each experiment. Three dishes were pooled per treatment, and all experiments were repeated three or four times. PMA (Calbiochem, La Jolla, CA; 100 nM) or vehicle (dimethylsulfoxide) was added to dishes for 0, 1, or 4 h. One milliliter of medium was collected from individual dishes and frozen at the time of harvest for RIA of GnRH. Polysomes were prepared as described previously (12). Briefly, cells were harvested and spun down for 5 min at 800 × *g*, and the cell pellet was resuspended in 400 μl lysis buffer (0.3 M sucrose; 0.25% sodium deoxycholic acid; 10 mM Tris, pH 7.4; 1.5 mM $MgCl_2$; and 0.5% Nonidet P-40) (13). This fraction was layered over 350 μl cushion buffer (0.4 M sucrose; 10 mM Tris, pH 7.4; and 1.5 mM $MgCl_2$) and centrifuged at 800 × *g*. The cytoplasmic fraction was further centrifuged at 16,000 × *g* for 20 min (12). This postmitochondrial supernatant was layered over a continuous

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sucrose gradient (10–40%) and spun for 4.5 h at 124,000 × *g* in a 12-ml Sorvall polyallomer ultracentrifuge tube (Sorvall, Norwalk, CT). For the EDTA and ribonuclease (RNase) treatments, EDTA (100 mM) or RNase (1 mM Tris, pH 7.4; 30 mM NaCl; 4 mg/ml RNase-A; and 0.2 mg/ml RNase T_1) was added to the postmitochondrial fraction and incubated at 30 C for 10 min before layering over the sucrose gradient (12). RNA was fractionated into eight 1.4-ml fractions and precipitated with 2.5 vol ethanol at −20 C. These fractions were centrifuged and washed with 70% ethanol, and the pellets were resuspended in NETS buffer (100 mM NaCl, 2.5 mM EDTA, 1% SDS, and 20 mM Tris, pH 7.4), extracted with phenol-chloroform-isoamyl alcohol and then with chloroform-isoamyl alcohol, and precipitated with 2.5 vol ethanol. RNA was centrifuged and washed with 70% ethanol, and the pellets were resuspended in 10 μl H_2O. Of this, 2 μl were aliquoted for RNase protection assay, 2 μl were electrophoresed on an ethidium bromide-stained 0.8% nondenaturing agarose gel, and 6 μl were diluted in 600 μl TE (10 mM Tris and 1 mM EDTA) to determine the total RNA content using absorbance at 260 nm.

RNase protection assay

RNA (2 μl) was diluted in 20 μl hybridization solution (4 M guanidine thiocyanate and 0.1 M EDTA, pH 8). We used a mouse 443-basepair complementary DNA (cDNA) clone coding for the GnRH-associated peptide region and spanning the *Eco0109*I and *Xba*I restriction sites inserted into the *Eco*RI site of a Bluescript SK(+) vector (produced by Dr. KeWen Dong in our laboratory), and a cyclophilin cDNA clone (kindly provided by Dr. J. Douglass) spanning the *Pst*I and *Xmn*I restriction sites, 111 bp of which were subcloned by Dr. Moshe Jakubowski in our laboratory into a Bluescript KS(+) vector. Plasmids were linearized and RNA probes synthesized with [α-^{32}P]UTP to high specific activity, 1 ng of which was added to each sample. For standard curves, probes were mixed with increasing known amounts of mouse GnRH or cyclophilin cDNA reference RNA. The assay was performed as described previously (13, 14). Samples were electrophoresed through a 5% nondenaturing polyacrylamide gel and exposed to x-ray film for 12 h or to a Phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA) for 6 h 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 or cyclophilin in each sample. Data were calculated as picograms of GnRH mRNA per pg cyclophilin mRNA (as cyclophilin was found to be unaffected by PMA treatment) to ensure that changes in GnRH mRNA levels were specific to GnRH and not generalized for all RNAs.

Determination of RNA profiles in sucrose gradients

Polysomes were prepared as described above, and after serial centrifugations, the ultracentrifuge tube was punctured at the bottom with a 20-gauge needle (12). Fractions (eight drops) were collected using a microfractionator (Gilson, Middleton, WI) and frozen at −20 C until analysis. The amount of RNA in each fraction was determined by combining 50 μl of each sample with 850 μl TE and 100 μl ethidium bromide (stock concentration, 100 μg/ml), and reading the fluorescence at 590 nm in a spectrofluorometer (Perkin-Elmer, Norwalk, CT). Samples from the top of the gradient exhibited quenching due to salts and other cytoplasmic components contaminating these fractions.

Analysis of ribosome-associated GnRH mRNA distribution

To calculate differences between treatments, weighted means were calculated from all of the profiles (n = 3–4) as follows (12): Σ[(fraction number)(p)], where p = (pg GnRH mRNA/Σ(pg GnRH mRNA). The difference in the means of the distributions was analyzed by analysis of variance, followed by Fisher's protected least significant difference *post-hoc* test.

GnRH RIA

GnRH in the medium was measured in 150-μl aliquots by RIA in a single assay using antiserum LR-10, which was kindly provided by Dr. R. Guilleman. Synthetic GnRH (Richelieu Laboratory, Montreal, Canada) was used for the radiolabeled antigen and the reference standard. The antigen-antibody complex was precipitated with a goat antirabbit γ-globulin (Calbiochem). The sensitivity of the assay was 0.5 pg/tube in a final volume of 500 μl without secondary antibody. The intraassay coefficient of variation was 6.0%. Differences in GnRH release between treatment groups were calculated by analysis of variance, followed by Fisher's protected least significant difference test.

Results

Spectrofluorometric analysis

The distribution of ribosomes associated with each fraction was determined by spectrofluorometric analysis. Mild RNase treatment resulted in a profile with a single large peak, corresponding to 80S monosomes. EDTA treatment dissociated the ribosomal subunits, resulting in peaks corresponding to the 40S and 60S subunits (data not shown). The positions of the 40S, 60S, and 80S peaks thus obtained could be aligned with the control gradient for comparison. For the control gradients, peaks were observed corresponding to the 40S and 60S ribosomal subunits, 80S monosomes, and several discrete peaks corresponding to increasing numbers (two, three, or four) of ribosomes associated with the mRNA (Fig. 1).

Polysome analysis

Sucrose fractionation yielded a distribution of RNA throughout the sucrose gradient. Figure 2A shows the electrophoresis through an agarose gel of RNA obtained from a representative sucrose fractionation. The lightest fraction of each gradient (lane 1) contained transfer RNA (tRNA) and other small RNAs. In lane 2, 18S and 28S ribosomal RNAs begin to appear, but are not in equal molar proportions, as expected for ribosomes, indicating dissociated ribosomal subunits. In lanes 3–8, 18S and 28S ribosomal RNAs appear in the correct molar ratios (~2:1 for a 1:1 molar ratio of 28S:18S), indicating the presence of intact ribosomes in mono- or polyribosome structures. EDTA treatment shifted

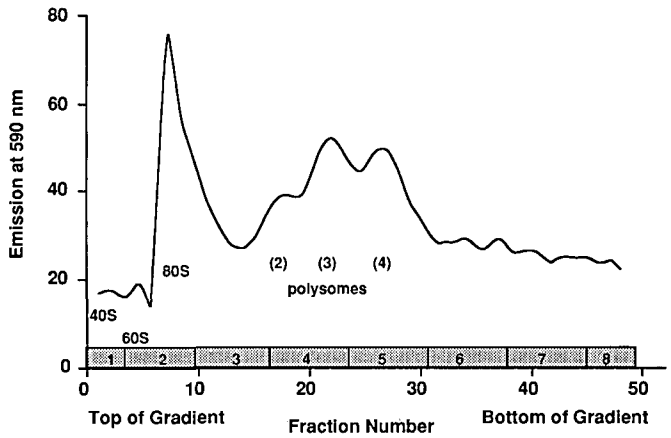

FIG. 1. Spectrofluorometric analysis of the distribution of polysomes through a sucrose gradient. Peaks representing 40S and 60S subunits, and 80S monosomes were observed as were several small peaks, which indicate increasing numbers of ribosomes (two, three, and four) associated with the mRNA. The approximate locations of the eight fractions aliquoted for the other experiments are indicated in the *shaded region above the abscissa.*

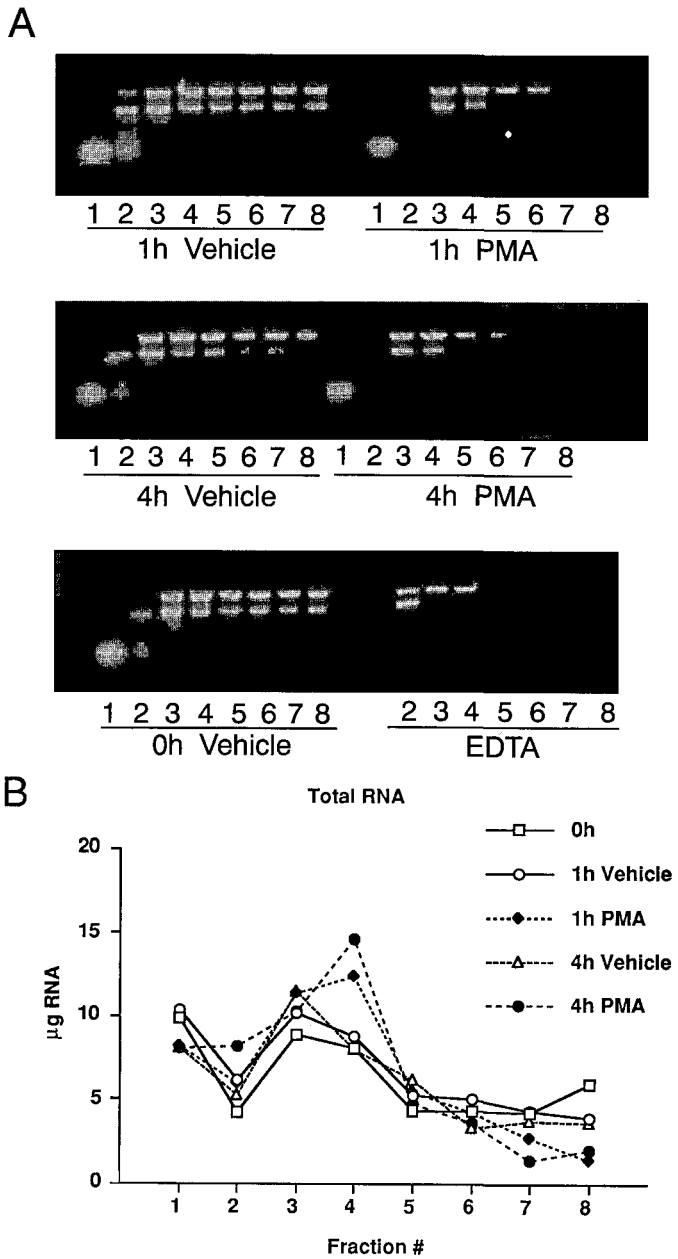

FIG. 2. Distribution of RNA extracted from fractionated polyribosomes through a representative agarose gel. Lane 1 indicates the lightest fraction and lane 8 the heaviest. A, Gel electrophoresis demonstrated that in control or PMA-treated GT1–7 cells, the lightest fraction (lane 1) contained exclusively tRNA. The 18S and 28S ribosomal subunits appear in lane 2, but with a greater proportion of the 18S subunit. In lanes 3–8, 18S and 28S subunits appear in the correct (~2:1) ratio. EDTA treatment shifted the 18S and 28S subunits to lanes 2–4, and the subunits were completely dissociated by EDTA. The high salt concentration in lane 1 prevented collection of sufficient RNA to run on the gel. B, The total amount of RNA, of which 20% was run on the agarose gel, was calculated for each fraction. There were no overall effects of PMA on RNA content, although there was a slight decrease in the heavier fractions (enough to make these undetectable by ethidium bromide on the agarose gel) and a slight increase in fraction 4.

the 18S and 28S ribosomal RNAs into the lighter fractions, with a predominance in fraction 2, demonstrating that the 40S and 60S ribosomal subunits had been dissociated. The high salt concentrations in lane 1 of the EDTA treatment prevented collecting sufficient amounts of RNA to analyze. Although the appearance of the PMA-treated lanes in the agarose gel appears different from that of the vehicle controls, in fact the amount of RNA in each treatment group was only slightly affected by PMA, as is shown in Fig. 2B, with a slight decrease in the heavier lanes and a small increase in fraction 4. This small decrease in RNA levels in the heaviest lanes after PMA treatment was sufficient to bring these lanes below the level of detectability for ethidium bromide staining.

RNase protection assay

Analysis by RNase protection assay indicated that PMA treatment caused a shift in the distribution of ribosome-associated GnRH mRNA through the sucrose gradient. PMA had no significant effect on cyclophilin mRNA. An autoradiogram of a representative assay for GnRH mRNA is shown in Fig. 3. The standard curve and the corresponding regression analysis obtained from the curve are shown in Fig. 3, A and B. A shift in the distribution of ribosome-associated

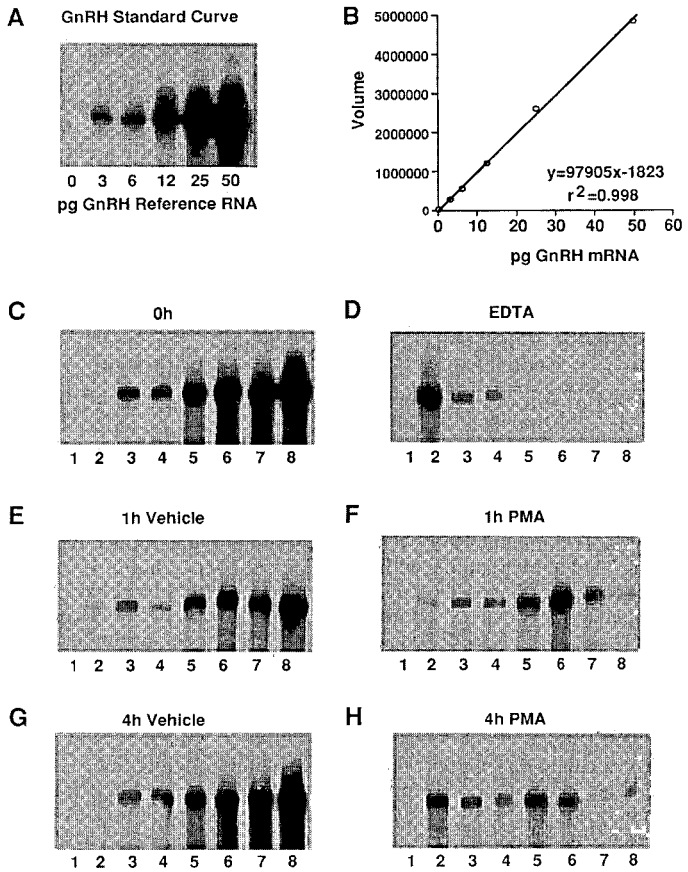

FIG. 3. A representative autoradiogram of an RNase protection assay showing GnRH mRNA. A and B show the standard curve and the regression line calculated from this curve (see *Materials and Methods*). C–H show GnRH mRNA in each of the eight fractions, from lightest (lane 1) to heaviest (lane 8). For control (C, E, and G) gradients, GnRH mRNA peaked in lanes 6–8. For PMA (F and H) gradients, GnRH mRNA peaked in lanes 5 and 6, and 2 and 3 (H only). EDTA (D) shifted the GnRH mRNA to a peak in lane 2.

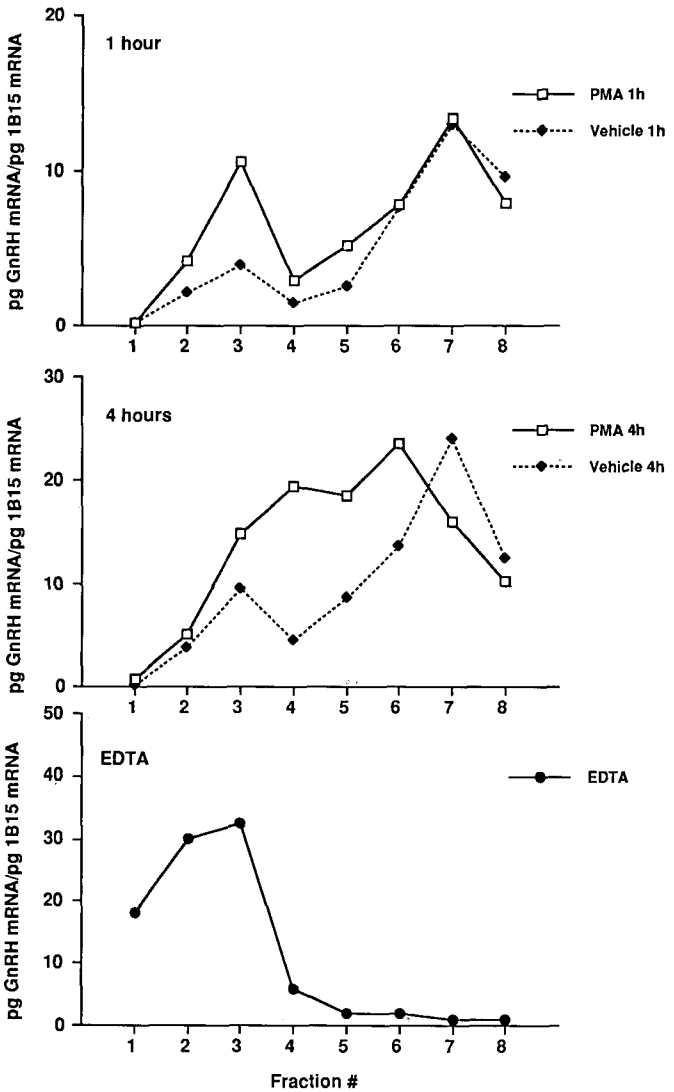

FIG. 4. A representative example of ribosome-associated GnRH mRNA through sucrose gradients. Lane 1 is the lightest fraction and lane 8 the heaviest. A, PMA or vehicle treatment for 1 h are shown. Both profiles showed a peak in fraction 7, but PMA treatment induced a secondary peak in fraction 3. B, PMA or vehicle treatment for 4 h is shown. The vehicle-treated gradient peaked in fraction 7, whereas PMA induced a shift to lighter fractions with a less-defined peak. C, EDTA treatment shifted the peak to fractions 2 and 3.

GnRH mRNA after PMA treatment was observed. The most GnRH mRNA is in lanes 6–8 in control treatments (Fig. 3, C, E, and G), whereas the peak GnRH mRNA after PMA treatment is observed in lanes 5 and 6 after 1 h of PMA (F) and in lanes 2 and 3 as well as 5 and 6 after 4 h of PMA (H). A representative distribution of the ribosome-associated GnRH mRNA, normalized to cyclophilin mRNA, is presented graphically in Fig. 4. For this experiment, a peak was observed in fraction 7 for 1-h vehicle and 1-h PMA treatment (Fig. 4A) as well as for 4-h vehicle treatment (Fig. 4B) and no treatment (data not shown). PMA treatment for 1 h resulted in a second prominent peak in fraction 3 (Fig. 4A). PMA treatment for 4 h resulted in an overall shift to lighter fractions (Fig. 4B). EDTA treatment caused a peak in fractions 2–3 (Fig. 4C).

A weighted mean was calculated for each polyribosome profile (see *Materials and Methods*) and was found to be fractions 5.8, 5.7, and 5.6 for 0, 1, and 4 h of vehicle treatment, respectively (Fig. 5). The weighted means were 5.2 and 4.8 for 1 and 4 h of PMA treatment, respectively; the value was not significantly different at 1 h, but was different at 4 h of PMA treatment ($P < 0.001$) from the corresponding vehicle control. EDTA treatment caused a shift in the distribution of polyribosome-associated GnRH mRNA with a peak in fraction 3.1 (data not shown) that was different from all other groups ($P < 0.001$).

Effects of PMA on GnRH release in GT1–7 cells

PMA treatment stimulated significant increases in GnRH release ($P = 0.0038$; Table 1), as determined by analysis of variance. *Post-hoc* analysis indicated that GnRH levels were elevated 1 and 4 h after PMA treatment compared to vehicle controls ($P < 0.05$ and $P < 0.01$, respectively).

Discussion

GnRH gene expression in GT1–7 cells is negatively regulated by PKC activators. It has been reported, using the transcription run-on assay, that PMA inhibits transcription of the GnRH gene (15). Levels of GnRH primary transcript and mRNA are also suppressed by PKC and another phorbol ester, TPA, as indicated by Northern hybridization and RNase protection assay (8, 10). We also found that mRNA stability, as reflected by poly(A) tail length, is decreased by PMA treatment (11). Therefore, in the present study we examined whether PMA had a similar inhibitory effect on translation of GnRH mRNA.

Before studying the effects of PMA on translational efficiency, it was necessary to examine the distribution of polyri-

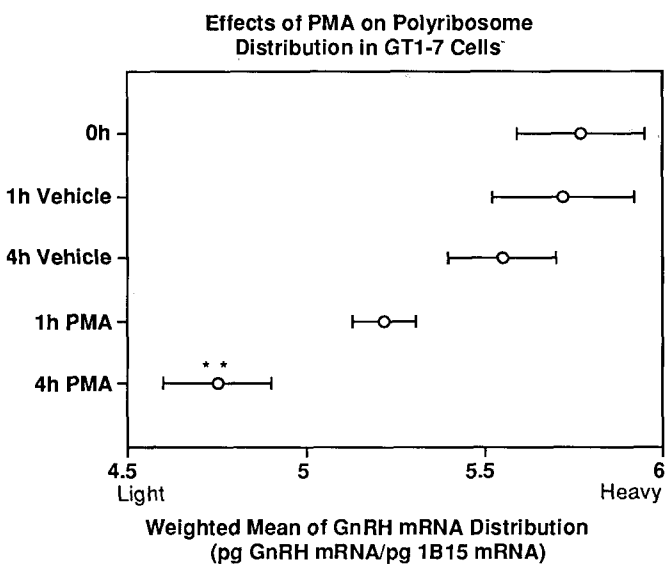

FIG. 5. The weighted mean of GnRH mRNA distribution was calculated for each treatment. Control gradients had a weighted mean of approximately fraction 5.7, whereas PMA shifted this mean to fraction 5.2 (1 h PMA) or 4.8 (4 h PMA). **, $P < 0.001$ *vs.* corresponding vehicle treatment.

TABLE 1. Effects of PMA on GnRH release in GT1–7 cells

Treatment	Mean + SEM
No treatment	47 ± 6
1 h vehicle	44 ± 6
1 h PMA	93 ± 31[a]
4 h vehicle	56 ± 14
4 h PMA	123 ± 12[b]

Results are expressed as picograms of GnRH per tube.
[a] $P < 0.05$ *vs.* corresponding vehicle treatment.
[b] $P < 0.01$ *vs.* corresponding vehicle treatment.

bosomes extracted from GT1–7 cells through a sucrose gradient. Spectrofluorometric analysis indicated that the entire complement of ribosomal subunits, monosomes, and polysomes was present in control gradients. The gradient concentration (10–40% sucrose) and speed and duration of centrifugation were chosen to focus on fewer ribosomes (<10), because GnRH mRNA is small with only 273 nucleotides from the start to stop codon. Thus, GnRH mRNA could accommodate a maximum of only 7–8 ribosomes (16).

We confirmed the observations from the spectrofluorometric analysis using electrophoresis of fractionated RNA through an ethidium bromide-stained agarose gel. In the absence of EDTA, polyribosomes were distributed throughout the gradient. In the lightest two fractions (no. 1 and 2), only tRNA and dissociated ribosomal RNAs were observed. Intact polyribosomes were seen in fractions 3–8, indicating that the mRNA can be actively translated in these heavier fractions. In the presence of EDTA, the majority of the material (dissociated ribosomal subunits) shifted to much lighter fractions.

A significant shift in the distribution of ribosomes associated with GnRH mRNA occurred after PMA treatment. Some of this may have been due to a generalized effect of PMA on all mRNAs, as manifested in the agarose gel as a slight shift of RNAs to lighter fractions after PMA treatment. However, we believe that most of the effect of PMA is specific to the GnRH mRNA, because cyclophilin mRNA was not similarly affected by PMA treatment. When weighted means were calculated, the peak distribution of ribosome-associated GnRH mRNA in untreated or vehicle-treated GT1–7 cells occurred in fraction 5.7. Four-hour treatment with PMA shifted this peak significantly to fraction 4.8, and treatment for 1 h shifted the peak to fraction 5.2; however, this was not significantly different from the control values. We observed in the agarose gel that both 18S and 28S ribosomal RNAs exist in fractions 3–8, indicating that mRNA in these fractions can be actively translated. Thus, PMA shifts the ribosome-associated mRNA from one translatable pool to another. Spectrofluorometric analysis indicates that fraction 6 is associated with approximately four to six ribosomes, whereas fractions 4–5 contain only approximately two to four ribosomes. All of these fractions have the capability of being translationally active. It has been reported in other systems, for example ferritin (17), that mRNA can be shifted between translationally active and inactive states. However, this does not appear to be the case for the GnRH system.

There are two levels at which translational efficiency can be regulated: initiation of the translational complex and elongation of the peptide chain. With regard to initiation, the binding of a ribosomal initiation complex to the 5′-end of the mRNA allows translation to begin. A decrease in the rate of initiation would result in a decreased number of ribosomes attaching to the mRNA, and would be evidenced by a shift in the polyribosome profile from heavier to lighter fractions, as occurred for the GnRH system in the present study. This would also cause a decrease in translational efficiency, resulting in a decrease in pro-GnRH/GnRH peptide content, as we observed in a previous study (10). Indeed, initiation is a common level at which translation is regulated (18, 19). Translational initiation can be modulated by the phosphorylation of initiation factors by protein kinases (18); this is a potential mechanism by which phorbol esters, which activate the PKC system, affect translation of GnRH mRNA in the present study. Another mechanism by which a shift in the polyribosome profile to lighter fractions could occur is by an increase in the rate of elongation without a concomitant change in initiation. This would result in no change in translational efficiency; however, we do not believe that this mechanism applies to the GnRH system because it is not consistent with the decrease in peptide content observed after phorbol ester treatment (10).

Despite the negative effects of PMA on the biosynthesis and processing of GnRH mRNA, GnRH levels increase in the medium after phorbol ester treatment in the present and other studies (8–10). The finding that transcription and post-transcriptional processes are inhibited by PMA suggests that the increase in secreted GnRH induced by PMA is probably not due to *de novo* synthesis of the peptide, but, rather, to the processing and release of preexisting stores of GnRH. This is supported by the finding that pro-GnRH levels in the medium and intracellular GnRH decrease after prolonged phorbol ester treatment (9, 10), indicating that these stores of peptide, had in fact, been released, but not replenished.

In conclusion, we report that the phorbol ester PMA decreases the translational efficiency of GnRH mRNA. Thus, GnRH gene expression is negatively regulated at a multiplicity of levels, including transcription, primary transcript and mRNA levels, mRNA stability, and translational efficiency.

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