Dissociation between Release and Gene Expression of Gonadotropin α-Subunit in Gonadotropin-Releasing Hormone-Stimulated αT3-1 Cell Line†

David Ben-Menahem,‡ Zurit Shraga,‡ Hadas Lewy,‡ Rona Limor,§ Ilan Hammel,‖ Reuven Stein,‡ and Zvi Naor*,‡

Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel, Timsit Institute of Reproductive Endocrinology, Sackler School of Medicine, Tel Aviv University, Ramat Aviv 69978, Israel, and Department of Pathology, Sackler School of Medicine, Tel Aviv University, Ramat Aviv 69978, Israel

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ABSTRACT: The αT3-1 cell line which was derived by targeted tumorigenesis in transgenic mice [Windle et al. (1990) *Mol. Endocrinol. 4*, 597–603] possesses high-affinity binding sites for GnRH analogs coupled to enhanced phosphoinositide turnover and phospholipase D activity. Incubation of αT3-1 cells with [D-Trp^6]-GnRH analog (GnRH-A) resulted in a rapid increase in gonadotropin α-subunit mRNA levels which was detected already at 30 min of incubation (0.1 nM GnRH-A, 3-fold, $p < 0.01$). The effect diminished with time to reach basal levels at about 12 h of incubation, with a secondary rise in α mRNA levels between 12 and 24 h of incubation. Addition of the protein kinase C activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 100 ng/mL) or the Ca^{2+} ionophore ionomycin (1 μM) to αT3-1 cells also resulted in a rapid increase in α-subunit mRNA levels. Surprisingly, GnRH-induced α-subunit release was detected only after a lag of 4 h of incubation. Thus, dissociation between exocytosis and gene expression can be demonstrated in GnRH-stimulated αT3-1 cell line.

Gonadotropin-releasing hormone (GnRH)[1] action upon gonadotropin secretion involves several steps following the binding to specific receptors located exclusively on pituitary gonadotrophs [see Naor (1990) for review]. The signaling events include the following: interaction with a G-protein (Perrin et al., 1989; Limor et al., 1989); enhanced phosphoinositide turnover (Naor et al., 1986; Morgan et al., 1987); Ca^{2+} mobilization and influx (Chang et al., 1986; Limor et al., 1987; Naor et al., 1988); activation of phospholipase D (Netiv et al., 1991); translocation and activation of protein kinase C (Naor et al., 1985a; Hirota et al., 1985); release of arachidonate and formation of active lipoxygenase products (Naor & Catt, 1981; Naor et al., 1985b; Kiesel et al., 1991; Dan-Cohen et al., 1992). Although a large body of literature exists regarding gonadotropin release [see Naor (1990) for review], much less is known about GnRH-induced gonadotropin synthesis [see Gharib et al. (1990) for review].

LH, FSH, and TSH are heterodimer glycoproteins sharing a common α-subunit and specific β-subunits bound in noncovalent association (Gharib et al., 1990; Pierce & Parsons, 1980). With the isolation of the cDNAs for common α- and the specific β-subunits from several species (Godine et al., 1982; Nilson et al., 1983; Chin et al., 1983; Maurer, 1987), analysis of GnRH and steroid effects on common α and LHβ or FSHβ mRNA levels was explored in vivo and in vitro [see Gharib et al. (1990) for review]. Administration of GnRH in various animal models as well as castration resulted in a 2–3-fold increase in LH subunit mRNA steady-state levels (Papavasiliou et al., 1986; Haisenleder et al., 1987, 1988; Leung et al., 1987; Childs et al., 1987; Mercer et al., 1988; Saade et al., 1989). In vitro experiments, utilizing primary rat pituitary cell cultures, are difficult to interpret since controversy exists concerning GnRH regulation of gonadotropin subunit mRNA levels [see Gharib et al. (1990), Mercer (1990), and Counis and Jutisz (1991) for reviews]. While some investigators found increased α- but not β-subunit mRNA levels after GnRH challenge (Hubert et al., 1988; Weiss et al., 1990a), others found increased LHβ mRNA levels by GnRH (Andrews et al., 1988), while still others found that both α and LHβ mRNA levels were elevated after GnRH challenge (Starzec et al., 1989; Attardi et al., 1989). On the other hand, using transcription run-on and S-1 nuclease protection assays, it was shown that GnRH-stimulated LH release is not coupled to changes in the rate of LHβ gene transcription or to the cytosolic mRNA levels, while GnRH affected the level of LHβ primary transcripts and processing intermediates, and a rapid decrease in the level of fully processed LHβ mRNA in the nucleus was observed (Salton et al., 1988).

The recent availability of a gonadotroph-like cell line (αT3-1; Windle et al., 1990), which was derived by targeted tumorigenesis in transgenic mice and is capable of producing the common α-subunit, enables studies on GnRH action in homogeneous cell population. Recently, the same group has demonstrated that GnRH elevates common α-mRNA levels in a 16-h incubation protocol (Horn et al., 1991). More recently, the GnRH receptor has been cloned and its cDNA was isolated from the αT3-1 cell line (Tsutsumi et al., 1992; Reinhart et al., 1992). We therefore decided to initiate studies on GnRH action in αT3-1 cells. Here we report an interesting

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* Address correspondence and requests for reprints to this author at the Department of Biochemistry, Tel Aviv University, Tel Aviv 69978, Israel. FAX: (972)-3-6415053.

‡ The George S. Wise Faculty of Life Sciences, Tel Aviv University.

§ Timsit Institute of Reproductive Endocrinology, Sackler School of Medicine, Tel Aviv University.

‖ Department of Pathology, Sackler School of Medicine, Tel Aviv University.

[1] Abbreviations: GnRH, gonadotropin-releasing hormone; GnRH-A, [D-Trp^6]GnRH analog; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TRH, thyrotropin-releasing hormone; BSA, bovine serum albumin; dCTP, deoxycytidine 5′-triphosphate; IP_1, IP_2, and IP_3, inositol phosphate, bisphosphate, and trisphosphate, respectively; DMEM, Dulbecco's Eagle's medium; FCS, fetal calf serum; HS, horse serum; RIA, radioimmunoassay; PBS, phosphate-buffered saline; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PKC, protein kinase C.

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dissociation between GnRH-induced α-mRNA levels (30 min) and α-subunit release (4 h).

EXPERIMENTAL PROCEDURES

Materials

αT3-1 cells were kindly provided by Dr. P. Mellon (La Jolla, CA). GnRH and TRH were purchased from Peninsula Laboratory (San Carlos, CA). Buserelin, a stable GnRH analog ([D-Ser(*t*-Bu)^6]Pro^9-NEt-GnRH), was kindly provided by Dr. J. Sandow (Frankfurt, Germany). [^{125}I]Buserelin was kindly provided by Dr. Y. Koch (Rehovot, Israel). The GnRH analog [D-Trp^6]GnRH (GnRH-A) was a gift from Dr. R. Millar (Cape Town, South Africa). The calcium ionophore ionomycin was purchased from Boehringer (Mannheim, Germany). Bovine serum albumin (BSA), 12-*O*-tetradecanoylphorbol 13-acetate (TPA), and other chemicals were purchased from Sigma (St. Louis, MO). A potent GnRH antagonist ([D-pGlu^1,pClPhe^2,D-Trp^{3,6}]GnRH) was kindly provided by Dr. D. Coy (New Orleans, LA). The rat α-subunit and LHβ cDNA probes were kindly provided by Dr. W. W. Chin (Boston, MA). Rat FSHβ cDNA was kindly provided by Dr. R. A. Maurer (Iowa City, IA). β-Tubulin cDNA was kindly provided by Dr. I. Ginzburg (Rehovot, Israel). All media and sera for cell culture were from Biological Industries (Kibbutz Beth Ha'Emek, Israel). [α-^{32}P]dCTP was purchased from NEN (Boston, MA).

Methods

Cell Culture. αT3-1 cells were subcultured into 60-mm tissue culture dishes (Sterilin, Hounslow, U.K.). Cells were grown in 5 mL of Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS), 5% horse serum (HS), penicillin (100 units/mL), and streptomycin (0.1 mg/mL). After 3–4 days, when cells were 70–80% confluent, the cultures were washed three times with fresh DMEM, and stimulants were added in 5 mL of DMEM at the indicated concentrations for the given length of time. For short period incubations (up to 1 h) 10 mM HEPES was added to the medium. When the stimulation period was longer than 9 h, the medium was supplemented with 0.1% BSA.

RNA Isolation and Analysis. At the end of the stimulation period, total RNA was isolated from cells by extraction in guanidinium thiocyanate containing 8% 2-mercaptoethanol by the LiCl method as previously described (Cathala et al., 1983). Total RNA (10 μg) was fractionated on 1% denaturing agarose gel and transferred onto GeneScreen membranes (NEN, Boston, MA). Alternatively, 4 μg of total RNA samples were slot blotted onto GeneScreen using a slot blot manifold (Schliecher and Schull, Dassal, Germany), and the slots in each lane were separated into two. Following baking and prehybridization, the membranes were hybridized overnight with the specific cDNA probe labeled to high specific activity using a random primer labeling kit (Boehringer, Mannheim, Germany). One of the two halves of each sample derived from the slot blot analysis was hybridized with common α-cDNA, and the second corresponding half was hybridized with β-tubulin cDNA which was used as an internal control. Thereafter, filters were washed at high stringency and autoradiographed at −70 °C. Steady-state levels of mRNAs were quantitated with densitometric scanning of autoradiograms. The data were corrected for variability in loading by calculation as a ratio to β-tubulin.

Release of α-Subunit. αT3-1 cells (2×10^5) were grown in 24-well plates (Sterilin, Hounslow, U.K.) in 1 mL of the culture medium described above for 3 days. The cells were then washed, incubated with 1 mL of DMEM, and stimulated with GnRH-A as indicated. Medium was collected and the cells were solubilized in 1 mL of H_2O containing 0.1% Triton X-100. The α-subunit content of the cells and the amount secreted to the medium were determined using RIA. Reagents for rat α-subunit RIA were kindly provided through the NIDDK, NIH, and the National Hormone and Pituitary Program (NHPP) of the University of Maryland School of Medicine (Baltimore, MD). Extracts of mouse anterior pituitary showed close parallelism for displacement of labeled α-subunit compared to purified rat LHα.

Binding Assays of GnRH Receptors. For GnRH binding studies we utilized a stable GnRH analog, buserelin, as the radioiodinated ligand (Naor et al., 1980). Buserelin was iodinated by the lactoperoxidase method (Meidan & Koch, 1981). αT3-1 cells (2×10^6 cells/dish) were washed three times with the assay buffer (medium 199, 0.1% BSA, 25 mM HEPES) and then incubated for 60 min at room temperature (25 °C) with ^{125}I-buserelin (10^5 cpm) and increasing concentrations of unlabeled GnRH-A in a total volume of 0.2 mL of the assay buffer. Incubation was terminated by washing of each well three times with phosphate-buffered saline (PBS, pH 7.4). NaOH (1 N, 0.3 mL) was then added at room temperature, and after 60 min the cells were collected. Following the addition of 0.3 mL of 1 M Tris-HCl (pH 7.4), the ^{125}I counts were determined using a γ counter (LKB).

Phosphoinositide Turnover. Cells (5×10^6/dish) were prelabeled (3 days) with *myo*-[2-^{3}H]inositol (1 μCi/mL). Cells were then washed with Krebs–Henseleit buffer, treated briefly (15 min) with Li^+ (10 mM) to block inositol 1-monophosphatase activity, and treated later with GnRH. Reactions were stopped by aspiration of the medium and addition of 0.25 mL of H_2O. Cells were scraped and transferred to tubes, to which 1 mL of chloroform/methanol (1:2) was added. Following incubation for 30 min at room temperature, 350 μL of chloroform and 350 μL of H_2O were added and the cells were centrifuged for phase partition. The water-soluble inositol phosphates were collected (upper phase) and separated by ion-exchange chromatography on Dowex AG-1×8 (chloride form). The eluants used were H_2O (inositol); 5 mM sodium tetraborate and 60 mM sodium formate (glycerolphosphoinositol); 0.1 M formic acid and 0.2 M ammonium formate (IP_1); 0.1 M formic acid and 0.4 M ammonium formate (IP_2); 0.1 M formic acid and 1 M ammonium formate (IP_3). In parallel, samples of the chloroform phase were dried and counted. The ^{3}H content of each fraction was determined by liquid scintillation counting (Naor et al., 1986).

Electron Microscopy. αT3-1 (4×10^6 cells/dish) were grown in 60-mm dishes as described. After 3 days monolayers were rinsed with PBS and fixed overnight at 4 °C in a cold solution of 3.2% glutaraldehyde and 1.6% formaldehyde in 0.1 M sodium cacodylate (pH 7.4). The cells were scraped, collected, and centrifuged. The cell pellet was washed twice by 0.1 M sodium cacodylate and postfixed in a solution of 1% osmium tatraoxide in 0.1 M sodium cacodylate buffer. The fixed cells were dehydrated in ethanol, washed twice by propylene oxide, and embedded in Poly Bed 812 kit (Polysciences, Warrington, PA). Ultrathin sections, post stained with uranyl acetate and lead citrate, were observed using JEOL 100B transmission electron microscope (JEOL, Tokyo, Japan).

RESULTS

Binding to GnRH Receptors in αT3-1 Cells. We have utilized a stable analog of GnRH, buserelin ([D-Ser(*t*-Bu)^6]-

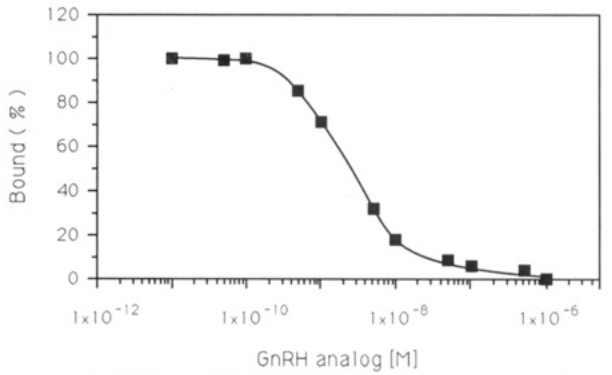

FIGURE 1: Inhibition of [^{125}I]iodobuserelin ([D-Ser(*t*-Bu)^6]Pro^9-NEt-GnRH) binding by increasing concentrations of unlabeled [D-Trp^6]-GnRH analog. αT3-1 cells (2×10^6 cells/well) were subjected to the binding assay as described in Methods. A representative competition curve is shown here.

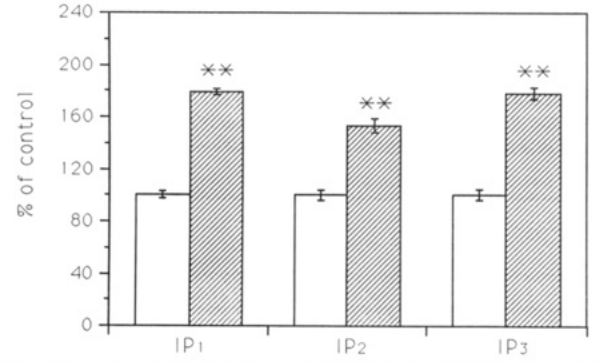

FIGURE 2: Effect of GnRH on phosphoinositide turnover. αT3-1 cells (5×10^6 cells/well) were prelabeled with *myo*-[2-^{3}H]inositol (1 μCi/mL) for 3 days. Cells were then treated with Li^+ (10 mM, 15 min) and later with (striped bars) or without (empty bars) GnRH (1 nM) for 7 min. [^{3}H]Inositol phosphates were separated on Dowex 1×10 columns as described in Methods. $^{**}p < 0.01$. Bars represent the mean ± SEM.

Pro^9-Net-GnRH), as the iodinated ligand to demonstrate GnRH receptors in αT3-1 cells (Figure 1). Binding inhibition by unlabeled ligand [[D-Trp]^{6}GnRH analog (GnRH-A)] revealed an EC_{50} value of 2.5 nM, which is relatively close to that observed in normal pituitary cells (Naor et al., 1980).

Effect of GnRH on Phosphoinositide Turnover. In order to demonstrate that the observed GnRH binding sites are coupled to a signaling apparatus, we examined the neurohormone effect on phosphoinositide turnover (Figure 2). Cells were prelabeled with *myo*-[2-^{3}H]inositol for 3 days, then treated briefly with Li^+ to block inositol 1-monophosphatase activity, and later treated with GnRH. All three water-soluble [^{3}H]inositol phosphates examined here (IP_1, IP_2, IP_3) were elevated by GnRH (1.8-, 1.5-, and 1.8-fold, respectively). Time response analysis revealed significant elevation of [^{3}H]inositol phosphates as early as 30 s after GnRH challenge. Hence the data are similar to the effect observed in normal pituitary cells (Naor et al., 1986), indicating activation of phospholipase C by GnRH.

GnRH also stimulated phosphatidylethanol accumulation, a specific product of phospholipase D phosphatidyltransferase activity in αT3-1 cells (Netiv et al., 1991). However, stimulation of phospholipase D by GnRH was detected after a lag of 1–2 min, suggesting sequential activation of phospholipase C followed by phospholipase D by the neurohormone.

Identification of α-Subunit mRNA in αT3-1 Cells. Figure 3 shows Northern blot analysis of RNA isolated from pituitary glands, liver, and αT3-1 cell line. As indicated in Figure 3, steady-state levels of mRNAs for common α, LHβ, FSHβ,

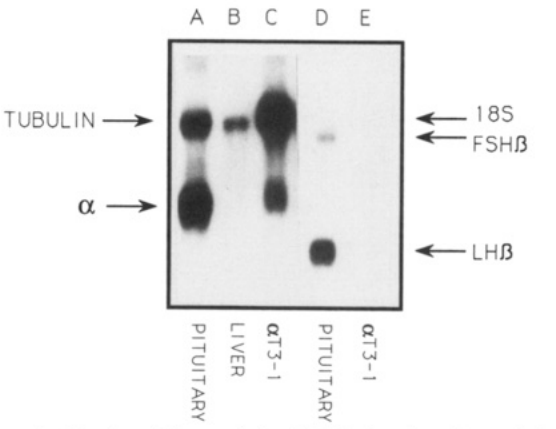

FIGURE 3: Northern blot analysis of RNA fractions from pituitary, liver, and αT3-1 cell line. Total RNA (10 μg/lane) was prepared from adult rats (pituitaries, liver) or αT3-1 cell line and hybridized to cDNA probes of common α and β-tubulin (lanes A–C) or LHβ and FSHβ (lanes D and E).

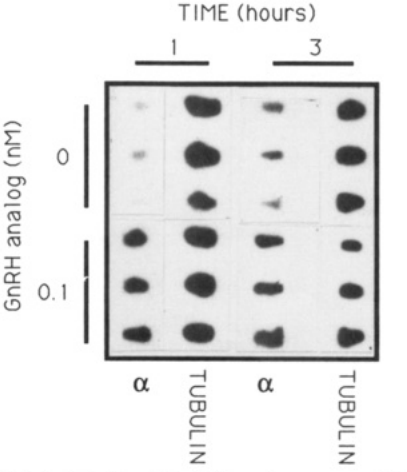

FIGURE 4: Effect of [D-Trp^6]GnRH analog on α-mRNA levels in αT3-1 cells. Cells in triplicates were treated with or without GnRH analog (0.1 nM) for 1 or 3 h of incubation. Autoradiograph of slot-blot hybridization with probes to common α and β-tubulin is shown. β-Tubulin serves as a marker for RNA loading.

and β-tubulin are present in the pituitary, whereas αT3-1 cells express only the α-subunit and β-tubulin. The liver gland, as expected, expresses only the β-tubulin mRNA.

Effect of GnRH Analog on α-mRNA Levels in αT3-1 Cells. The regulation of α-subunit mRNA was determined after treatment of αT3-1 cells with [D-Trp^6]GnRH analog (GnRH-A) using slot-blot analysis. As shown in Figure 4, GnRH-A (0.1 nM) increased α-mRNA levels at both the 1- and 3-h time points examined here. Quantitation of the effect observed at various time points and with low and high doses of GnRH-A is shown in Figure 5. A very rapid increase in α-mRNA levels is detected already after 30 min of incubation with 0.1 nM GnRH-A (3-fold, $p < 0.01$, Figure 5A). The effect diminishes gradually to reach basal levels at about 12 h of incubation. A secondary rise is observed (12–24 h) with a higher dose of GnRH-A (10 nM, Figure 5B), suggesting partial degradation of GnRH-A during prolonged incubation periods. The effect of GnRH-A as well as the effect of the natural hormone GnRH are dose-dependent (Figure 6A and data not shown), and the stimulatory effect is blocked by the potent antagonist [D-pGlu^1,pClPhe^2,D-Trp^{3,6}]GnRH (Figure 6B). TRH (10^{-6} M) had no effect on α-subunit mRNA levels under the above conditions.

Effect of TPA and Ionomycin on α-mRNA Levels in αT3-1 Cells. The potential candidates for mediating the GnRH effect

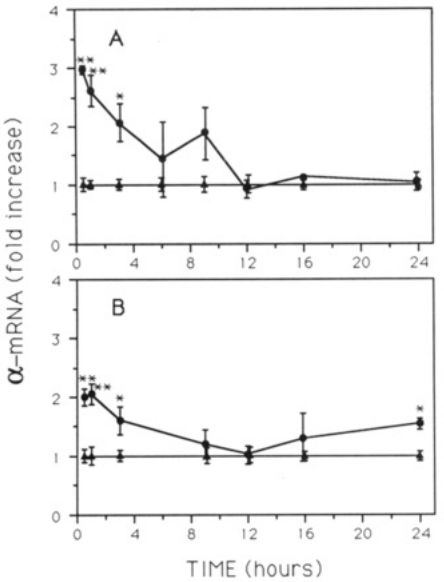

FIGURE 5: Time response of the effect of [D-Trp^6]GnRH analog on α-mRNA levels in αT3-1 cells. αT3-1 cells were incubated with GnRH analog (0.1 and 10 nM in panels A and B, respectively; circles) for the time points indicated, and α-mRNA levels were determined by slot-blot analysis as described in Methods. An arbitrary unit of 1 represents the control values (triangles) at each time point obtained by densitometry. Results are expressed as mean ± SEM (n = 5–12). *p < 0.05; **p < 0.01.

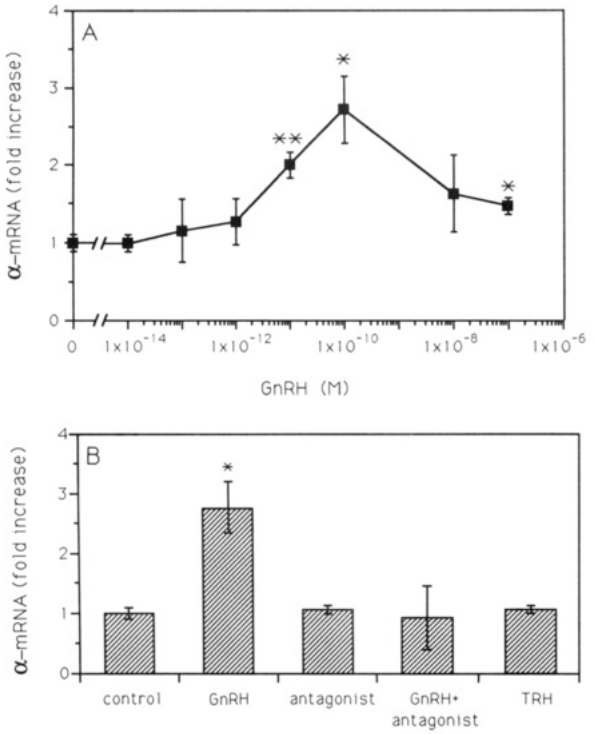

FIGURE 6: Dose response effect of GnRH (A) and its inhibition by the GnRH antagonist [D-pGlu^1,pClPhe^2,D-Trp^{3,6}] (B) on α-mRNA levels in αT3-1 cells. The cells were incubated with the indicated concentration of GnRH for 1 h (A), or with GnRH (10^{-10} M) and the antagonist (10^{-9} M) or with TRH (10^{-6} M) (B). The cells were pretreated with the antagonist for 15 min and later incubated with or without GnRH for 1 h. Values are mean ± SEM (n = 4). *p < 0.05.

are PKC and Ca^{2+} [see Naor (1990) for review]. We therefore incubated αT3-1 cells with TPA (100 ng/mL) and ionomycin (1 μM) for a short period of incubation time and analyzed α-subunit mRNA levels (Figure 7). While TPA reached its maximal response (2-fold) very rapidly (30 min, $p < 0.01$), the effect of ionomycin was slower, with marked stimulation

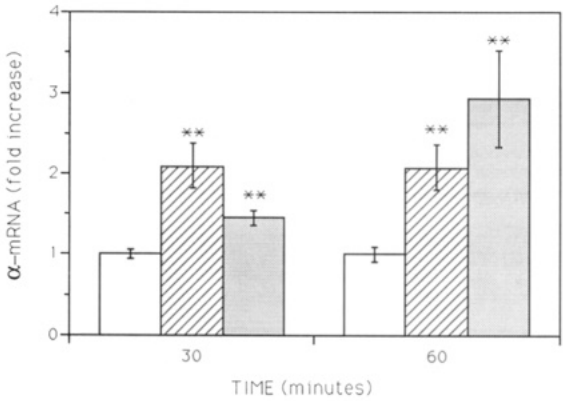

FIGURE 7: Effect of TPA and ionomycin on α-mRNA levels in αT3-1 cells. The cells were incubated with TPA (striped bars; 100 ng/mL) or with ionomycin (dotted bars; 1 μM) for 30 or 60 min. Results are mean ± SEM (n = 4–6). **p < 0.01.

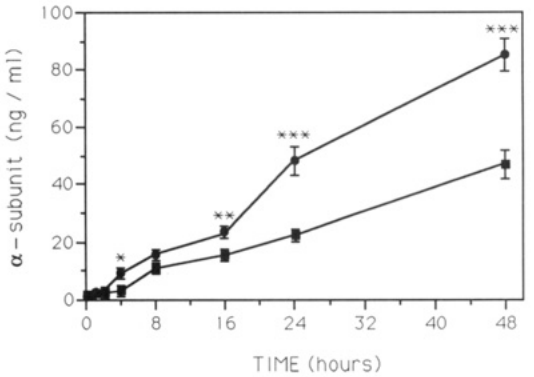

FIGURE 8: Time response of the effect of [D-Trp^6]GnRH analog (GnRH-A) on α-subunit release from αT3-1 cells. Cultured cells were incubated with GnRH-A (1 nM) for the time points indicated, and α-subunit release was determined by RIA. Values are mean ± SEM (n = 12). *p < 0.05; **p < 0.01; ***p < 0.001. Control, squares; GnRH-A, circles.

observed at 60 min of incubation (3-fold, $p < 0.01$). Nevertheless, both TPA and ionomycin significantly elevated α-mRNA levels after 30 min of incubation, implicating both PKC and Ca^{2+} as potential messengers for GnRH action on α-subunit mRNA accumulation.

Effect of GnRH Analog on α-Subunit Release. The rapidity of the GnRH response upon α-subunit gene expression prompted us to examine also the release of α-subunit following hormonal challenge. Incubation of αT3-1 cells with GnRH-A (1 nM) resulted in enhanced α-subunit release to the medium which was detected only after a lag of 4 h (Figure 8). Analysis of content at time zero revealed the presence of α-subunit in nonstimulated cells (8 ± 1.3 ng/3 × 10^5 cells, n = 6). Further analysis of content in control and GnRH-treated cells at time points ranging from zero to 48 h of incubation showed that most of the hormone released between 8 and 48 h could not be accounted for by the content observed and apparently represents newly synthesized α-subunit. In addition, analysis by EM confirmed the presence of storage secretory granules in αT3-1 cells, albeit fewer than in normal pituitary cells [data not shown and Naor and Childs (1986)]. Furthermore, the EM analysis revealed that the number of secretory granules did not increase even at time points when significant exocytotic response to GnRH challenge had been initiated. Hence, enhancement of α-subunit mRNA levels by GnRH-A preceded α-subunit release by several hours in the αT3-1 gonadotroph-like cell line.

DISCUSSION

Studies on GnRH action upon gonadotropin secretion and synthesis have been complicated because of the heterogeneity of pituitary cells, of which only about 10% are gonadotrophs [see Naor and Childs (1986) for review]. The recent development of a gonadotroph-like cell line producing α-subunit, which was derived by targeted tumorigenesis in transgenic mice (Windle et al., 1990; Horn et al., 1991), enabled us to study the effect of GnRH upon α-mRNA levels and α-subunit release in an homogeneous cell preparation. The αT3-1 cell line possesses GnRH receptors coupled to phospholipase C (PLC) and phospholipase D (PLD) activation [present results and Horn et al. (1991) and Netiv et al. (1991)], making it an appropriate model to study the regulation of common α gene expression. Indeed, binding affinities for GnRH analogs and stimulation of phosphoinositide turnover in the αT3-1 cells observed here and elsewhere (Horn et al., 1991) resemble the findings in normal pituitary cells (Naor et al., 1980, 1986; Loumaye et al., 1982; Naor & Childs, 1986; Morgan et al., 1987; Horn et al., 1991). Thus, the αT3-1 cell line is a suitable model for investigations concerning the signal transduction cascade involved in GnRH induced α-subunit release and synthesis. The mRNA for the α-subunit is rapidly and significantly elevated by GnRH analog (3-fold at 30 min). To the best of our knowledge, such a rapid response is unique for induction of trophic hormone mRNAs but common for induction of the immediate early genes such as the protooncogenes c-*fos* and c-*jun* which might participate by means of the AP-1 complex in induction of α-mRNA by GnRH. The stimulatory effect declines thereafter to basal levels at around 12 h of incubation, rising again (1.5-fold; 24 h) at higher analog concentrations. The results differ from previous reports which found increased α-mRNA levels at 8–16 h of incubation with high doses of GnRH agonist (Windle et al., 1990; Horn et al., 1991). The discrepancy might be explained by the different concentrations of GnRH agonist used for the time response studies. While we used here 0.1 nM of a weak agonist [D-Trp^6]GnRH, Mellon and her co-workers utilized 100 nM of nafarelin or [D-Nal^6,Pro^9-NEt]GnRH, which are superagonistic analogs (Windle et al., 1990; Horn et al., 1991). As with LHβ mRNA levels reported elsewhere (Andrews et al., 1988), pituitary cells might respond differently to low doses of GnRH in terms of gonadotropin synthesis. A possible explanation for the effectiveness of low doses of GnRH emerges from recent studies on $[Ca^{2+}]_i$ changes in single gonadotrophs (Leong & Thorner, 1991; Iida et al., 1991). Low doses of GnRH induce Ca^{2+} oscillation, while high doses favor the spike/plateau type of response. Since the oscillatory cells did not respond to GnRH in terms of LH release, it was suggested that oscillatory cells might mediate GnRH-induced gonadotropin synthesis and up-regulation of GnRH receptors (Leong & Thorner, 1991). On the other hand, the cells responding to GnRH in a spike/plateau fashion were implicated in exocytosis (Leong & Thorner, 1991; Iida et al., 1991). Heterogeneity among gonadotrophs was also observed recently when we noted that not all the gonadotrophs reacted with PKC antibodies (Garcia-Navarro et al., 1991). Hence the idea of subspecialization (synthesis vs exocytosis) among stimulated gonadotrophs undergoing various stages and exposed to varying doses of GnRH cannot be ruled out.

Suprisingly, we found here that elevation by GnRH of common α-mRNA levels preceded α-subunit release by several hours, indicating uncoupling of secretion and gene expression. Since the pattern of free α-subunit secretion in response to GnRH mimics that of LH in normal pituitary cells and is very rapid (Weiss et al., 1990b), it could be argued that the delay observed here is a phenotype of the cell line. Nevertheless, the data observed here indicate the presence of a repressor of exocytosis, or a defect in the exocytotic apparatus which is removed after several hours of incubation. Indeed, α-subunit gene regulation has been reported to involve a labile repressor in HeLa cells (Cox et al., 1990), and an analogous repressor might inhibit exocytosis in αT3-1 cells. Alternatively, the possibility of a defect in the exocytotic apparatus is extremely interesting since it might open a vista to enable us to identify this particular crucial step in exocytosis. Although the number of secretory granules observed here is small when compared to that of normal pituitary cells (Naor & Childs, 1986), this could not account for the lack of an exocytotic response during the first 4 h of incubation with GnRH-A. The number of secretory granules did not increase during the GnRH challenge, while exocytotic response was observed after 4 h of incubation and time points thereafter. Also, analysis of α-subunit content at time zero showed the presence of the hormone in quantities sufficient to account for the exocytotic response observed at 4 h of incubation (see Results). In addition, other pituitary cell lines known to have a small number of secretory granules (e.g., GH_3 cells) respond rapidly to TRH in terms of prolactin release (Albert & Tashjian, 1984). Thus the observations confirm the uncoupling phenomenon between exocytosis and gene expression.

It is commonly throught that ligand-induced exocytosis in secretory cells occurs first, followed later by gene expression and synthesis to refill the secretory granules. The data presented here demonstrate that the two events can be dissociated. An inverse dissociation between secretion and gene expression has been demonstrated also in normal pituitary cells where GnRH-stimulated LH release was not followed by an increase in βLH gene transcription or cytoplasmic elevation of mRNA levels (Salton et al., 1988). Hence, bidirectional uncoupling in GnRH responses upon release and synthesis can be demonstrated.

It was previously demonstrated that growth hormone-releasing factor stimulates gene transcription independently GH release, while concentrations of high K^+ or TPA caused GH release with no effect on the rate of GH transcription (Barinaga et al., 1985). In the case of GH, it was suggested that while cyclic AMP might mediate gene transcription, Ca^{2+} is involved in the release process. Such a dissection cannot be applied to GnRH action since cyclic AMP has been ruled out as a potential messenger in GnRH-induced gonadotropin secretion and α-subunit mRNA elevation in rat and mouse (Naor et al., 1975, 1978; Naor, 1990; Horn et al., 1991). It could be argued that Ca^{2+} and PKC mediate GnRH stimulation of gonadotropin release and synthesis, respectively, as suggested in some studies (Andrews et al., 1988). However, our own studies (Naor & Eli, 1985; Naor & Childs, 1986; Limor et al., 1987; Naor et al., 1988; Naor, 1990) and those of others (Chang et al., 1986; Stojilkovics et al., 1991) implicate both Ca^{2+} and PKC in mediating GnRH-induced gonadotropin release. Since PKC was also reported to be involved in GnRH-induced α (Horn et al., 1991) and LHβ mRNA accumulation (Andrews et al., 1988), a simple dissection of messenger molecules which are separately involved in release vs synthesis cannot be proposed at this stage. Furthermore, the observation reported here that TPA and ionomycin induced a rapid elevation of α-mRNA levels in αT3-1 cells suggests that both Ca^{2+} and PKC might be responsible for the early response of the neurohormone. Further studies are therefore required to elucidate the intracellular cascade of signaling events involved

in gonadotropin subunit release and synthesis after GnRH challenge in normal and transformed gonadotrophs.

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Registry No. LHRH, 9034-40-6; Ca^{2+}, 7440-70-2; phospholipase C, 9001-86-9; phospholipase D, 9001-87-0; protein kinase C, 141436-78-4.