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Studies of Gonadotropin-Releasing Hormone (GnRH) Action Using GnRH Receptor-Expressing Pituitary Cell Lines*

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I. Introduction

THE regulation of normal mammalian sexual maturation and reproductive function requires the integration and precise orchestration of hormonal regulation at the hypothalamic, pituitary, and gonadal levels. GnRH is a decapeptide synthesized in neurosecretory cells in the preoptic area of the hypothalamus. GnRH is secreted into the hypophysial portal circulation and is transported to the anterior pituitary gland, where it binds to receptors on a specific pituitary cell type, the gonadotrope, to modulate the synthesis and secretion of the gonadotropins, LH and FSH. Gonadotropins, in turn, are secreted into the systemic circulation and act on the gonads to regulate steroidogenesis and gametogenesis. LH stimulates ovulation and corpus luteum formation in females and androgen secretion in males; FSH stimulates the growth and maturation of ovarian follicles in females and spermatogenesis in males. Gonadal steroids and peptides, in turn, are secreted into the systemic circulation and act to modulate hypothalamic and pituitary function in both positive and negative feedback loops (1, 2).

Research into the neuroendocrine control of reproductive function by GnRH has undergone an explosion in the past 25 yr, marked first by the isolation and chemical characterization of GnRH (3–5). This led to the development of both agonist and antagonist analogs, resulting in rapid advances in our basic understanding as well as clinical applications to the treatment of disorders such as prostate cancer, endome-

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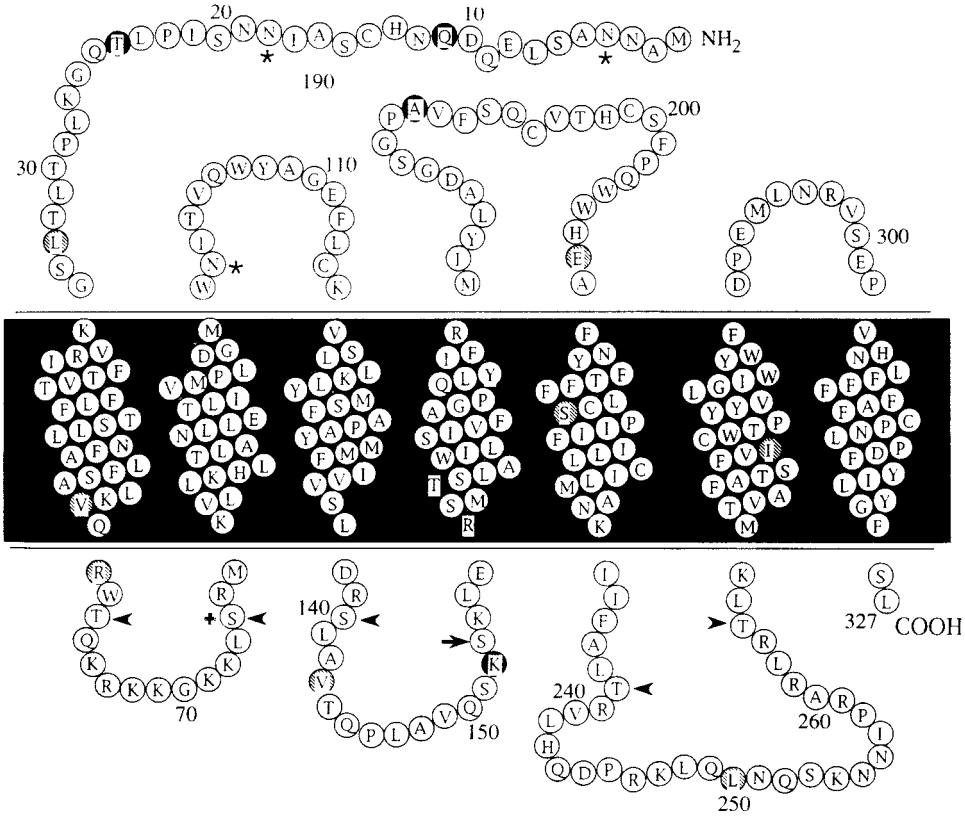

FIG. 1. Model of the rat GnRHR. Amino acid residues in *black* represent nonconserved amino acids between the rat and mouse GnRHR; *shaded* amino acid residues are nonidentical but conserved between the two species. *Asterisks* denote potential glycosylation sites. Potential phosphorylation sites are indicated for protein kinase C (*arrowheads*), casein kinase II (*arrow*), and protein kinase A (*cross*). [Reprinted with permission from U. B. Kaiser *et al*: *Biochem Biophys Res Commun* 189:1645–1652, 1992 (13) (Fig. 2A).]

triosis, precocious puberty, and infertility (6, 7). More recently, the molecular cloning of cDNAs encoding receptors for GnRH (GnRHR)[1] was achieved, first in mouse (8, 9) and subsequently in human, rat, cow, and sheep (10–17). The availability of the GnRHR cDNA has allowed studies leading to further understanding of the mechanisms of GnRH action.

Primary anterior pituitary cells are comprised of a heterogeneous population of well differentiated, secretory cell types. These include somatotropes, which secrete GH; lactotropes, which secrete PRL; corticotropes, which secrete ACTH as well as other hormones derived from the peptide precursor, POMC, including MSH, lipotropins, endorphins, and enkephalin; thyrotropes, which secrete TSH; and gonadotropes, which secrete LH and/or FSH (18, 19). Several anterior pituitary cell types produce more than one of the anterior pituitary hormones; for example, LH and FSH are often colocalized to the same cell, as are GH and PRL. More recently, there has been evidence of colocalization of GH with LH or FSH (20) .

A major hindrance to progress in our understanding of the mechanisms of neuroendocrine control of reproduction at the hypothalamo-pituitary level is the lack of an ideal cell model for these studies. Historically, such studies have been performed *in vivo* in a variety of animal models and *in vitro* in dispersed primary pituitary cell cultures. These studies are limited by the heterogeneity of anterior pituitary cell types; gonadotropes make up only 6–15% of anterior pituitary secretory cells in adult animals (21). In addition, anterior pituitary cells cannot be propagated in culture systems, thus limiting the feasibility of many studies. Recently, a number of immortalized pituitary cell lines have been used as models for studies of the mechanisms of action of GnRH and its receptor.

Several aspects of the GnRHR and its signaling properties have been reviewed previously (22–30). Past reviews have focused on the molecular mechanisms of action of GnRH and the signaling properties of the GnRHR in primary pituitary cells. In this review, we will focus on studies of GnRH action using GnRHR-expressing pituitary cell lines as model systems. The results of these studies will be compared with what

[1] Abbreviations used: GnRHR, GnRH receptor; TRHR, TRH receptor; SV40, simian virus-40; PMA, phorbol myristic acid; PKC, protein kinase C; PKA, protein kinase A; PLC, phospholipase C; IP, inositol phosphate; $[Ca^{2+}]_i$, ionized intracellular calcium concentration; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; GSE, gonadotrope-specific element; SF-1, steroidogenic factor-1; MIS, Mullerian inhibiting substance; PGBE, pituitary glycoprotein hormone basal element; GnRH-RE, GnRH-responsive element; αLUC, α-subunit gene promoter/luciferase reporter fusion gene; PRLLUC, PRL gene promoter/luciferase reporter fusion gene; LHβLUC, LHβ subunit gene promoter/luciferase reporter fusion gene; FSHβLUC, FSHβ subunit gene promoter/luciferase reporter fusion gene; CAT, chloramphenicol acetyltransferase; E_2, estradiol.

is known about GnRH signaling in primary pituitary cells. In addition, we will focus on the role of the GnRHR pathway in the regulation of gene expression.

II. GnRHR Structure Analysis

The GnRHR cDNA encodes a 327- to 328-amino acid protein with seven putative membrane-spanning domains, characteristic of the family of G protein-coupled receptors (Fig. 1) (31). Interestingly, the GnRHR lacks the typical intracellular carboxyl terminus, making it one of the smallest receptors with the seven-transmembrane segment motif. The lack of a carboxyl-terminal tail domain is a unique feature of the GnRHR among G protein-coupled receptors.

Northern blot analysis using the mouse GnRHR cDNA as a probe reveals the presence of at least two hybridizing mRNAs, approximately 4.3 kb and 2 kb in size, in the murine gonadotrope-like cell line, αT3-1 (described below) (8, 9, 32). mRNAs of similar sizes are present in other species as well. An additional mRNA approximately 5.0–5.5 kb in size is present in rat and sheep pituitaries, and a smaller 1.3-kb mRNA is also detected in sheep pituitaries (13, 16). It is not clear whether the differences between αT3-1 cells and rat and sheep pituitaries reflect species differences or differences between primary gonadotropes and an immortalized cell line. The presence of these multiple transcripts raises the possibility that alternative functional forms of the GnRHR may exist.

Cloning of the mouse and human GnRHR genes reveals the presence of two introns (Fig. 2) (33, 34). The introns in the mouse gene occur in the sequences encoding the fourth transmembrane helix and the third intracellular loop. The human gene has the same structure, with the introns interrupting the coding sequences at the same locations, although the introns appear to vary in size. Both the human and the mouse appear to have only a single GnRHR gene, as determined by Southern blot analysis. Analysis of multiple cDNA clones obtained from αT3-1 cells revealed the presence of at least four alternative transcripts, derived largely by alternate splicing (34). It is possible that these alternative transcripts account for some of the additional bands seen on Northern blot analysis. However, these alternative transcripts are less abundant than the original cDNA clone and appear to encode nonfunctional, truncated GnRHRs.

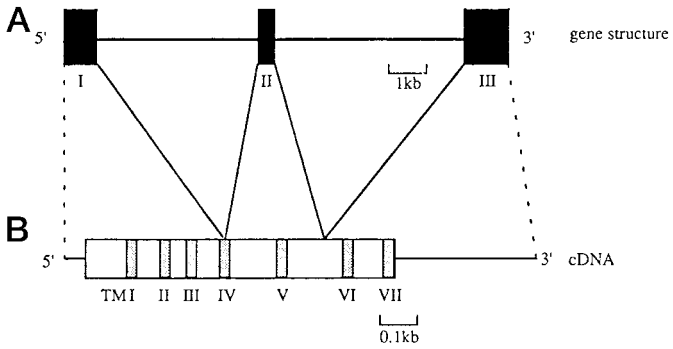

FIG. 2. Schematic representation of the human GnRHR gene. A, Exon-intron localization. The *shaded boxes* indicate exons and the *intervening lines* indicate introns. B, The structure of the human GnRHR cDNA. The *open box* indicates the protein-coding regions, and *hatched boxes* are the putative transmembrane domains. [Reprinted from *Mol Cell Endocrinol* 103:R1-R6, (Fig. 1, C and D), N. C. Fan *et al.*, "The human gonadotropin-releasing hormone (GnRH) receptor gene: cloning, genomic organization and chromosomal assignment" 1994 (33) with kind permission from Elsevier Science Ireland Ltd., Bay 15K, Shannon Industrial Estate, Co. Clare, Ireland.]

The 5′-flanking region of the mouse GnRHR gene has been cloned, and its transcriptional start sites have been defined (35). A major transcriptional start site was identified 62 nucleotides upstream of the translational start site, which does not appear to use a TATA box. Other minor transcriptional start sites were also detected; 1.2 kb of the 5′-flanking sequence fused to a luciferase reporter gene appears to be sufficient to direct high levels of expression when transiently transfected into αT3-1 cells. Some expression also occurred in the rat somatolactotropic GH_3 pituitary cell line, whereas only low levels of expression occurred in a placental cell line, JEG-3, and in a kidney fibroblast cell line, CV-1. These data suggest that this region of the GnRHR gene confers pituitary-specific, and, to a large extent, gonadotrope-specific expression. 5′-Deletion analyses indicate the presence of sequences between −500 and −400 relative to the translational start site that appear to activate GnRHR gene expression in the αT3-1 cell line (36).

The 5′-flanking region of the human GnRHR gene has also been cloned and sequenced (37). Five consensus TATA boxes were identified, distributed within a 700-nucleotide region, and multiple transcriptional start sites were detected associated with these TATA sequences. These transcriptional start sites reside further upstream than the major transcriptional start site identified in the mouse, although the mouse 5′-flanking sequence also reveals several putative TATA boxes. These findings raise the possibility of species-specific or tissue-specific transcription initiation sites. The 3′-end of the human GnRHR gene has also been sequenced, revealing five classical polyadenylation signals (37). The large 3′-untranslated sequence likely accounts for the greatest portion of the major mRNA species observed by Northern blot analysis.

III. Studies of GnRH Action in αT3-1 Cells

A. Derivation of αT3-1 cells

A fusion gene containing 1.8 kb of 5′-flanking sequences of the human glycoprotein hormone α-subunit gene linked to the protein-coding sequences of the simian virus-40 (SV-40) T antigen oncogene was used to generate transgenic mice. Mice carrying this fusion gene developed tumors of the anterior pituitary. The αT3-1 cell line was derived from a pituitary tumor in such a mouse. Cells from this tumor were dispersed and maintained in monolayer culture. Stable cultures were established, and monoclonal cell lines were derived and characterized (38). These cells have provided a continuous cell model system for the study of the GnRHR and GnRH action, as well as for cell-specific expression of the α-subunit; indeed, the availability of αT3-1 cells was critical for the molecular cloning of cDNAs encoding the GnRHR (8, 9).

B. Characterization of αT3-1 cells

αT3-1 cells express α-subunit mRNA. In addition, α-subunit protein is synthesized and secreted by these cells. The cells do not express TSHβ, GH, PRL, or POMC genes, the hormones expressed in other, nongonadotrope anterior pituitary cell types. However, neither LHβ nor FSHβ subunit mRNA, expressed in primary pituitary gonadotropes, is expressed in the αT3-1 cells. The cells respond to GnRH with an increase in α-subunit mRNA levels, whereas levels remain unchanged after exposure to TRH. The GnRH response is time- and dose-dependent and blocked by a GnRH antagonist, consistent with action through the GnRHR (38). Furthermore, GnRH binding and expression of GnRHR mRNA in αT3-1 cells have been shown (39). αT3-1 cells also bind activin A and express mRNAs for the activin receptor types I, II, and IIB, as well as for the inhibin βB-subunit (40, 41). The expression of the gonadotropin α-subunit and GnRHR in αT3-1 cells is consistent with their derivation from the gonadotrope lineage; however, they fail to express the full complement of gonadotrope-specific proteins, specifically the LHβ and FSHβ subunits. This suggests that αT3-1 cells are derived from precursor cells that were not fully differentiated into gonadotropes. This is supported by observations that α-subunit expression occurs early in ontogeny before LHβ or FSHβ (42, 43). The presence of GnRH responsiveness indicates that these cells likely arose after the expression of GnRHR; GnRH-binding sites have been reported to appear, albeit at very low levels, several days earlier in development than the β-subunits (44).

C. GnRH binding

Specific, high-affinity binding sites for GnRH have been identified in αT3-1 cell membrane preparations (39). A GnRH analog binds to these sites with a dissociation constant of 0.50 nM, similar to that measured in normal mouse (0.51 nM) and rat (0.20 nM) anterior pituitary. The total number of binding sites for GnRH is 1.6 pmol/mg protein, about 5 times higher than in normal mouse (0.33 pmol/mg) and rat (0.31 pmol/mg) anterior pituitary (Table 1) (39). However, one must take into account that αT3-1 cells represent a homogeneous cell population, in which all the cells express the GnRHR and bind the GnRH analog, whereas anterior pituitary cells are a heterogeneous cell population, in which only approximately 10% of the cells, the gonadotropes, express the GnRHR. Therefore, the estimated number of GnRH-binding sites on αT3-1 cells is approximately 50% of the number on primary gonadotropes.

TABLE 1. Comparison of the GnRH receptor on mouse, αT3-1, and rat anterior pituitary membrane homogenates

	Mouse	αT3-1	Rat
K_d (nM)	0.51 (0.22–1.2)	0.50 (0.38–0.67)	0.20 (0.14–0.30)
R^o (pmol/mg)	0.33 (0.19–0.67)	1.6 (1.3–1.9)	0.31 (0.24–0.41)

K_d, Dissociation constant of [D-Ala^6,Me-Leu^7,Pro^9-NEt]-GnRH; R^o, total number of binding sites. The 95% confidence limits are given in *parentheses*. [Adapted with permission from F. Horn *et al.*: *Mol Endocrinol* 5:347–355, 1991 (39) (Table 1) © The Endocrine Society.]

D. GnRHR regulation

1. Homologous regulation by GnRH. Homologous ligand regulation of the GnRHR has been shown to occur *in vivo* in rats (45, 46) as well as *in vitro* in cultured rat anterior pituitary cells (47). Similarly, exposure of αT3-1 cells to 10^{-10} or 10^{-8} M GnRH for 20 min has been shown to induce a 50% increase in the number of GnRHRs 24 h later, as determined by GnRH-binding studies (32). This appears to occur at a posttranscriptional level, as GnRHR mRNA levels were unchanged. Interestingly, treated αT3-1 cells with increased GnRH-binding capacity showed a corresponding increase in cellular GnRHR mRNA "activity." That is, αT3-1 RNA was injected into *Xenopus* oocytes, and the GnRH-stimulated Cl^- current was quantitated by voltage clamp recording of the response to GnRH. The evoked current, a measure of the levels of functional GnRHR translated from the injected mRNA, was almost 2-fold higher in oocytes injected with RNA from treated αT3-1 cells compared with controls. These data suggest that GnRH regulates GnRHR numbers in αT3-1 cells by altering GnRHR mRNA translational efficiency. Similarly, prolonged exposure of αT3-1 cells to continuous high concentrations of GnRH, 1 μM for 24 h, resulted in a decrease in GnRH-binding sites to 25% of control levels, no change in GnRHR mRNA levels, but a decrease in GnRH-induced currents in oocytes injected with RNA isolated from the down-regulated cells (48). The changes in GnRH binding in response to GnRH are qualitatively similar to those seen in primary pituitary cells, but this novel mechanism has not yet been shown to occur in primary gonadotropes; indeed, it has been shown that GnRH can regulate GnRHR mRNA levels in primary pituitary cells (49). Hence, it is unclear whether this mechanism of modulation of GnRHR mRNA translational efficiency is unique to αT3-1 cells or is a generalized phenomenon. Alarid and Mellon (50) also found no change in GnRHR mRNA levels in αT3-1 cells in response to continuous exposure to a GnRH agonist for 1–24 h. In contrast, Catt and co-workers (51) showed that exposure of αT3-1 cells to GnRH or a GnRH agonist resulted in a time- and dose-dependent reduction in the level of GnRHR mRNA. Nevertheless, the reductions in mRNA levels were less pronounced than the decreases in receptor number, consistent with the involvement of additional, posttranscriptional mechanisms.

2. Regulation by gonadal steroid hormones. Estradiol has been shown to reduce GnRHR number in αT3-1 cells, as determined by GnRH-binding studies, without significantly altering the dissociation constant (K_d) (52). This inhibitory effect of estradiol is dose- and time-dependent. A reduction in GnRHR number was measurable after 24 h of exposure to estradiol and was maximal after 4–5 days. The EC_{50} of the estradiol effect was approximately 10^{-11} M. In primary cultures of rat pituitary cells, estradiol can both increase (chronic exposure) and decrease (short-term exposure) GnRH binding (53, 54). In ovine pituitary cultures, estradiol increased GnRH binding by 10 h, and this increase was maintained up to 48 h (55). Thus, there appear to be some differences in the responses of αT3-1 cells and primary gonadotropes to estradiol. These discrepancies may be attributable to differences between physiological cellular responses of αT3-1 cells and primary gonadotropes; alternatively, the up-regulation of

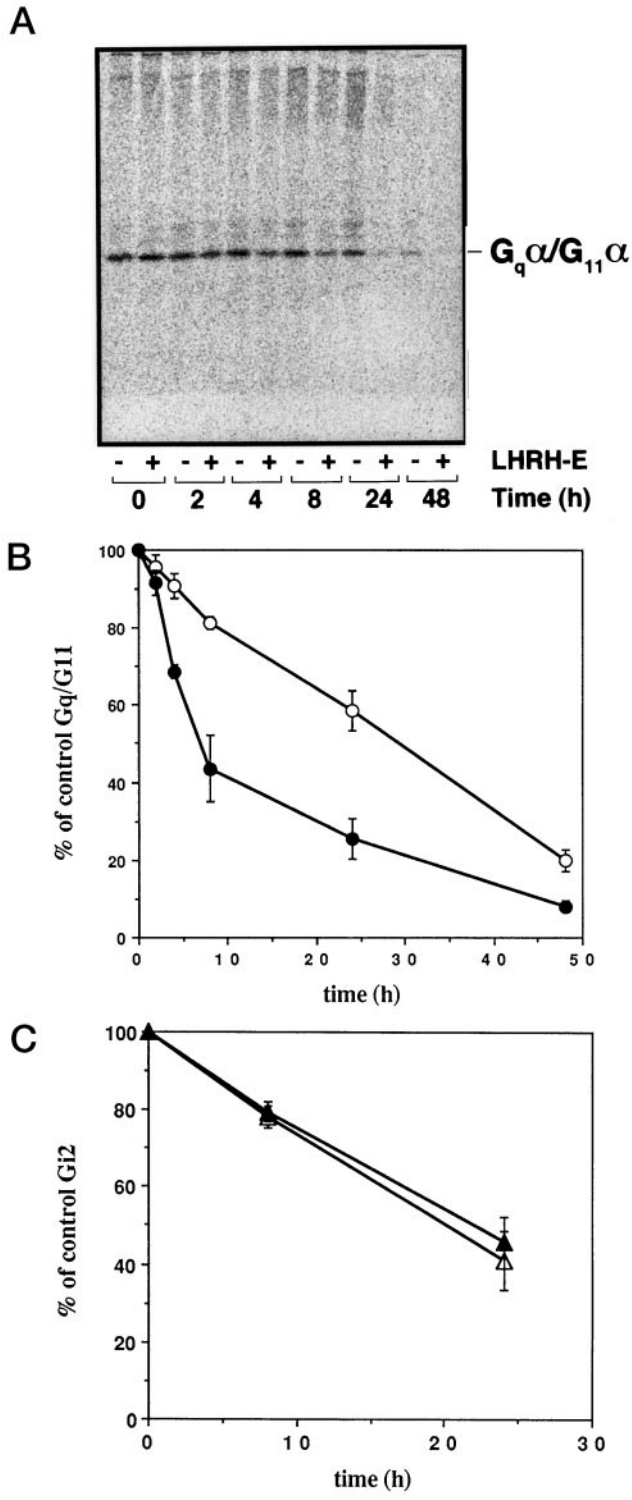

FIG. 3. The turnover of $G_q\alpha/G_{11}\alpha$ and $G_i2\alpha$ in control and LHRH-E-treated αT3-1 cells. A, Autoradiograph of a pulse-chase experiment with [^{35}S]methionine in αT3-1 cells treated or not for various times with LHRH-E. The turnover of $G_q\alpha/G_{11}\alpha$ was monitored in αT3-1 cells in the presence (+) or absence (−) of LHRH-E (1 μM) as described. Immunoprecipitates using antiserum CQ ($G_q\alpha/G_{11}\alpha$) were subjected to SDS-PAGE, and the resulting gel was exposed to a phosphor storage plate for 48 h. The indicated ^{35}S-labeled band was not present in immunoprecipitations done with preimmune serum (data not shown). B, Quantitative analysis of the effect of LHRH-E on the turnover of $G_q\alpha/G_{11}\alpha$. Data such as that presented in panel A were quantitated and are displayed as means ± SEM of four individual experiments. ○, Control; ●, LHRH-E treated. C, LHRH-E treatment does not alter the turnover of $G_i2\alpha$. Samples such as those of panel A were immunoprecipitated with the anti-$G_i2\alpha$ antiserum, SG, and exposed to a phosphor storage plate; the images were analyzed as for panel B. Data represent the means ± SEM of three experiments. △, Control; ▲, LHRH-E treated. LHRH-E, des-Gly^{10}-[D-Ala^6] LH-releasing hormone ethylamide. (The term LHRH used in this figure is synonymous with GnRH used elsewhere.) [Reprinted with permission from B. H. Shah *et al*: *Proc Natl Acad Sci USA* 92:1886–1890, 1995 (64) (Fig. 3).].

GnRHR number seen in primary cultures may occur indirectly, involving steroid hormone effects on cells other than gonadotropes.

3. Regulation by gonadal peptides. Activin A increases GnRHR mRNA levels in αT3-1 cells in a time- and dose-dependent fashion, with maximal stimulation occurring after 24–48 h of exposure (40). This stimulation of GnRHR mRNA levels by activin A occurs at the transcriptional level, as indicated by nuclear run-off and transient transfection experiments. Furthermore, pretreatment of αT3-1 cells with activin A is able to enhance GnRH-induced activation of the gonadotropin α-subunit promoter, suggesting that activin A may have a functional role in modulating the responsiveness of the gonadotrope to GnRH by increasing the expression of the GnRHR. Follistatin is able to block the effects of activin on the GnRHR gene, possibly by binding to and inactivating activin. These data are consistent with data in primary pituitary cells, demonstrating stimulation of the synthetic rate of GnRHRs by activin A (56). In contrast, recent data demonstrated that activin A blocked the stimulatory effect of GnRH on α-subunit promoter activity in αT3-1 cells; whether this was a receptor or postreceptor effect was not determined (57) .

4. Regulation by second messenger activators. In an attempt to identify possible regulators of GnRHR, αT3-1 cells were treated with the second messenger activators, phorbol myristic acid (PMA) and forskolin (50). These agents activate the signal transduction pathways of a multitude of potential effectors that might regulate GnRHR. PMA, a phorbol ester that activates protein kinase C (PKC), had no effects on GnRHR mRNA levels in αT3-1 cells. However, forskolin, which activates adenylyl cyclase, leading to increases in intracellular cAMP levels and hence activation of protein kinase A (PKA), decreased GnRHR mRNA levels by up to 6-fold. This effect was maximal after 8 h, but was transient, with GnRHR mRNA levels returning to control levels by 24 h after treatment. Correlation with GnRH binding is not yet known. Thus, in αT3-1 cells, factors that activate the PKA pathway may decrease GnRHR mRNA levels, whereas activation of the PKC pathway appears to have no effect. In contrast, activation of PKC appears to play a role in mediating up-regulation of the GnRHR by GnRH in primary rat pituitary cells (27, 58, 59).

E. Intracellular second messengers

Studies of signal transduction pathways activated by GnRH in αT3-1 cells have included studies of G protein coupling, generation of inositol phosphates, stimulation of increases in intracellular calcium concentration, activation of

PKC, generation of cAMP, and activation of mitogen-activated protein kinases. The majority of studies have observed the responses to a single pulse of GnRH or to continuous GnRH; the responses to pulsatile administration of GnRH have not yet been described.

1. G protein coupling. Activation of the GnRHR by GnRH has long been known to result in the activation of heterotrimeric GTP-binding (G) proteins. Therefore, when the GnRHR cDNA was cloned, it was no surprise to find that it encoded a protein predicted to be a member of the family of cell surface, seven-transmembrane domain, G protein-coupled receptors (31). Because GnRH actions are generally not affected by cholera or pertussis toxin, a novel G protein (G_p) was suggested to mediate receptor activation. Using an antibody to the common $G_{q\alpha}/G_{11\alpha}$ carboxy-terminal sequence, it has been shown that GnRH activation of phospholipase C (PLC) in αT3-1 cells requires GnRHR coupling to G_q, G_{11}, or both (60). Sustained exposure of αT3-1 cells to a GnRH agonist results in the specific down-regulation of cellular levels of both $G_{q\alpha}$ and $G_{11\alpha}$ (Fig. 3) (61–63). This was attributable to enhanced proteolysis of the activated G proteins; there was no change in $G_{q\alpha}$ or $G_{11\alpha}$ mRNA levels (64). Sustained activation of PKC with the phorbol ester, PMA, was unable to mimic the GnRH agonist-mediated down-regulation of $G_{q\alpha}$ and $G_{11\alpha}$, and inhibition of PKC with the selective inhibitor chelerythrine did not prevent this effect of GnRH, suggesting that the down-regulation of the G protein α-subunits is a direct result of activation of the G protein, and does not require activation of a downstream second messenger-activated protein kinase. Interestingly, the rate of decay of $G_{q\alpha}/G_{11\alpha}$ in the presence of GnRH agonist had two components: an initial rapid rate and a slower secondary phase. It is possible that the initial fast decay rate occurring upon receptor occupancy is reduced to a lower rate with desensitization of the receptor response; alternatively, the fast decay rate may be dependent on the fraction of the cellular G protein that becomes activated upon occupancy of the GnRHR, whereas the lower decay rate depends on the residual G protein pool. The down-regulation of $G_{q\alpha}$ and $G_{11\alpha}$ may, in turn, be a component of the desensitization of the cellular response to GnRH upon sustained exposure to GnRH or to an agonist.

2. Inositol phosphates (IPs). Activation of the pertussis toxin-insensitive G proteins of the G_q family results in stimulation of PLCβ activity, leading to the breakdown of phosphoinositide to inositol phosphates and diacylglycerol. Therefore, the coupling of the GnRHR to G_q and G_{11} would lead one to expect that activation of the GnRHR by GnRH or GnRH agonists would give rise to elevated intracellular concentrations of IPs. Indeed, intracellular concentrations of IPs increased within 30 sec following exposure of αT3-1 cells to a GnRH agonist and continued to accumulate, reaching a maximum after 20 min (Fig. 4) (39, 61). The IP responses were pertussis toxin-insensitive. Levels of inositol 1,4,5-trisphosphate, the immediate product of the cleavage of phosphatidylinositol 4,5-bisphosphate (the major substrate of PLCβ), were rapidly but transiently stimulated after exposure of αT3-1 cells to GnRH. Levels increased within 10 sec, reached

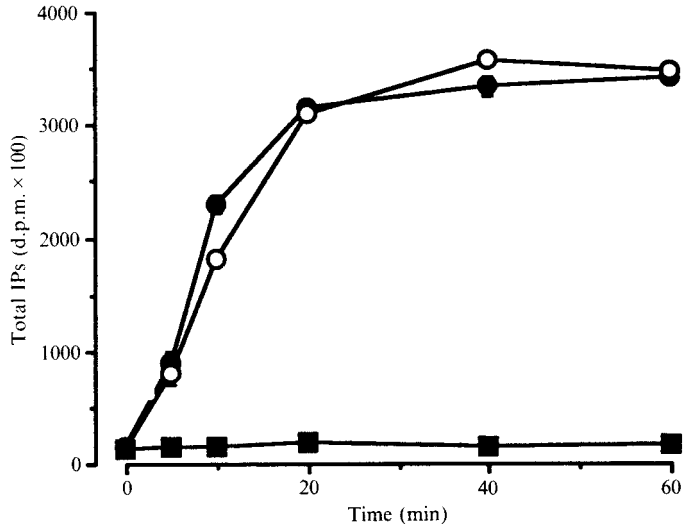

FIG. 4. Time course of total IP production in unstimulated αT3-1 cells (■) or cells stimulated with either GnRH (GnRH; 10 μmol/liter (●) or the GnRH agonist, buserelin (10 nmol/liter; (○). Monolayer cultures were incubated for the times indicated and total IPs were measured. Results are the mean ± SD of triplicate determinations in three separate experiments. [Reproduced by permission of The Journal of Endocrinology, Ltd. From L. Anderson *et al*: *J Endocrinol* 136:51–58, 1993 (61) (Fig. 1).].

a maximum after 30 sec, and returned to basal values after 60 sec. The accumulation of IPs in response to GnRH was inhibited by estradiol. The maximum levels of IPs attained were decreased, and estradiol caused a rightward shift in the dose-response relationship for GnRH-stimulated IP accumulation. This suggests that estradiol reduces GnRHR number and also reduces the efficiency with which the residual receptors are able to activate PLC (52). Estradiol has been shown to regulate levels of G proteins in rat pituitaries; hence, down-regulation of $G_{q\alpha}$ and $G_{11\alpha}$ levels may contribute to this effect (65).

3. Intracellular calcium. Intracellular calcium concentrations ($[Ca^{2+}]_i$) increase rapidly in αT3-1 cells after exposure to GnRH. $[Ca^{2+}]_i$ started to increase by 5 sec following GnRH exposure, with the majority of cells showing a maximal response within 15 sec. Thereafter, $[Ca^{2+}]_i$ decreased, although there was a prolonged secondary phase of the GnRH-induced calcium response, with levels increased up to 11 min after the addition of GnRH (Fig. 5) (61, 66). Thus, GnRH augments calcium currents in αT3-1 cells, with a functionally similar response to that reported in primary gonadotropes. Primary gonadotropes have at least two types of voltage-sensitive calcium channels, resembling T- and L-type calcium channels and giving transient and sustained currents, respectively (67). Like T-type current, the transient current in αT3-1 cells was activated by low voltage and rapidly inactivated, and, like L-type current, the sustained current was activated by high voltage and dihydropyridine-sensitive (39, 68). Precise measurements of $[Ca^{2+}]_i$ have been done in single, fura-2-loaded αT3-1 cells by dual wavelength fluorescence microscopy, as well as in cell suspension by spectrofluorometric analysis, and in single indo-1 AM-loaded cells (66, 69). These studies revealed a biphasic rise in $[Ca^{2+}]_i$

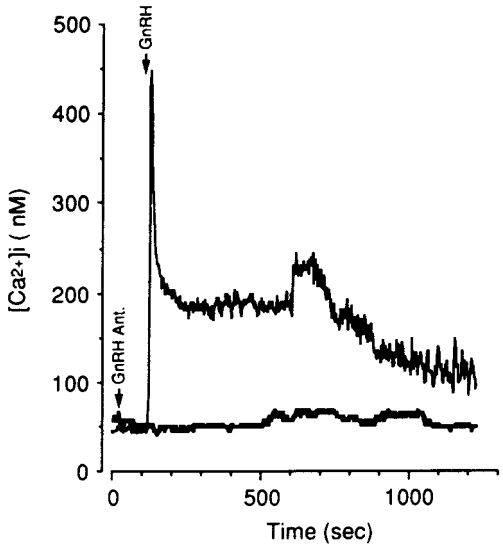

FIG. 5. The effect of GnRH (10^{-8} M, t = 118 s, n = 6, *upper trace*) alone or after pretreatment with a GnRH antagonist (10^{-6} M for 2 min, *lower trace*, n = 10) on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$, Ionized intracellular calcium concentration. [Reprinted from *Mol Cell Endocrinol* 86:167–175, Fig. 1, L. Anderson *et al*., "Characterization of the gonadotropin-releasing hormone calcium response in single αT3-1 pituitary gonadotroph cells" 1992 (66) with kind permission from Elsevier Science Ireland Ltd., Bay 15K, Shannon Industrial Estate, Co. Clare, Ireland.].

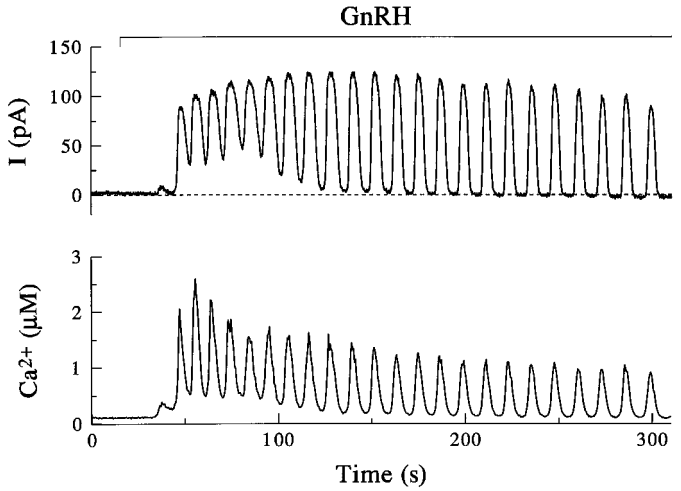

FIG. 6. GnRH-induced oscillations of outward K^+ current and $[Ca^{2+}]_i$. The K^+ current is measured under voltage clamp conditions at −50 mV, and $[Ca^{2+}]_i$ is measured simultaneously with 50 μM indo-1 in the pipette. GnRH (2 nM) is perfused in the bath during the period marked with a *bar*. The opening of K^+ channels is strictly synchronous with $[Ca^{2+}]_i$ elevations. I, Current; Ca^{2+}, ionized calcium concentration. [Reprinted with permission from B. L. Hille *et al*: *Recent Prog Horm Res* 50:75–95, 1995 (25) (Fig. 3).]

in response to 10^{-8} to 10^{-7} M GnRH. The initial calcium response was complete within seconds and involved primarily an IP_3-mediated rise in cytosolic calcium due to release from intracellular stores. Importantly, the peak elevation in $[Ca^{2+}]_i$ was around 500 nM, above the threshold for activation of exocytosis (24). The smaller secondary plateau phase lasted several minutes and primarily involved the influx of extracellular calcium through specific, dihydropyridine-sensitive, L-type, PKC-activated channels. The biphasic nature and duration of the calcium response in αT3-1 cells is similar to the response obtained in studies using enriched gonadotrope preparations. In single αT3-1 cells exposed to increasing doses of GnRH, from 10^{-10} to 10^{-6} M, amplitude-modulated calcium responses were elicited, with no indication of $[Ca^{2+}]_i$ oscillations or frequency modulation. This finding contrasts with observations in primary pituitary gonadotropes, in which GnRH induces prominent $[Ca^{2+}]_i$ oscillations and frequency-modulated calcium signaling (Fig. 6) (25, 70). An additional difference observed in the calcium response of αT3-1 cells compared with primary gonadotropes is that activation of PKC exerts only a negative feedback effect on calcium entry in αT3-1 cells, whereas in cultured primary pituitary gonadotropes, PKC activators cause transient activation of calcium entry, followed by an inactivation phase (69, 70). This effect in αT3-1 cells is similar to that observed in the rat somatolactotropic GH_3 cell line (71). Although the reasons for these differences between the immortalized cell lines and primary pituitary cells are not known, it is possible that calcium channels in αT3-1 cells and GH_3 cells are spontaneously active and undergo inactivation in a Ca^{2+}- and PKC-dependent manner.

4. Protein kinase-C (PKC). The cleavage of phosphoinositides by phospholipase C produces 1,2-diacylglycerols in addition to inositol trisphosphates. Diacylglycerols activate PKC, which results in the translocation of PKC from the cytosol to the plasma membrane. After exposure to GnRH, a portion of intracellular PKC is translocated in αT3-1 cells (39). PMA, a potent activator of PKC, caused an even more pronounced translocation of the enzyme. The effects of GnRH on PKC in αT3-1 cells is similar to that observed in primary pituitary cells *in vivo* and *in vitro* (72, 73). αT3-1 cells contain PKC α-, ε-, and ζ-isoforms, as detected by immunostaining (74). By Northern blot analysis, mRNAs for PKCα and -β, but not -γ, were detected. Exposure of αT3-1 cells to a GnRH agonist resulted in a dose-dependent increase in PKCβ, but not PKCα, mRNA levels. This response was mimicked by PMA. The calcium ionophore, ionomycin, stimulated the expression of both PKCα and PKCβ mRNA levels. Removal of intra- or extracellular calcium or inhibition of PKC abolished the effect of GnRH, indicating that GnRH-induced PKCβ gene expression is Ca^{2+}-dependent and autoregulated by PKC (75).

5. cAMP. No significant change in cAMP levels could be detected in αT3-1 cells after treatment with a GnRH agonist, even in the presence of a phosphodiesterase inhibitor to prevent the degradation of cAMP (39). This is in contrast to the rise in cAMP levels that has been observed in whole pituitaries (76). This difference may lie in the possible need for the presence of testosterone for this response; the GnRH-induced rise in cAMP levels was observed in intact male rats only (77). Others have not been able to detect significant changes in cAMP levels after GnRH treatment of primary gonadotropes (78).

6. Mitogen-activated protein kinases (MAPKs). MAPKs, also known as extracellular signal-related kinases (ERKs), are a family of serine/threonine protein kinases that are rapidly activated in response to a wide variety of stimuli (Fig. 7)

(79–83). Several members of the MAPK family have been identified, including $p42^{mapk}$ (ERK2) and $p44^{mapk}$ (ERK1). Stimuli for their activation include growth factors, many of which have receptors with intrinsic protein tyrosine kinase activity. MAPKs are involved in transmitting extracellular growth and differentiation signals into the cell nucleus, resulting in an array of transcriptional and mitogenic effects. Recent evidence indicates that some G protein-coupled receptors can activate the MAPK family of enzymes and that MAPKs may also be involved in nonproliferative signaling cascades (84–87). G protein-coupled receptors appear to activate MAPK through Ras-dependent and -independent pathways, and both Gα- and Gβγ-subunits appear to be variably involved. These findings have led several investigators to study the ability of the GnRHR to activate MAPK and the role of MAPK in mediating cellular effects of GnRH (88–93).

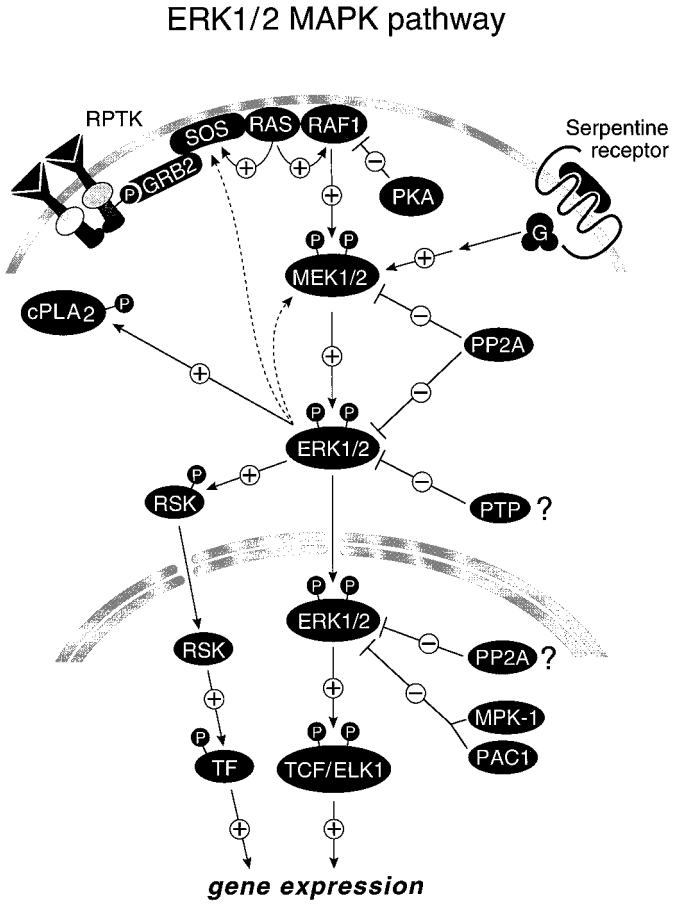

FIG. 7. ERK1/ERK2 MAPK pathway. A schematic illustration of the MAPK pathway. RPTK, receptor protein tyrosine kinase; ERK, extracellular signal-related kinase; MEK, mitogen activated protein kinase (MAPK)/ERK; PKA, protein kinase A; $cPLA_2$, cytosolic phospholipase A_2; PP2A, protein phosphatase 2A; G, G protein; PTP, protein tyrosine phosphatase; RSK, ribosomal S6 kinase; TF, TCF, ELK1, transcription factors; MKP1, PAC1, protein phosphatases. [Reprinted with permission from T. Hunter: *Cell* 80:225–236, 1995 (79) (Fig. 1). © 1995 by Cell Press].

Stimulation of αT3-1 cells with GnRH resulted in phosphorylation of both ERK1 and ERK2, and rapid and sustained activation of both, as assayed by their ability to phosphorylate myelin basic protein (91, 92, 94). Stimulation of enzyme activity was detected within 5 min after the addition of GnRH and remained elevated for 60 min. A maximal activation of 4- to 5-fold was achieved, at a GnRH concentration of 100 nM. Activation of ERK1 and ERK2 was blocked by treatment of αT3-1 cells with a GnRHR antagonist, Antide, demonstrating that activation of the MAPK signal transduction cascade by GnRH is receptor-mediated (92). Activation of MAPK by GnRH was comparable to that observed in response to PMA. Furthermore, PMA pretreatment for 24 h to deplete phorbol ester-sensitive forms of PKC blocked the activation of ERK1 by GnRH. These data suggest that the activation of MAPK by GnRH may involve activation of PKC (91). MAPK activity was also stimulated, although to a lesser extent, by GnRH in primary cultures of male rat pituitary cells. The lower level of activation probably reflects the heterogeneity of the pituitary cell population. Thus, it appears that the MAPK signal transduction pathway is activated by GnRH in both αT3-1 cells and primary pituitary gonadotropes. Interestingly, treatment of αT3-1 cells with pertussis toxin blocked GnRH-induced MAPK activation, suggesting that this signaling pathway is coupled to the pertussis toxin-sensitive G_i or G_o pathway. This provides evidence for G_i/G_o-mediated signal transduction by GnRHR in addition to G_q-mediated signal transduction (88, 90).

F. α-Subunit gene expression

1. Cell-specific expression. αT3-1 cells have proven to be a useful cell model for the isolation and characterization of transcription factors that appear to be involved in mediating gonadotrope-specific expression of the α-subunit gene (Fig. 8). Some of these factors may be involved in mediating stimulation of α-subunit gene expression by GnRH as well. However, because these factors appear to be more important for basal or tissue-specific α-subunit gene expression rather than GnRH-stimulated expression, they will be mentioned only briefly here.

The element in the α-subunit promoter that has been best

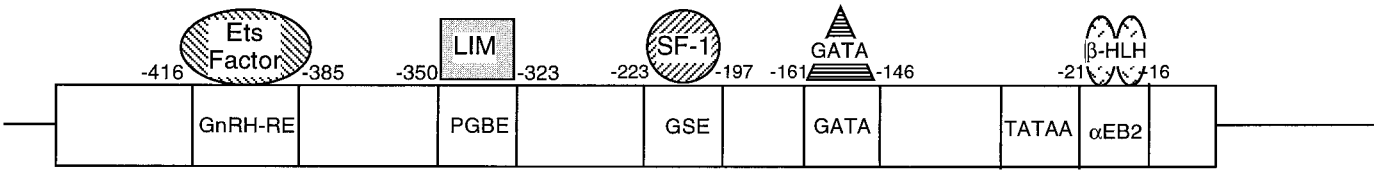

FIG. 8. *cis*-Acting elements and transcription factors important for cell-specific and regulated expression of the glycoprotein hormone α-subunit gene that have been characterized in αT3-1 cells. GnRH-RE, GnRH response element; PGBE, pituitary glycoprotein hormone basal element; GSE, gonadotrope-specific element; αEB2, E-box; LIM, LIM homeodomain protein; SF-1, steroidogenic factor 1; βHLH, basic helix-loop-helix protein.

characterized as a basal, tissue-specific enhancer is the gonadotrope-specific element (GSE). The GSE sequence, TGACCTTG, occurs upstream of the placenta-specific elements, at positions −215/−208 in the mouse α-subunit gene, and is highly conserved among mouse, human, rat, cow, and horse species (95). The GSE is bound by a 54-kDa protein, steroidogenic factor-1 (SF-1) (96). SF-1 was first identified by its ability to bind to and coordinately regulate the expression of genes encoding enzymes in the corticosteroid biosynthetic pathway (97, 98). Subsequently, it has also been shown to bind to and regulate the aromatase and Mullerian-inhibiting substance genes in gonadal tissues (99, 100). Disruption of the gene encoding SF-1 in mice precludes adrenal and gonadal development and also results in the selective loss of expression of gonadotrope-specific markers, including LHβ, FSHβ, and GnRHR mRNAs, and a reduction in α-subunit mRNA levels (101, 102). Thus, SF-1 appears to be important for function of the reproductive axis at multiple levels. Treatment of SF-1-deficient mice with exogenous GnRH stimulates expression of LHβ and FSHβ, suggesting that SF-1 is not necessary for GnRH stimulation of gonadotropin gene expression (103) .

An additional putative basal enhancer, referred to as the pituitary glycoprotein hormone basal element (PGBE), has been identified at −344/−300 of the mouse α-subunit gene (104). The PGBE is able to direct expression of the α-subunit promoter to cells of both gonadotrope and thyrotrope lineages, but not to placenta. A member of the LIM (lin-11, isl-1, mec-3)-homeodomain family of transcription factors, LH-2, binds to a 14-bp imperfect palindrome within the PGBE domain *in vitro* (105). This element and factor are discussed further below.

Other elements that have been identified to play a role in expression of the α-subunit gene in αT3-1 cells include a GATA element, bound by GATA-binding proteins (106), and two E boxes, which bind members of the family of basic-helix-loop-helix-zipper proteins (107). The optimum level of α-subunit gene expression in gonadotropes is probably determined by the combined actions of widely expressed, pituitary-restricted, and gonadotrope-specific transcriptional activators that act in combination and synergistically.

2. GnRH-stimulated expression. Although a number of factors that may be necessary for maintenance of basal levels of gonadotrope-specific gene expression have been identified in αT3-1 cells, the identification of mechanisms for GnRH-stimulated expression have been less forthcoming. Windle *et al.* (38) have demonstrated that αT3-1 cells respond to GnRH by elevating α-subunit gene expression. A similar increase of α-subunit mRNA levels was observed in response to PMA, and this increase was not additive with GnRH, suggesting that PKC may play a role in transducing the GnRH signal to the nucleus (39). The calcium ionophore, ionomycin, also stimulates α-subunit mRNA levels. In contrast, an inhibitor of cAMP-dependent protein kinase did not affect the ability of GnRH or PMA to stimulate expression of an α-subunit promoter/luciferase reporter gene (αLUC), indicating that cAMP-dependent protein kinase is not required for transcriptional activation by GnRH (104).

The increase in α-subunit mRNA levels in response to GnRH was maximal at 12–24 h and maintained for a further 24 h (Fig. 9) (108). The observed increase in mRNA levels appears to be mediated by both an increase in α-subunit gene transcription and mRNA stability. Nuclear run-off assays demonstrated an increase in α-subunit gene transcription of 2- to 3-fold within 1 h after exposure to GnRH but returned to baseline by 12 h. GnRH also stimulated the activity of αLUC, apparent after 1 h, maximal after 4–6 h, but back to baseline by 24 h of GnRH treatment (Fig. 9). Thus, GnRH appears to stimulate a burst of α-subunit gene transcription lasting less than 4–6 h. The persistent elevation of α-subunit mRNA levels for at least 48 h suggests that the mRNA has a long half-life and/or that GnRH stabilizes the mRNA in addition to its transcriptional effects. Indeed, pulse-chase experiments showed that the half-life of the α-subunit mRNA increased from 1.2 h in the absence of GnRH to 8 h in the presence of GnRH in αT3-1 cells. Whether this mechanism also occurs in primary gonadotropes is unclear, as the half-life of α-subunit mRNA in primary pituitary cultures is 6.5 h; however, in this case both gonadotropes and thyrotropes contribute to α-subunit mRNA levels (109). Interestingly, while the stimulatory effects of GnRH on α-subunit gene transcription and mRNA levels were evident very rapidly, within 1 h after exposure to GnRH, GnRH-induced α-subunit release was detected only after a lag of 4 h of incubation (110). Thus, there appears to be dissociation between the stimulation of gene expression and exocytosis.

Transient transfection studies in αT3-1 cells with mouse or human αLUC have been used to determine DNA sequences

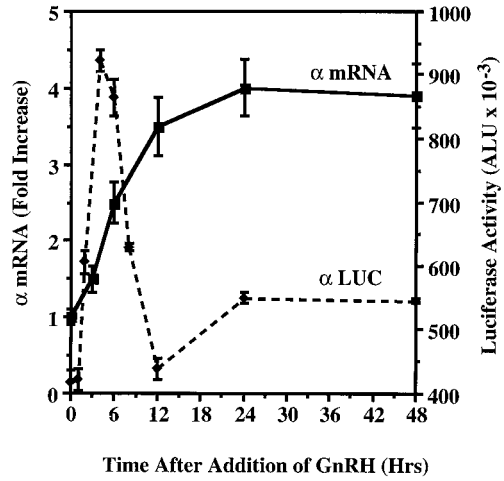

FIG. 9. Effect of GnRH on αLUC expression and α-subunit mRNA levels. αT3 cells stably transfected with αLUC were incubated in the absence or presence of GnRH (10^{-7} M) for the indicated periods of time. Cells were harvested and assayed for luciferase activity. Luciferase activity (αLUC) is expressed in arbitrary light units (ALU) and is the mean ± SEM of triplicate plates of cells. Basal expression of αLUC was 415,000 ALU. Background luciferase activity was below 120 ALU. Total RNA (5 μg) from triplicate plates of αT3 cells treated in the absence or presence of GnRH was analyzed by Northern blot for α-subunit and GAPDH mRNAs. mRNA levels were quantitated using scanning densitometry, and α-subunit mRNA levels were corrected for hybridization to GAPDH mRNA. The mean ± SEM of three separate experiments are expressed relative to the basal α-subunit mRNA level in the absence of GnRH. [Reprinted with permission from P. J. Chedrese *et al*: *Endocrinology* 134:2475–2481, 1994 (108) (Fig. 1). © The Endocrine Society.]

of the α-subunit gene that mediate transcriptional responses to GnRH. Deletion analyses indicated that deletion of sequences between −507 and −205 of the mouse α-subunit gene resulted in a decrease in responsiveness to GnRH, as well as to PMA and to cAMP (104). This region, when linked to a heterologous promoter, was capable of supporting responses to GnRH, PMA, and cAMP. Further mutational analysis revealed that mutations at positions −406/−399 and −337/−330 resulted in a decrease in the response to GnRH. Multimers of −416/−385, when linked to a minimal promoter upstream of the luciferase gene, responded to GnRH with a stimulation of luciferase activity (Fig. 10). In contrast, multimers of −344/−300 enhanced basal transcription but did not respond further to GnRH. These data suggest that GnRH responsiveness requires the cooperative interaction of two distinct sequences, an upstream GnRH-responsive element (GnRH-RE) at −416/−385, and a downstream element at −344/−300, corresponding to the location of the PGBE described above. The upstream GnRH-RE was also responsive to PMA, further supporting the role of the PKC pathway in mediating the effects of GnRH on expression of the α-subunit gene. The need for a complex response unit for the mediation of GnRH stimulation may provide a mechanism for the maintenance of appropriate, tissue-specific expression and regulation of the α-subunit gene. The involvement of a tissue-specific basal element may restrict α-subunit gene expression to the appropriate cell type, and the involvement of two elements in mediating GnRH responses may prevent the α-subunit gene from responding to activation of the PKC-signaling pathway in nongonadotrope cells and tissues.

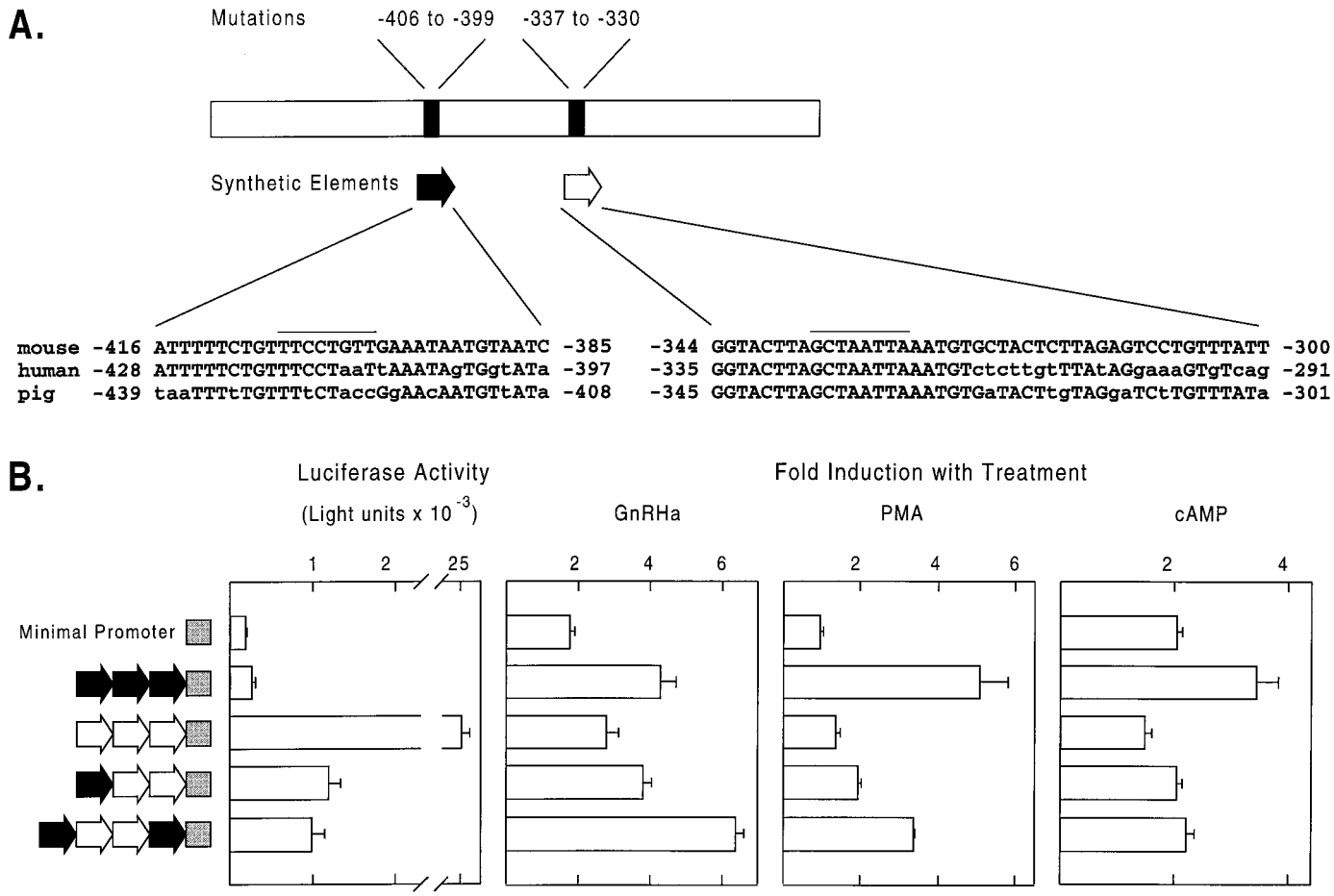

FIG. 10. Multimers of the −416 to −385 region function as a GnRH-responsive element. A, Synthetic DNA elements were prepared that included the sequences that were shown to be important by mutation analysis. The sequence of the mouse α-subunit gene, which was used as a synthetic DNA element, is aligned with the corresponding region of the human and pig α-subunit genes. Positions in which the human or pig sequence are identical to the mouse sequence are indicated by *uppercase letters*. The locations of the α-subunit sequences where mutations reduced GnRH and phorbol responses are indicated by *overbars*. B, To assess the functional properties of these elements, multimers of the synthetic DNA elements were placed upstream of a minimal promoter, which was linked to luciferase, and the reporter genes were transfected into αT3-1 cells. Cells were treated with vehicle alone, 10^{-5} M buserelin (GnRHa), 10^{-7} M phorbol myristic acid (PMA), or 0.5 mM 8-(4-chlorophenylthio)cAMP (cAMP) 18 h after transfection. Cells were collected 24 h after transfection (6 h after treatment), and luciferase activity was determined. All values are means ± SE from two to four separate experiments; each experiment included three transfections for each DNA construct. The luciferase data were normalized for transfection efficiency between experiments. Responses to different agents are indicated as the ratio of luciferase activity in the treated cells to that in vehicle-treated cells. A schematic representation of the organization of each of the constructs is shown at the *left*. The −416 to −385 element is indicated by a *black arrow*; the −344 to −300 element is indicated by a *white arrow*; the minimal promoter sequences are indicated by *gray shading*. [Reprinted with permission from W. E. Schoderbek *et al*: *J Biol Chem* 268:3903–3910, 1993 (104) (Fig. 7).].

As mentioned above, a member of the LIM-homeodomain family of transcription factors, LH-2, binds to a 14-bp imperfect palindrome within the PGBE domain *in vitro* (Fig. 8) (105). LIM-homeodomain proteins contain both a zinc finger (the LIM domain) and a homeodomain (111). The homeodomain of these factors is sufficient for specific DNA binding; the LIM domains appear not to be DNA-binding domains, but rather may function as protein-protein interaction domains to facilitate homo- or heterodimer formation. LH-2 has a restricted tissue distribution, being most abundant in αT3-1 and αTSH cells, cell lines of gonadotropic and thyrotropic origin, respectively, and in mouse brain; less abundant in whole rat pituitaries, corticotropic AtT20 cells, and somatolactotropic GH_3 cells; and undetectable in placental JEG-3 cells and in mouse liver. Cotransfection of LH-2 into COS cells showed that LH-2 is able to activate specifically the α-subunit promoter 2-fold and a 3XPGBE reporter construct 5- to 6-fold. These studies suggest that the LIM-homeodomain protein LH-2 is an activator of the glycoprotein hormone α-subunit gene in gonadotropes and thyrotropes. It is possible that another transcription factor, binding to the upstream GnRH-RE, may interact with LH-2 bound to the PGBE to mediate GnRH-induced expression of the α-subunit gene.

Another candidate factor for a role in mediating α-subunit gene expression by binding to the PGBE is mLim-3, a related member of the family of LIM-homeodomain proteins. mLim-3, also known as P-Lim or Lhx3, is a mouse gene expressed in the pituitary throughout development and in the adult, as well as transiently in the spinal cord, pons, and medulla oblongata, but with no detectable expression elsewhere. mLim-3 expression was detected in cell lines of pituitary origin, including cells representative of somatolactotropes (GH_3, GH_4C1, GC), thyrotropes (αTSH), gonadotropes (αT3), and corticotropes (AtT-20), but not in cell lines derived from peripheral, other endocrine, or neural tissues (112, 113). mLim-3 is able to bind to the PGBE sequence *in vitro* and is a strong activator of transcriptional activity of the α-subunit promoter, as well as the PRL, TSHβ, and Pit-1 promoters (112). Interestingly, it was recently reported that targeted disruption of the mLim-3 gene in mice leads to failure of growth and differentiation of the anterior and intermediate lobes of the pituitary (114). The development of all pituitary cell lineages, except the corticotropes, was affected. This suggests that mLim-3 plays an important role not only in α-subunit gene expression, but in differentiation and proliferation of nearly all the pituitary cell lineages.

Further studies of the putative GnRH-RE in the mouse α-subunit promoter have identified a core Ets factor (a family of transcription factors that have been implicated in mediating transcriptional responses to MAPK activation) binding site within the GnRH-RE, which appears to be important in mediating GnRH stimulation of α-subunit gene expression (Fig. 8) (92). Recent evidence that GnRH activates the MAPK signal transduction pathway, as discussed above, is relevant in terms of the mechanisms of transcriptional stimulation of the α-subunit gene by GnRH. Activation of the MAPK cascade by a constitutively active form of Raf kinase in αT3-1 cells leads to stimulation of the α-subunit promoter. Furthermore, inhibition of MAPK activity by kinase-defective ERK1 or ERK2, or overexpression of MAPK phosphatase 2, which dephosphorylates and inactivates MAPK, leads to the attenuation of GnRH-induced activation of the α-subunit promoter. The DNA-binding domain of Ets-2 was able to bind specifically to a site within the GnRH-RE, and a dominant negative Ets-2 expression vector reduced the ability of GnRH to stimulate expression of αLUC. These findings suggest that the Ets factor-binding site in the GnRH-RE may contribute to transcriptional stimulation of the α-subunit gene by GnRH, via activation of the MAPK pathway. In contrast, however, Sundaresan *et al.* (91) found that dominant negative mutant forms of Ras, ERK1, and ERK2 reduced basal expression of a human αLUC but had no effect on GnRH-stimulated expression. The reasons for the differences between these two studies are not clear, although Roberson *et al.* (92) used the mouse α-subunit promoter, whereas Sundaresan *et al.* used the human gene.

In addition to the studies characterizing GnRH-responsive DNA sequences in the mouse α-subunit gene using αT3-1 cells as described above, a GnRH-responsive region in the human gene was identified by transfection analyses in primary rat pituitary cell cultures (115). Deletion analyses suggested that one or more GnRH-responsive sequences reside between −346 and −244 in the human α-subunit promoter. This GnRH-responsive region does not include the GnRH-RE defined in the mouse α-subunit promoter. In contrast to the findings with the mouse α-subunit gene in αT3-1 cells, the regions of the human α-subunit gene that are important for the GnRH response appear to be distinct from those required for basal activity. Basal expression appeared to be primarily mediated through the proximal promoter and cAMP-responsive regions. These differences may reflect different mechanisms of GnRH stimulation of the human *vs.* the mouse α-subunit gene or differences in the mechanisms of regulation in αT3-1 cells *vs.* primary pituitary gonadotropes.

G. Desensitization

GnRH is secreted from the hypothalamus in a pulsatile fashion, and pulsatile GnRH stimulates LH and FSH biosynthesis and secretion (116). In contrast to the stimulatory effects of pulsatile GnRH, sustained exposure to high concentrations of GnRH reduces the response of gonadotropes to subsequent stimulation with GnRH (homologous desensitization), leading to suppression of gonadotropin secretion (117). This homologous desensitization to GnRH can occur rapidly, within the time frame of endogenous GnRH pulses (118). The mechanism of this desensitization is not known, and both receptor (119) and postreceptor (120, 121) mechanisms have been proposed. For a number of other G protein-coupled receptors, early desensitization events are thought to involve the uncoupling of the receptor from its regulatory G protein, with loss of downstream-signaling events (122). Rapid desensitization appears to involve phosphorylation by specific intracellular kinases of the third intracellular loop or the C-terminal tail (123, 124). However, the GnRHR lacks the C-terminal cytoplasmic tail as well as the third intracellular loop sequences implicated in the desensitization of other receptors (31).

αT3-1 cells have been used as a model for the study of mechanisms of desensitization to GnRH. Stimulation of αLUC activity in transfected αT3-1 cells was maximal 4–6 h after exposure to GnRH but thereafter declined, returning to levels in unstimulated control cells by 12–24 h. αLUC activity was also stimulated by a PKC activator, PMA, a calcium channel agonist, BAY K 8644, and an activator of the PKA pathway, 8-bromo-cAMP. Maximal responses to these agents also occurred after 4–6 h of exposure, although the maximal levels of activity were less than those observed in response to GnRH. A decline in αLUC activity over time with continuous exposure to these agents was particularly marked for PMA, but was also seen with BAY K 8644, whereas stimulation by 8-bromo-cAMP was maintained for at least 24 h. Pretreatment of αT3-1 cells with GnRH blocked subsequent stimulation of αLUC activity by either GnRH or PMA. In contrast, both 8-bromo-cAMP and BAY K 8644 were still able to stimulate αLUC activity after pretreatment with GnRH. These data suggest that the transcriptional stimulation of the α-subunit gene by GnRH is mediated by the PKC pathway, and that this pathway can be desensitized in αT3-1 cells by continuous exposure to GnRH. The kinetics of desensitization are difficult to infer from these studies; exposure to GnRH may incite a short burst of transcriptional activity of the α-subunit promoter, which then leads to a more gradual accumulation of the luciferase product. However, the addition of the GnRH antagonist, Antide, after treatment of the cells with GnRH resulted in a reduction of luciferase activity compared with exposure to GnRH alone, even when Antide was added up to 6 h after GnRH, indicating that some stimulation of the α-subunit promoter by GnRH was still occurring, *i.e.* the cells were not fully desensitized to GnRH. Continuous exposure of primary pituitary cells to GnRH causes rapid desensitization at the secretory level for free α-subunit as well as intact LH and FSH, evident within 15 min (125). The differences in kinetics for transcriptional and secretory desensitization may reflect different cellular mechanisms or differences between the αT3-1 cell line and primary gonadotropes.

Regulation of α-subunit gene transcription is a relatively downstream endpoint for the study of homologous GnRH desensitization. Measurements of second messengers may lead to insights into early or short-term desensitization events. GnRH treatment led to a linear increase in total IP production in αT3-1 cells over 0–15 min (126–128). Furthermore, GnRH pretreatment for 5 min did not alter subsequent stimulation of IP_3 production by GnRH 15 min later. These data indicate a lack of desensitization of the rapid GnRH-induced IP_3 response in αT3-1 cells. Pretreatment with GnRH for 1 h did reduce subsequent cellular IP accumulation in response to GnRH, but this may be attributable to a reduction in GnRHR numbers. GnRH pretreatment of αT3-1 cells for short times (5–15 min) had no effect on GnRHR number; however, treatment for 1 h with 10^{-7} M GnRH reduced GnRHR number by 48%. The affinity for GnRH was not altered. Desensitization of both the extracellular Ca^{2+}-dependent and -independent phases of the Ca^{2+} response to GnRH were observed after pretreatment with 10^{-7} M GnRH for 1 h (128). Thus, one mechanism of intermediate desensitization to GnRH may be receptor loss.

However, this does not account for rapid or early desensitization or the degree of desensitization of the Ca^{2+} response. An additional uncoupling event may occur during the pretreatment, which reduces the ability of the agonist-occupied GnRHR to elevate intracellular Ca^{2+}. Treatment of αT3-1 cells with 5-min pulses of GnRH every 15 min resulted in desensitization of the Ca^{2+} response after the first pulse in a dose-dependent manner, being evident at GnRH concentrations greater than 2×10^{-9} M (126). The mechanisms underlying this desensitization are not known but could include loss of IP_3 receptors, depletion of intracellular Ca^{2+} stores, and inactivation of Ca^{2+} channels, as has been suggested in studies of primary pituitary cells (120). The dissociation of IP production and calcium stimulation suggests that desensitization of GnRH-induced calcium mobilization is a postreceptor phenomenon occurring distal to PLC activation. The lack of the C-terminal cytoplasmic tail, implicated in the desensitization of other G protein-coupled receptors, in the GnRHR therefore appears to correlate with a lack of receptor desensitization; rather, desensitization to GnRH appears to be primarily a postreceptor phenomenon. Alternatively, αT3-1 cells may be lacking a factor(s) necessary for mediating rapid receptor desensitization in primary gonadotropes.

H. Summary of GnRH action in αT3-1 cells

The development of the αT3-1 gonadotropic cell line has enabled significant advances in our understanding of gonadotrope function and gonadotropin regulation, particularly in the areas of α-subunit gene expression and GnRHR structure and function. αT3-1 cells were critical for the initial cloning of GnRHR cDNAs as well as for elucidation of the GnRHR gene structure, confirming previous findings in primary pituitary cells which suggested that the GnRHR was a member of the G protein-coupled receptor family. The absence of a carboxy-terminal intracellular tail on the receptor was a surprising finding, which makes questions about the mechanisms of gonadotrope desensitization to GnRH all the more intriguing.

αT3-1 cells have been used to elucidate a number of components of the GnRH signal transduction pathway (Fig. 11). The GnRHR in αT3-1 cells is coupled to G proteins of the G_q/G_{11} family, leading to production of IPs and increases in intracellular calcium levels, which, in turn, leads to activation of PKC. While cAMP has, in some studies, been suggested to be activated by GnRH, and has been shown to lead to increases in expression of the α-subunit gene, there is no evidence for increases in cAMP levels in response to GnRH in αT3-1 cells. Furthermore, there is now evidence that the MAPK pathway is activated by GnRH in αT3-1 cells and may be important in the stimulation of α-subunit gene expression by GnRH.

While αT3-1 cells have proven to be invaluable for the study of GnRH action, there are some differences between αT3-1 cells and primary pituitary gonadotropes. The regulation of the GnRHR in αT3-1 cells is different from primary gonadotropes; in particular, the receptor does not appear to be markedly regulated by GnRH itself in αT3-1 cells, especially at the level of gene expression, whereas it is markedly

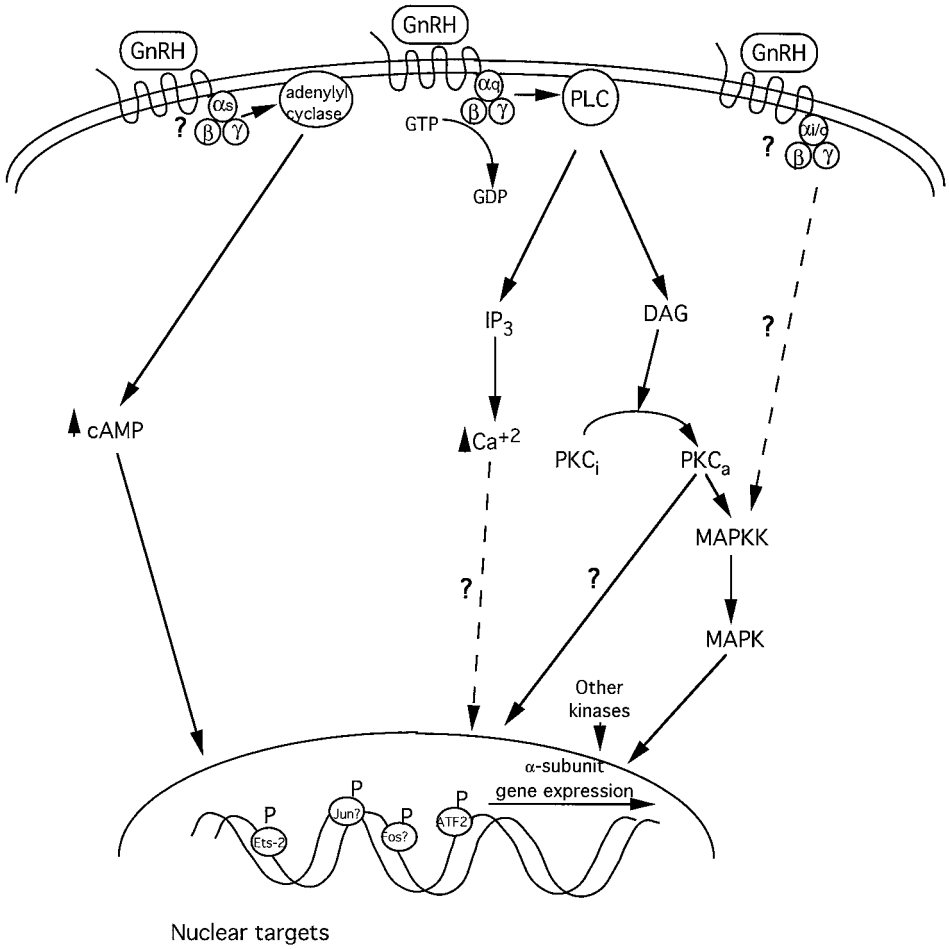

FIG. 11. Summary of known GnRH actions on α-subunit gene expression in αT3-1 cells. GnRH binds to the seven-transmembrane domain GnRHR, which is coupled to G_q/G_{11}. Activation of G_q/G_{11} activates phospholipase C, which stimulates the production of inositol triphosphate and an increase in $[Ca^{2+}]_i$, leading to activation of PKC. PKC, in turn, leads to stimulation of α-subunit gene expression, either directly, or indirectly by activating the MAPK cascade. GnRHR may also be coupled to G_s, leading to activation of adenylyl cyclase and stimulation of cAMP production, which may also influence α-subunit gene expression. Third, activation of the GnRHR may also activate the MAPK cascade via G_i.

regulated in primary pituitary cells. In addition, detailed studies of intracellular calcium profiles in response to GnRH reveal that amplitude-modulated intracellular calcium responses occur in αT3-1 cells, in contrast to primary gonadotropes, in which GnRH induces calcium oscillations and frequency-modulated calcium signaling. A major difference between αT3-1 cells and primary gonadotropes, however, is the lack of expression of the gonadotropin β-subunit genes by αT3-1 cells.

IV. Studies of GnRH Action in GH_3 Cells Transfected with the GnRH Receptor (GGH_3 Cells)

A. Derivation of GH_3 cells

The GH_3 cell is a well characterized pituitary cell strain established from a GH-producing rat pituitary tumor, MtT/W5, that was propagated as a transplantable rat pituitary tumor. By a method of alternate culture and animal passage, several clonal strains of epithelial cells were established (129, 130).

B. Characterization of GH_3 cells

These cells are somatolactotropic in origin. They secrete large amounts of GH into culture medium and stimulate body weight gain and growth after injection into normal or hypophysectomized rats (129, 130). They express PRL and GH genes and also secrete PRL and GH in a regulated fashion. GH_3 cells express TRH receptors (TRHR) and respond to TRH with an increase in PRL biosynthesis and secretion, and a reduction in GH production (131, 132). GH_3 cells do not express α-subunit, TSHβ, LHβ, FSHβ, and POMC genes, hormones expressed in other, nonsomatolactotropic anterior pituitary cell types. However, they are capable of supporting the expression of exogenous α- and TSHβ-subunit genes, introduced into the cells by transient transfection (133–138). In addition to TRHR, GH_3 cells also express somatostatin, galanin, and pituitary adenylate cyclase-activating polypeptide receptors (139–141), as well as activin types I and II receptors (142). However, GH_3 cells appear to lack functional dopamine receptors (143).

C. Derivation of GH_3 cells transfected with the GnRHR (GGH_3 cells)

GH_3 cells stably expressing the rat GnRHR (GGH_3 cells) were developed by cotransfecting GH_3 cells with a rat GnRHR expression vector (pcDNA1-GnRHR) and a pSVneo plasmid, which expresses the neomycin resistance gene, by electroporation (144). Control cells were transfected with pSVneo and pcDNA1 vector. The cells were grown in the presence of neomycin, to select for neomycin-resistant cell clones, and monoclonal cells were then expanded and tested for binding of GnRH and GnRH responsiveness. The general morphology of the stably transfected GGH_3 cell lines is indistinguishable from that of the parent GH_3 cells and of control, vector-transfected cells (145).

D. GnRH binding

Specific, high-affinity binding sites for GnRH and GnRH analogs are present on GGH_3 cells but not on the parental GH_3 cell line (127, 144, 146). Scatchard analysis of the binding of [^{125}I]Buserelin, a metabolically stable analog of GnRH, shows a K_d of $4.1 \pm 1.0 \times 10^{-8}$ M using GGH_3-1′ cells, one of the clonal strains of GH_3 cells that have been stably transfected with the rat GnRHR cDNA. Each GGH_3-1′ cell has 11,000 ± 2,800 receptors. Both GnRH agonists and antagonists are able to displace binding of [^{125}I]Buserelin to GGH_3-1′ cells (Table 2), whereas chemically unrelated peptides such as PRL, GH, and TRH do not significantly displace binding, even at high concentrations. The relative affinities of the GnRH agonists and antagonists are similar to those observed in cell cultures derived from rat pituitaries, suggesting that this receptor is similar to those expressed in primary gonadotropes, although the absolute affinities are slightly lower. Another clonal strain of stably transfected GH_3 cells, GGH_3-2 cells, had 13,000 ± 1,000 binding sites for [^{125}I]Buserelin per cell. In comparison, this cell line had 64,800 ± 3,700 specific [^{3}H]MeTRH binding sites per cell, representing binding to the TRHR, which is expressed endogenously in these cells.

The GnRHR contains Asn^{87} and Asp^{318} rather than the highly conserved Asp^{87} and Asn^{318} found in other G protein-coupled receptors. Site-directed mutagenesis was used to introduce a Asn^{318} mutation and $Asp^{87}Asn^{318}$ double mutation into the mouse GnRHR, and the mutant receptors were stably expressed in GH_3 cells. Both mutant receptors were able to bind [^{125}I]Buserelin, but IP production was attenuated (147). Furthermore, while cell surface levels of wild-type GnRHR are down-regulated upon exposure to GnRH (see below), this down-regulation was not observed for either mutant receptor. These data suggest that these mutations impair the coupling between the GnRHR and Gq, the G protein believed to be involved in IP production. Similar studies have been done using COS cells. In these studies, mutation of Asn^{87} to Asp resulted in loss of ligand binding, whereas mutation of Asp^{318} to Asn or the $Asp^{87}Asn^{318}$ double mutant were able to bind GnRH, but IP production was attenuated, similar to the results in GH_3 cells (148). However, in a similar study, also in COS cells, the $Asp^{87}Asn^{318}$ double mutant was unable to bind ligand, similar to the Asp^{87} mutant (149). The reason for the differences between these studies is not clear.

TABLE 2. Displacement of [^{125}I]buserelin binding to GGH_3 cells by GnRH analogs and other peptide and protein hormones

Agent	IC_{50} (nM) ± SEM
GnRH	18.2 ± 0.88
Lupron	0.8 ± 0.02
Lystide	0.2 ± 0.01
PRL	>1000
GH	>1000
TRH	>1000

Lupron, D-Leu^6-des-Gly^{10}-Pro^9-ethylamide-GnRH; Lystide, [Ac-D-Qal^1-D-Cpa^2-D3Pal^3-Ser^4-NicLys^6-Leu^7-ILys^8-Pro^9-D-Ala^{10}]NH_2.
[Adapted with permission from D. Kuphal *et al.*: *Endocrinology* 135: 315–320, 1994 (146) (Table 1) © The Endocrine Society.]

E. GnRHR regulation

The expression of the GnRHR in GGH_3 cells is driven by a cytomegalovirus promoter, which is not regulated by GnRH (144). The concentration of GnRHR is therefore unaffected at the transcriptional level by hormonal manipulation. This cell model may be useful, therefore, for distinguishing transcriptional from posttranscriptional regulation of cell surface GnRHR concentrations. Many G protein-coupled receptors are down-regulated by their ligands (123). Receptors are sequestered from the plasma membrane and internalized, followed by proteolytic degradation. This leads to a reduction in receptor number (homologous down-regulation) over a period of hours. In addition, the receptor may be down-regulated at the level of gene expression as well, leading to a reduction in receptor number over a period of several hours to days. Because GnRH will not affect the transcriptional rate of the GnRHR gene in GGH_3 cells, changes in cell surface GnRHR numbers would be expected to reflect translational or posttranslational regulation of the receptor by GnRH.

Indeed, the GnRHR in GGH_3 cells does undergo homologous down-regulation followed by recovery after continuous exposure to 10 nM GnRH, as determined by GnRH-binding studies (145). Down-regulation of the GnRHR was evident by 1 h of GnRH treatment, reached a nadir of 50–80% by 2–5 h, and returned to baseline levels by 7 h. This biphasic regulation of GnRHR is similar in time course and extent to that reported in primary pituitary cells (119). The ability of the receptor to be down-regulated in GGH_3 cells suggests that down-regulation does not require cell-specific components other than the receptor itself, does not involve transcriptional down-regulation (which may occur in primary gonadotropes, but over a longer time course), and does not require an intracellular C-terminal region. The mechanisms of this down-regulation of the GnRHR in GGH_3 cells remain to be elucidated. It will be interesting to determine whether the GnRHR is internalized and degraded or recycled after exposure to its ligand, as is the case for other receptors of this family, such as the TRHR (150, 151) and the β-adrenergic receptor (152).

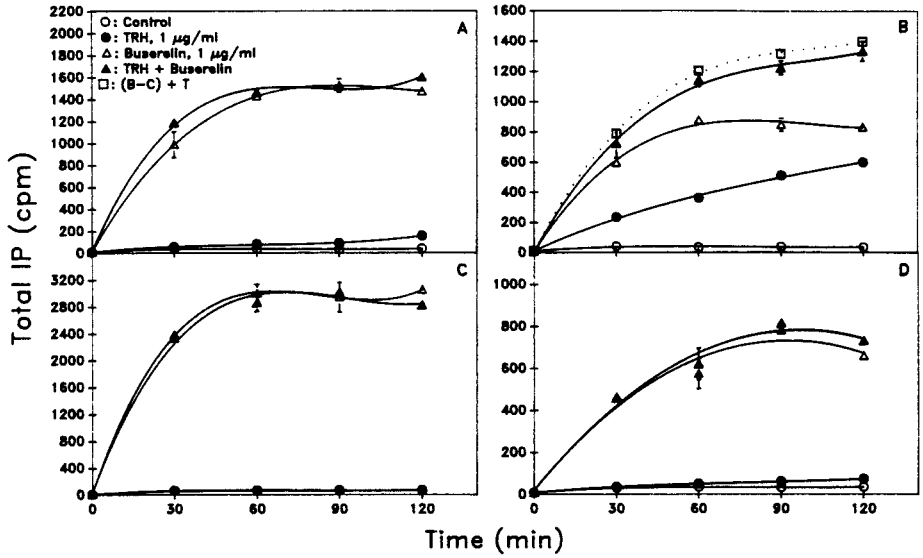

FIG. 12. Time course of IP production in untreated cells (○) or cells treated with 1 μg/ml TRH (●), 1 μg/ml of the GnRH agonist Buserelin (△), or both peptides (▲). GGH_3-1′ (A), GGH_3-2 (B), GGH_3-6 (C), or GGH_3-12 (D) cells (GGH_3 clonal cell lines) were plated and preincubated in [^{3}H]inositol. At the indicated times after the addition of peptides, total IPs were determined by ion exchange chromatography. (B − C) + T is the arithmetic result of the response measured in the presence of Buserelin (1 μg/ml) alone (B) less that in unstimulated cells (C) plus the response in the presence of TRH (1 μg/ml) alone (T). [Reprinted with permission from J. A. Janovick and P. M. Conn: *Endocrinology* 135:2214–2219, 1994 (162) (Fig. 1). © The Endocrine Society.]

F. Intracellular second messengers

1. G protein coupling. Direct studies of G protein coupling of the GnRHR in GGH_3 cells, as were done in αT3-1 cells, have not been reported. However, the TRHR, which is expressed endogenously by GH_3 cells, has been shown to be directly coupled to G proteins of the $G_{q/11}$ family (60, 153).

The βγ-subunits of a G protein can mediate signals as well as the α-subunit (154, 155). This confers to any G protein the potential for dual signaling. This has been best described for the G_i protein, *i.e.* the α-subunit inhibits adenylyl cyclase activity, whereas at higher concentrations the βγ-subunits can stimulate the activity of some adenylyl cyclase subtypes (156, 157). Similarly, G protein βγ-subunits have been shown to be capable of stimulating the β2-isoform of PLC (158). Recent data suggest that the βγ-complex of G proteins is involved in mediating GnRH effects. A 125-amino acid pleckstrin homology domain within the carboxyl terminus of β-adrenergic receptor kinase (βARK1-495/689) binds to $G_{\beta\gamma}$ (159). When transiently transfected and expressed in cell culture, this polypeptide has been shown to inhibit βγ-mediated signal transduction (160). Expression of this βARK1 fragment in GGH_3 cells inhibits basal and GnRH-stimulated IP production, cAMP release, and PRL release (161). The numbers and GnRH binding affinity of the GnRHR were unaffected by the expression of βARK1-495/689, indicating that the changes in signal transduction and PRL release are not due to a change in receptor expression or affinity. These data suggest that the βγ-complex of G proteins may play a role in mediating GnRH-stimulated signal transduction in GGH_3 cells.

2. Inositol phosphates. IP production was stimulated by a GnRH agonist (Buserelin) in GGH_3 clonal cell lines in a dose-dependent manner (145, 162). The EC_{50} was approximately 10^{-10} M GnRH. Production of IPs is an early response of GGH_3 cells to Buserelin, measurable at 15–30 min, maximal at 60 min, and maintained for at least 120 min after treatment (Fig. 12). GGH_3 cells also respond to TRH with an increase in IP production. Interestingly, the production of IPs by a GnRH agonist and by TRH were additive when maximal concentrations of both peptides were present. This suggests that GnRH and TRH may provoke IP production in GGH_3 cells by different means. This conclusion is supported by the finding that cholera toxin and pertussis toxin inhibit IP production in response to TRH, whereas they augment Buserelin-stimulated IP production. Alternatively, receptor number may be limiting the maximal IP production in response to either ligand. As is the case for αT3-1 cells, studies in GGH_3 cells have observed the responses to a single pulse of GnRH or to continuous GnRH; the responses to pulsatile administration of GnRH have not yet been described.

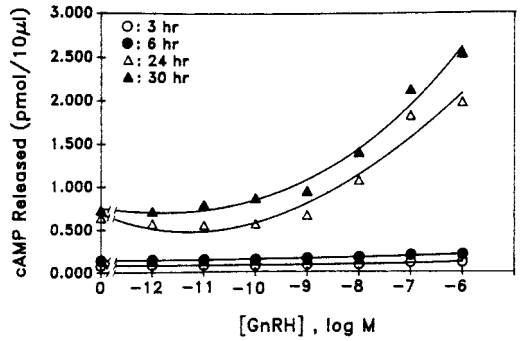

FIG. 13. cAMP production by GGH_3-1′ cells in response to GnRH. cAMP release from GGH_3-1′ cells was determined in response to GnRH for 3, 6, 24, or 30 h, as measured by RIA. [Reprinted with permission from D. Kuphal *et al*: *Endocrinology* 135:315–320, 1994 (146) (Fig. 7A). © The Endocrine Society.].

3. cAMP. Buserelin induced the release of cAMP in GGH_3 cells in a dose- and time-dependent manner (145, 146). Stimulation of cAMP production was not evident until 24 h after exposure to Buserelin (Fig. 13). The EC_{50} was approximately 10^{-8} M, about 100-fold higher than for IP generation. The stimulation of cAMP production by GnRH suggests that the GnRHR may be able to couple to G_s as well as G_q and is analogous to reports that GnRH can increase cAMP production in primary pituitary cells (76, 77).

Increased production of cAMP appears to mediate the desensitization of the PRL-secretory response to GnRH in GGH_3 cells. However, cAMP production in response to GnRH in GGH_3 cells persists even after the onset of desensitization. These data are consistent with a mechanism of desensitization that occurs distal to the production of cAMP (163).

G. Regulation of secretion

1. PRL. The TRHR and GnRHR are both members of the family of G protein-coupled receptors, and both are coupled to G proteins of the $G_{q/11}$ family (60, 153). The cellular responses to TRHR or GnRHR activation include enhanced phosphoinositide turnover, calcium mobilization, and PKC activation (24, 164, 165). Thus, the effects of TRH and GnRH appear to be mediated through the same or similar intracellular signal transduction pathways. Therefore, one might expect that GnRH would be able to stimulate PRL release in GGH_3 cells. Indeed, a GnRH agonist is able to stimulate PRL release from GGH_3 cells, whereas it does not in the parental GH_3 cells or in control cells, confirming that this response is mediated by the GnRHR, rather than by cross-activation of the TRHR by GnRH (145, 146, 162). The release of PRL in response to exposure of GGH_3 cells to a GnRH agonist was nearly linear to 96 h and was dose-dependent with an EC_{50} of approximately 10^{-8} M (Fig. 14). Cycloheximide inhibited the release of PRL in response to GnRH, indicating that this response is dependent on protein synthesis. This inhibition, and the slow time course for PRL release after GnRH stimulation, suggest that GnRH-stimulated PRL release is regulated at the level of PRL synthesis, rather than by release of stored hormone. The secretion of PRL in response to GnRH in these cells indicates that all of the components needed for coupling of the GnRHR to the activation of secretory events

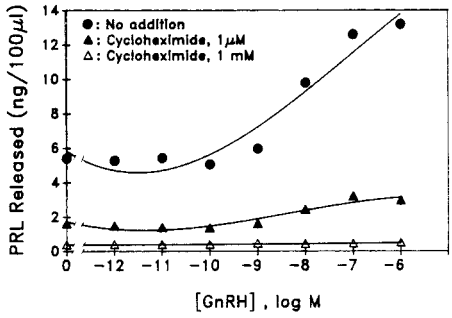

FIG. 14. Effect of the protein synthesis inhibitor cycloheximide on GnRH-stimulated PRL release from GGH_3 cells. Cells were treated with GnRH for 24 h in the presence or absence of cycloheximide. [Reprinted with permission from D. Kuphal *et al*: *Endocrinology* 135: 315–320, 1994 (146) (Fig. 8A). © The Endocrine Society.]

are present in the GGH_3 cell and suggests that no gonadotrope-specific components other than the secretory proteins themselves are necessary.

GnRH-stimulated PRL release in GGH_3 cells is potentiated by a phosphodiesterase inhibitor. This, coupled with the stimulation of cAMP production by GnRH and the stimulation of PRL release by 8-bromo-cAMP, suggests that cAMP may be a second messenger in GnRH-stimulated PRL release (146). Furthermore, GnRH-stimulated PRL release was sensitive to calcium channel inhibitors, suggesting a role for calcium as a second messenger as well (145).

The regulation of PRL by GnRH in GGH_3 cells could be interpreted to suggest that, at least in some instances, the hormonal regulation of gene expression in given pituitary cell subtypes is determined by the presence or absence of receptors for a particular hormone in that cell type, rather than or in addition to other tissue-specific intracellular factors or second messengers. A similar situation has, in fact, been observed *in vivo*. While LH and FSH are not normally regulated by TRH, in the case of gonadotrope adenomas secreting LH or FSH, their secretion is often stimulated by TRH (166, 167). Presumably, these adenomas express the TRHR, enabling a response to TRH, while normal gonadotropes do not.

2. LH and FSH (in GH_3 cells transfected with the α-, LHβ-, and FSHβ-subunit genes). GH_3 cells are able to secrete proteins through both constitutive and regulated pathways (168). To compare the mechanisms of storage and release of LH and FSH, expression vectors carrying the human LHβ or FSHβ gene in combination with the common gonadotropin α-subunit gene were transfected into GH_3 cells (169, 170). After transit and processing in the Golgi, the majority (>85%) of LH was retained intracellularly in Golgi/post-Golgi compartments, and decreased gradually with a $t_{1/2}$ of about 13 h. In contrast, FSH did not accumulate in GH_3 cells to the same extent as LH and was secreted with a $t_{1/2}$ of about 6 h. KCl or forskolin was able to stimulate LH secretion by 4- to 5-fold compared with unstimulated (basal) release, supporting the observation that LH enters a regulated secretory pathway in GH_3 cells and is stored in a secretagogue-releasable pool. FSH secretion was also stimulated by KCl or forskolin, but to a lesser extent (2.5-fold), possibly reflecting the smaller size of the stored, secretagogue-releasable pool. These studies indicate that in GH_3 cells, LH is secreted primarily through a regulated pathway, whereas the majority of FSH is released constitutively. These findings are consistent with observations in primary gonadotropes that FSH secretion is more tightly coupled to FSH biosynthesis, and that the magnitude of FSH secretion in response to secretagogues is smaller than that of LH (171–173). The effects of GnRH on LH and FSH secretion in this system, using cells in which the GnRHR is coexpressed, have not yet been studied.

3. Secretogranin-II. Secretogranin-II, an acidic glycoprotein that is a marker for the regulated pathway of secretion and a component of secretory granules, is synthesized in GGH_3 cells (174). It is released in a time- and dose-dependent manner in response to GnRH agonists, as well as to a cAMP analog. Release is inhibited by cycloheximide, an inhibitor of

protein synthesis, and by actinomycin-D, an RNA synthesis inhibitor. Interestingly, while secretogranin-II is generally thought to be a marker for the regulated pathway of secretion, it appears to be secreted in a constitutive manner in GGH_3 cells.

H. Regulation of PRL mRNA

In addition to stimulating PRL secretion, TRH also stimulates PRL gene transcription and mRNA levels, leading to an increase in PRL biosynthesis (175, 176). Analogously, PRL mRNA levels are stimulated in GGH_3 cells by GnRH (144). Treatment of GGH_3 cells with 100 nM of a GnRH agonist for 24 h resulted in the stimulation of PRL mRNA levels by 3- to 4-fold, whereas PRL mRNA levels were unchanged in response to the GnRH agonist in the parental GH_3 cells or in control cells. In the same cell line, TRH stimulated PRL mRNA levels by approximately 3-fold.

I. Regulation of expression of transiently expressed reporter genes

1. PRL vs. α-subunit gene. TRH stimulates PRL mRNA levels in GH_3 cells by increasing gene transcription, as determined by nuclear run-off assays (176). Time course experiments indicate that transcriptional activation is maximal 2 h after treatment with TRH. We therefore hypothesized that TRH and GnRH would stimulate the activity of a fusion gene comprised of the PRL promoter fused to a luciferase reporter gene (PRLLUC) in GGH_3 cells. Indeed, when PRLLUC was transiently transfected into GGH_3 cells, luciferase activity was stimulated by treatment with either TRH or a GnRH agonist in a dose- and time-dependent manner (144). In the parental GH_3 cells and in the control cell line, $pcGH_3$-1 (stably transfected with the pcDNA1 vector), luciferase activity was stimulated by TRH, but not by the GnRH agonist (Fig. 15). Time course studies using maximal doses of TRH and a GnRH agonist showed an increase in luciferase activity in response to both TRH and GnRH agonist by 2 h; maximal stimulation occurred at 4–6 h in both cases. The difference in the time courses for TRH response in nuclear run-off experiments and luciferase activities likely reflects the time needed for translation and accumulation of the luciferase enzyme in the cells after transcriptional activation. When a human αLUC was transiently transfected into GGH_3 cells, luciferase activity was also stimulated by treatment with either TRH or a GnRH agonist, whereas $pcGH_3$-1 cells and GH_3 cells responded only to TRH (144). Dose-response and time course studies for αLUC were similar to those for PRLLUC.

The stimulatory effects of TRH and the GnRH agonist, each at maximally effective doses, were additive on PRL mRNA levels and PRLLUC activity. In contrast, stimulation of αLUC by both TRH and GnRH agonist was no greater than with either one alone (144). If TRH and GnRH share the identical signal transduction pathway for the stimulation of gene expression, it would be expected that the induction of gene expression by maximally effective concentrations of one hormone would prevent any further stimulation by the other, as is the case for the α-subunit gene. Therefore, the

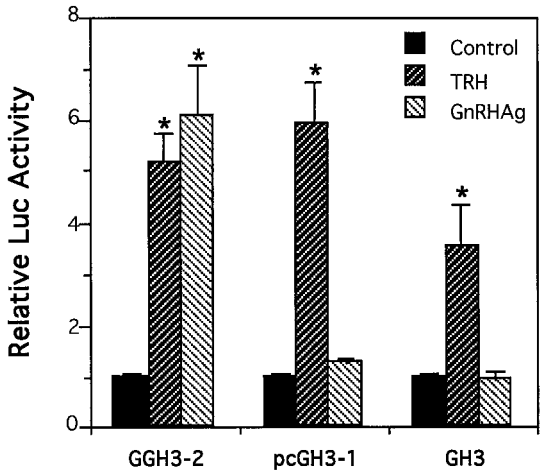

FIG. 15. Stimulation of luciferase activity by TRH and GnRHAg in GGH_3-2 cells, $pcGH_3$-1 cells, and GH_3 cells transiently transfected with PRLLuc. All cells were harvested 48 h after transfection. Cells were treated with 1 μM TRH or 100 nM GnRH agonist for the final 6 h before harvesting. Levels of luciferase activity are internally standardized according to levels of activity of RSV-β-galactosidase. Each *bar* represents the mean ± SEM for four samples. *, $P < 0.01$, significantly different from controls. [Reprinted with permission from U. B. Kaiser *et al*: *Mol Endocrinol* 8:1038–1048, 1994 (144) (Fig. 3A). © The Endocrine Society.]

signal transduction pathways used by TRH and GnRH in the regulation of PRL gene expression in GGH_3 cells may have some differences, particularly in the response-limiting step(s). These results also imply that the regulation of α-subunit and PRL gene expression by TRH and GnRH may not occur by identical pathways. The additivity of TRH and GnRH effects on PRL gene expression could also be accounted for if receptor number was the limiting factor in the pathway for either ligand. However, if this were the case, one might expect TRH and GnRH to be additive on α-subunit gene expression as well.

In time course studies, luciferase activity declined by 18–24 h of exposure to TRH or GnRH, similar to studies of αLUC in αT3-1 cells, suggesting that the signal transduction pathways became desensitized. To study this further, GGH_3 cells were pretreated with either TRH or a GnRH agonist, and then the responses of PRLLUC and αLUC to TRH and the GnRH agonist were tested. These studies show that homologous desensitization occurs, *i.e.* pretreatment with the GnRH agonist blocks subsequent stimulation of PRLLUC and αLUC activity by the agonist, and pretreatment with TRH blocks subsequent stimulation of PRLLUC and αLUC activity by TRH. In the case of the α-subunit promoter, heterologous desensitization also occurs. Stimulation of αLUC activity by TRH was blunted by pretreatment with a GnRH agonist, and conversely, there was loss of responsiveness to GnRH agonist after pretreatment with TRH. In contrast, heterologous desensitization of the PRL promoter did not occur (144). These data provide additional support that TRH and GnRH stimulate the α-subunit promoter by the same mechanism, whereas the mechanisms of transcriptional regulation of PRL by TRH and GnRH are not identical. It would be interesting to know whether similar effects would be ob-

served in αT3-1 cells transfected with the TRHR; however, these studies have not been performed.

2. α-, LHβ-, and FSHβ-subunit genes. The gonadotropin subunit genes, α, LHβ, and FSHβ, are not expressed endogenously in the somatolactotropic GH_3 cell line. As previously reported and discussed above, GH_3 cells are capable of supporting expression of transiently transfected luciferase reporter constructs driven by the rat, mouse, or human α-subunit promoter (133, 138). Furthermore, in GGH_3 cells, the α-subunit is stimulated in response to GnRH. Similarly, studies have shown that the TSHβ-subunit gene promoter, normally active in thyrotropes, can be expressed in GH_3 cells (134, 135). The rat LHβ gene promoter, fused to a chloramphenicol acetyltransferase (CAT) gene, was expressed at low levels when transiently transfected into GH_3 cells (177). Transcription was initiated correctly at the same LHβ transcriptional start site used in primary pituitary gonadotropes, as determined by RNase analysis. CAT activity was consistently induced by forskolin and by cAMP analogs, suggesting the presence of a cAMP-responsive *cis*-acting domain in the LHβ 5′-flanking region.

GH_3 cells transiently transfected with reporter genes in which regulatory regions of the human α-subunit, rat LHβ, and rat FSHβ genes are fused to the luciferase gene (αLUC, LHβLUC, and FSHβLUC, respectively) yield expression levels 250-fold higher than those of the promoterless luciferase vector for αLUC, 5-fold higher for LHβLUC, and 12-fold higher for FSHβLUC (178). Cotransfection of the GnRHR cDNA results in the additional specific stimulation of luciferase activity by a GnRH agonist, 10-fold for αLUC, 8-fold for LHβLUC, and 4-fold for FSHβLUC. TRH is also able to stimulate the expression of the gonadotropin subunit gene reporter constructs in these transfected cells, although to a lesser degree than the GnRH agonist (Fig. 16).

The magnitude of stimulation of gonadotropin subunit promoter activity in response to GnRH in these cells is influenced by the concentration of GnRHR expressed. When GH_3 cells are transiently transfected with progressively increasing amounts of GnRHR cDNA, the average number of GnRHRs expressed on the cell surface, as determined by a GnRH binding assay, also increases. When these cells are cotransfected with αLUC or LHβLUC and progressively increasing amounts of GnRHR cDNA, the degree of stimulation of luciferase activity by GnRH is increased in proportion to the numbers of GnRHR. In contrast, FSHβLUC activity is optimally stimulated when relatively low numbers of GnRHR are expressed; at higher concentrations of cell surface GnRHR, the magnitude of stimulation of FSHβLUC by GnRH is decreased (178).

Observations *in vivo* indicate that cell surface GnRHR numbers are regulated by varying GnRH pulse frequencies (47, 49, 179). The highest concentrations of cell surface GnRHR, as reflected by GnRH-binding activity, occur at a GnRH pulse frequency of every 30 min, which has also been shown to stimulate preferentially LH biosynthesis and secretion *in vivo* (180–184). Lower cell surface GnRHR numbers occur at a GnRH pulse frequency of every 2 h, which, in turn, has been shown to stimulate preferentially FSH biosynthesis and secretion. GnRHR concentration in primary pituitary cells is approximately 2- to 3-fold higher when the GnRH pulse frequency is every 30 min, compared with that at a frequency of every 2 h (179), a magnitude of change in GnRHR concentration similar to that in the GGH_3 cells which resulted in the maximal differences in LHβ and FSHβ gene expression. These observations support the hypothesis that varying GnRH pulse frequencies regulate differentially LH and FSH biosynthesis and secretion *in vivo* by regulating pituitary GnRHR numbers (Fig. 17). These data suggest that

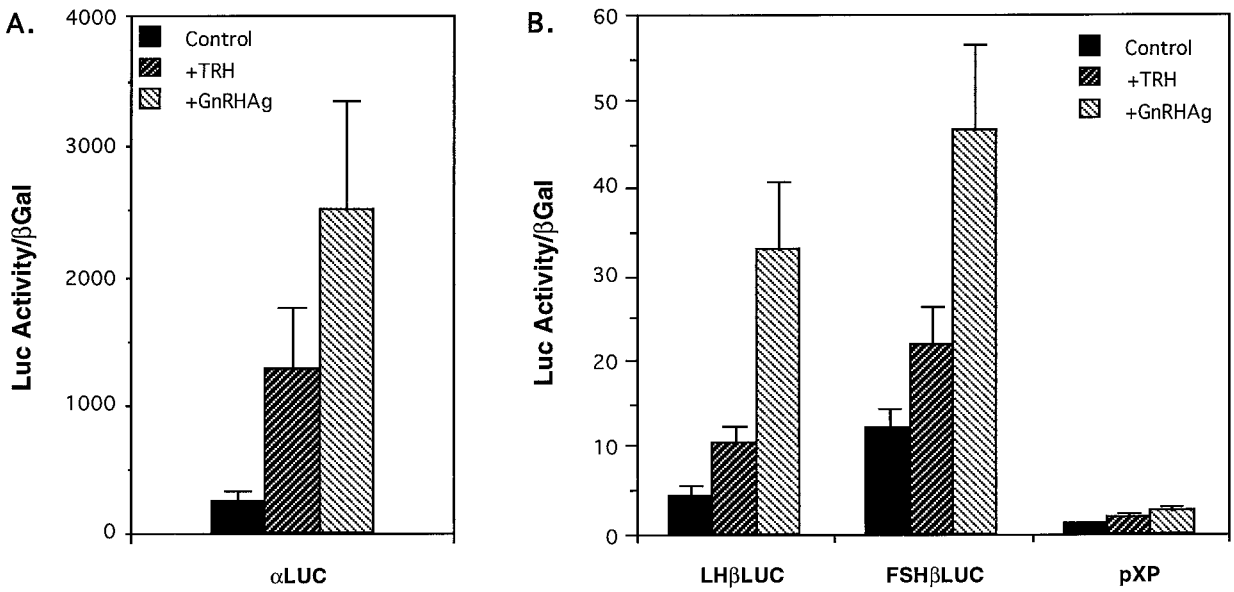

FIG. 16. Basal, TRH-, and GnRH-stimulated expression of (A) αLUC and (B) LHβLUC, FSHβLUC, and pXP2 in GH_3 cells. Cells were cotransfected with 20 μg αLUC, LHβLUC, FSHβLUC, or pXP2, 20 μg pcDNA1-GnRHR, and 9 μg RSV-βGAL. Cells were treated with 100 nM GnRHAg (GnRH agonist), 1 μM TRH, or control for 6 h before harvesting. All experiments were repeated at least three times. Each *bar* represents the mean ± SEM for nine samples, from three independent experiments. [Reprinted with permission from U. B. Kaiser *et al*: *Proc Natl Acad Sci USA* 92:12280–12284, 1995 (178) (Fig. 1).]

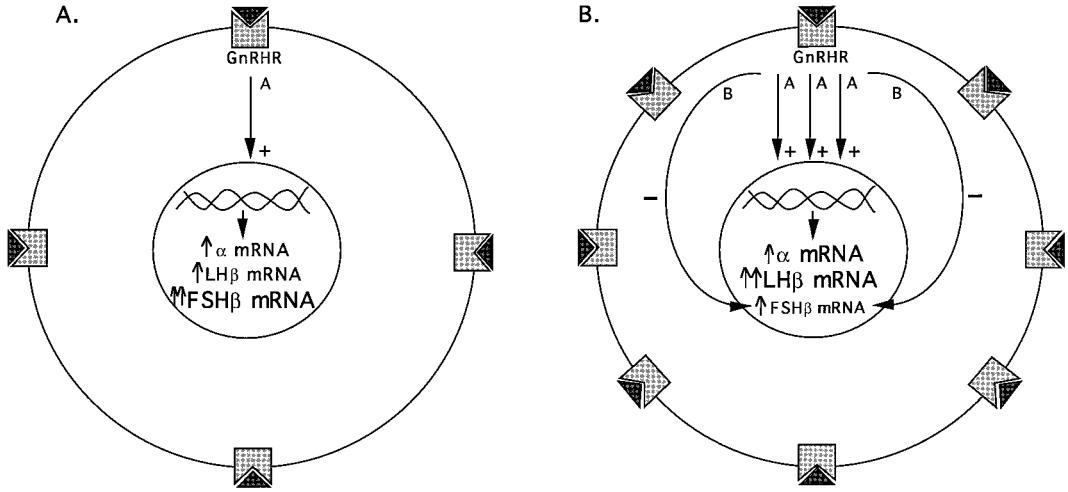

FIG. 17. Model of the mechanism of differential regulation of the gonadotropin subunit genes by GnRH at (A) low GnRH pulse frequencies, and (B) high GnRH pulse frequencies. A, At low GnRH pulse frequencies, every 2 h, GnRHR concentrations on the gonadotrope cell surfaces are relatively low. When GnRH binds to its receptors on gonadotropes, a signal transduction pathway, pathway A, is activated, resulting in the stimulation of the expression of all three of the gonadotropin subunit genes, α, LHβ, and FSHβ. B, At higher GnRH pulse frequencies, every 30 min, GnRHR concentrations on the gonadotrope cell surfaces are higher. When GnRH binds to the now greater receptor numbers on the gonadotropes, signal transduction pathway A is activated to an even greater extent, resulting in the greater stimulation of the α- and LHβ-subunit genes. In addition, a second signal transduction pathway, pathway B, is now also activated. Activation of pathway B results in the specific inhibition of the expression of the FSHβ gene, with no effects on the α and LHβ genes. The net effect is that α and LHβ gene expression is maximally stimulated at relatively high GnRH pulse frequencies, whereas FSHβ gene expression is optimally stimulated at lower GnRH pulse frequencies. [Reprinted with permission from U. B. Kaiser *et al*: *Proc Natl Acad Sci USA* 92:12280–12284, 1995 (178) (Fig. 4).]

the mechanisms by which GnRH regulates α and LHβ gene expression are distinct from those by which FSHβ gene expression is regulated. Furthermore, the signal transduction pathways activated by GnRH may be different at low *vs.* high GnRH receptor numbers. The details of the different intracellular signaling pathways activated by GnRH at low *vs.* high GnRHR numbers remain to be elucidated. It is possible that the GnRHR may couple to different G proteins at low *vs.* high cellular receptor numbers or that different signaling pathways are activated by the α- and $\beta\gamma$-subunits of a single G protein.

J. Summary of GnRH action in GGH_3 cells

GGH_3 cells have provided another cell model system for the study of the GnRHR and GnRH action. These cells bind GnRH and GnRH analogs specifically and with similar affinities to primary gonadotropes. Because they express both the GnRHR and the TRHR, they provide an opportunity to compare directly the signal transduction pathways and mechanisms of action of TRH and GnRH. GnRHR levels are modulated by GnRH treatment, suggesting that GnRH regulates its receptor at the posttranscriptional level. Unlike αT3-1 cells, GnRH appears to increase cAMP production as well as stimulating IP production and intracellular calcium levels in GGH_3 cells. The GnRHR may be coupled to more than one G protein in GGH_3 cells; additionally, there is evidence to suggest a role for G$\beta\gamma$-subunits in mediating GnRH responses as well. GnRH is able to stimulate gene expression as well as hormone release in GGH_3 cells.

GH_3 cells have both advantages and disadvantages for the study of GnRH action. Because the parental GH_3 cell line does not express the GnRHR gene, these cells are useful for structure-function studies of the GnRHR. Wild type and mutant receptors can be transfected into GH_3 cells and their function studied without interference from endogenously expressed receptors. Such studies to date have been largely done in nonpituitary cell lines, such as COS cells (148, 149). However, unlike the GGH_3 cells, COS cells do not support gonadotropin subunit promoter activity, so that domains of the GnRHR important for mediating signals for transcriptional stimulation cannot be defined. The transfected GnRHR is driven by a heterologous viral promoter, which is not regulated by GnRH. This means that GGH_3 cells cannot be used for studies of GnRHR gene expression. However, it allows effects of GnRHR concentration on GnRH action and effects of GnRH on GnRHR desensitization to be studied independently of regulatory effects at the level of gene expression. Finally, GH_3 cells support the expression of transfected reporter genes driven by regulatory regions of the LHβ- or FSHβ-subunit genes, making them the first homogeneous, immortalized cell population available for the study of gonadotropin β-subunit gene expression and regulation by GnRH.

Nonetheless, an important caveat to studies performed in GGH_3 cells is that this is a heterologous cell expression system, and the signal transduction pathways used in these cells may differ from those used in primary gonadotropes as well as in thyrotropes. It must be remembered that GH_3 cells, while they are pituitary in origin, are derived from somatolactotropes rather than from gonadotropes, and do not express the gonadotropin subunit genes endogenously. They may, therefore, lack gonadotrope-specific factors that may be important for cell-specific and/or regulated expression of gonadotropin subunit genes. Observations made using such a cell model should be confirmed in primary pituitary cells

or *in vivo* before physiological relevance is assigned to such observations.

V. Studies of GnRH Action in Other Pituitary Cell Lines

A. RC-4B/C cells

This anterior pituitary cell line was established from a pituitary adenoma that developed spontaneously in a 3-yr-old male rat (185). These cells have the ultrastructural appearance of well differentiated anterior pituitary cells. Immunocytochemical studies showed the presence of all known anterior pituitary secretory cell types, including gonadotropes. However, the proportion of different cell types was different from that observed in primary rat pituitaries. In particular, the percentage of LHβ cells was higher than in the normal male rat pituitary, and the percentage of GH cells was lower. The percentage of FSHβ-, PRL-, ACTH-, and TSHβ-staining cells was comparable to the normal male rat pituitary. GnRH receptors were also shown to be present, with the same binding affinity for a GnRH agonist, Buserelin, as in the pituitary gland, but with 2-fold lower capacity. Interestingly, dual-staining studies revealed that many of the cells are bihormonal, producing FSHβ and PRL or LHβ and PRL (186). This cell line thus represents an additional cell model for the study of the GnRHR and GnRH action, and for gonadotrope-specific expression of the FSH and LH subunit genes. This cell model has an advantage over αT3-1 cells and GGH_3 cells in that the FSHβ- and LHβ-subunit genes are expressed endogenously. However, a disadvantage of the RC-4B/C cell line is that the cells are a heterogeneous population, so only a fraction of the cells express the gonadotropin subunit genes. Furthermore, it has not yet been shown that the gonadotropins are synthesized and secreted in a regulated manner analogous to that which occurs *in vivo*. Keri *et al.* (187) were unable to obtain activity greater than that of a promoterless reporter for a fusion gene in which the bovine LHβ promoter was fused upstream of the CAT gene. Activity remained low even after the upstream addition of a Rous sarcoma virus enhancer. Also, surprisingly, although the proportion of cells staining for LHβ and/or FSHβ are higher than in normal pituitary, the FSH and LH content of RC-4B/C cells was 70- and 800-fold lower, respectively, than that of the normal male rat pituitary gland. Thus, the validity of this cell line as a physiological model has yet to be determined.

B. LβT2 cells

Recently, targeted expression of the SV40 T antigen with the rat LHβ-subunit gene regulatory region was used to generate transgenic mice. An immortalized cell line (LβT2 cells) was derived from a tumor generated in a LHβ-Tag mouse, in a manner analogous to the preparation of the αT3-1 cells (188). These cells express both the α- and β-subunits of LH as well as GnRHR, estrogen receptors, and estrogen-inducible progesterone receptors. However, the FSHβ-subunit is not expressed. LβT2 cells probably arose later in ontogeny than αT3-1 cells and thus represent a more mature gonadotrope precursor than do the αT3-1 cells.

LβT2 cells have been used to study the effects of GnRH on intracellular Ca^{2+} concentrations. Cells stimulated with 1 μM GnRH responded with an increase in intracellular Ca levels and also had a secretory response, as measured by changes in plasma membrane capacitance (189). Furthermore, LβT2 cells responded to a 15-min pulse of 1–100 nM GnRH with a dose-dependent increase in LH secretion (188, 190). Exposure of the cells to four, 15-min, 10 nM GnRH pulses every 90 min for 4 days led to an increase in LH secretion in response to the initial GnRH pulse on each succeeding day, independent of cell number. The stimulation of LH secretion by GnRH was enhanced by steroids: the LH-secretory response to GnRH by day 4 was 4-fold in the absence of added steroids, 7-fold in the presence of 0.2 nM estrogen (E_2), 14-fold in the presence of 20 nM dexamethasone, and 15-fold in the presence of both E_2 and dexamethasone. These changes in responsiveness to GnRH appeared to be due in part to changes in GnRHR number and in part to changes in the LH synthesis/secretory pathway independent of changes in the GnRHR.

Studies of $[Ca^{2+}]_i$ and exocytosis in individual LβT2 cells have been performed using the whole-cell perforated patch clamp technique to measure plasma membrane capacitance (191). These studies show that GnRH evokes dose-dependent increases in $[Ca^{2+}]_i$ and secretion. The $[Ca^{2+}]_i$ responses to GnRH are biphasic, as in αT3-1 cells and primary gonadotropes. However, the $[Ca^{2+}]_i$ oscillations observed in primary gonadotropes at low GnRH concentrations are not observed in the LβT2 cells. The extent of the changes in $[Ca^{2+}]_i$ and exocytosis in response to GnRH were dependent on the steroid hormone background. E_2 and dexamethasone caused an increase in the peak $[Ca^{2+}]_i$ stimulated by GnRH as well as a shift toward increased sensitivity of the Ca^{2+} dependency of exocytosis. The increased $[Ca^{2+}]_i$ response may be due to an increase in GnRHR numbers (see below). The increase in GnRH-induced secretion may be due to both an increase in $[Ca^{2+}]_i$ and an increase in the sensitivity of the secretory apparatus to $[Ca^{2+}]_i$, which may, in turn, be due to effects of other second messenger pathways activated by GnRH and/or steroids.

Studies of mRNA levels indicate that LβT2 cells respond to pulsatile GnRH administration with an increase in GnRHR mRNA levels (190). However, this increase is only approximately 2-fold, compared with the much greater increase observed in primary pituitary cells (49). This difference may be due to differences in the experimental paradigm of pulsatile GnRH delivery. GnRHR mRNA levels are also increased in LβT2 cells by estradiol and dexamethasone. LHβ mRNA levels are markedly induced by pulsatile GnRH in LβT2 cells, with no effect of steroids, and α-subunit mRNA levels are unaffected by either steroids or GnRH.

The LβT2 cell line thus exhibits some characteristics consistent with those of pituitary gonadotropes, including the expression of the α, LHβ, and GnRHR genes, the biphasic stimulation of $[Ca^{2+}]_i$ by GnRH, the secretion of LH via a regulated pathway, and the regulation of LHβ and GnRHR mRNA levels in response to GnRH and steroid hormones. These cells may be useful for studying the molecular and

cellular mechanisms involved in the regulation of LH subunit gene expression and LH secretion.

VI. Future Directions

The recent availability of immortalized cell models of gonadotrope function have allowed considerable advances in our understanding of the structure and function of the GnRHR, the signal transduction pathways activated by GnRH, and molecular mechanisms of action of GnRH in terms of regulation of gene expression, hormone biosynthesis, and hormone secretion, as well as of homologous regulation of the GnRHR at both transcriptional and posttranscriptional levels. In addition, these cell models have proven useful for the study of cell-specific and regulated expression of the gonadotropin α-subunit gene in particular, and to some extent the gonadotropin β-subunit genes as well. Nonetheless, further studies to clarify the molecular mechanisms by which GnRH regulates LH and FSH subunit gene expression, hormone biosynthesis, and secretion are needed. In addition, the mechanisms of the unique responses of gonadotropes to varying GnRH pulse frequencies and amplitudes are not well understood. For these studies, we eagerly await the development and availability of a novel, immortalized gonadotrope cell line that expresses the LHβ and FSHβ-subunit genes as well as the α-subunit and the GnRHR and that has the unique responses to different modes of administration of GnRH in a manner reflecting that which occurs in primary pituitary gonadotropes.

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