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GnRH RECEPTORS AND ACTIONS IN THE CONTROL OF REPRODUCTIVE FUNCTION

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Summary—The hypothalamic control of reproductive function is expressed through the receptor-mediated actions of GnRH on the pituitary gonadotroph. GnRH receptors in the pituitary gland exhibit prominent variations in number during the ovarian cycle and after changes in steroid feedback, and are modulated by the rate of GnRH secretion from the hypothalamus. In cultured pituitary cells, GnRH receptors undergo down-regulation during exposure to GnRH agonists, followed by a subsequent elevation of sites that is dependent on protein synthesis. GnRH antagonists do not cause receptor down-regulation, but high-affinity antagonist analogs bind for extended periods to cause receptor occlusion and prolonged inhibition of GnRH action. Analysis of the rat pituitary GnRH receptor by photoaffinity labeling reveals two binding subunits of mol. wt 53,000 and 42,000. The receptor-activated processes leading to gonadotropin secretion are highly calcium-dependent, and are initiated by rapid phospholipid hydrolysis with production of arachidonic acid metabolites, diacylglycerol, and inositol phosphates. The role of protein kinase C in gonadotropin secretion is indicated by the ability of phorbol esters and synthetic diacylglycerols to stimulate LH release, the inhibition of protein kinase C and LH release by retinal, and the redistribution of protein kinase C between cytosol and membrane fractions during stimulation of pituitary gonadotrophs by GnRH. It is likely that the effects of arachidonate metabolites are integrated with those of calcium-calmodulin and calcium, phospholipid-dependent protein kinases during the immediate and sustained phases of GnRH-induced gonadotropin secretion.

INTRODUCTION

The receptor-mediated mechanisms by which peptide hormones such as GnRH regulate the functions of their target-cells in the pituitary gland are common to those of many other calcium-dependent ligands. The concept that highly specific cell-surface receptor sites are responsible for the ability of peptide hormones to activate plasma-membrane "second message" systems, based on adenylate cyclase and calcium-calmodulin regulated processes, has been extended to include several other newly-defined effector systems. These include additional mechanisms that involve guanyl nucleotide coupling proteins [1], modulation of calcium channels [2], rapid changes in phospholipid metabolism and calcium mobilization [3], and the participation of recently discovered enzymes such as protein kinase C [4]. Several or all of these mechanisms may participate in the cellular responses to hormonal activation in endocrine-regulated target tissues. It is now recognized that most cells possess a variety of ligand-activated effector mechanisms through which specific target-cell responses can be elicited by locally-produced or systemic hormones. It is also apparent that the biochemical processes involved in hormonal regulation include a broad range of enzymatic reactions that lead to the formation of evanescent messenger molecules. The control of pituitary function by hypothalamic neuropeptides, and the regulation of gonadotropin secretion and actions in the testis and ovary, are mediated by mechanisms that involve a considerable degree of diversity among the pathways of hormonal activation within the individual target cells.

The actions of hypothalamic hormones on the pituitary gland involve both cyclic AMP and calcium-dependent processes, of which one or both mechanisms may be involved in the individual cell-types. The ability to utilize more than one activation pathway is clearly appropriate to cells which respond to multiple regulatory ligands, but it is not clear why certain peptide hormones act predominantly through one or other of the two major response pathways. In the case of GnRH, it is well established that calcium and phospholipid-dependent mechanisms are largely responsible for the immediate mobilization and release of stored gonadotropins [5–7]. The actions of GnRH upon its pituitary receptors and release of LH have been extensively analyzed in cultured rat pituitary cells, of which about 12% are gonadotrophs. Studies on GnRH-induced changes in potential intracellular mediators that are not unique to the gonadotroph are facilitated by the use of methods such as centrifugal elutriation [8] to prepare gonadotroph-enriched cell fractions. By this procedure, large members of dispersed pituitary cells can be resolved into fractions that are enriched in the individual cell types (Fig. 1), with an increase of about 5-fold in the concentration of gonadotrophs in the later-eluting fractions. Such cell preparations permit more precise definition of the biochemical mechanisms through

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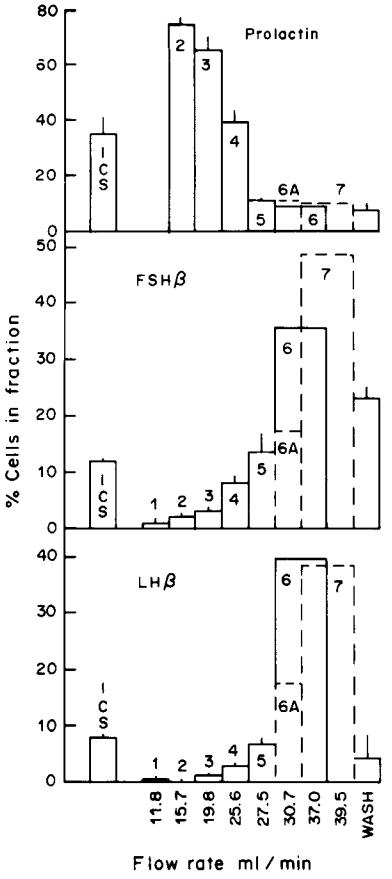

Fig. 1. Fractionation of trypsin-dispersed pituitary cells by centrifugal elutriation [8]. By this method, the proportion of gonadotrophs in the large-cell fractions eluted at higher flow rates can be increased by about 5-fold over that present in the original cell dispersion.

which the hypothalamus exerts its control of pituitary–gonadal function. Studies in enriched gonadotrophs are also essential to clarify the manner in which the calcium and phospholipid-mediated activation pathways in the gonadotrophs are integrated during the regulation of gonadotropin secretion by GnRH.

GnRH RECEPTORS: REGULATION BY AGONIST AND ANTAGONIST ANALOGS

The regulation of specific plasma-membrane receptors by changes in the ambient concentration of the homologous hormone is a common and notable feature of peptide hormone action. Such ligand-mediated changes in receptor number can lead to altered target-cell sensitivity or responsiveness to subsequent hormonal stimulation. GnRH receptor regulation may be particularly relevant to the control of reproductive function, since the secretion of biologically appropriate gonadotropin profiles is determined by the phasic and pulsatile secretion of GnRH from the hypothalamus, acting via specific receptor sites in the pituitary gonadotrophs. Such GnRH receptors, which have been identified and assayed by binding studies with radioiodinated GnRH superagonist analogs (GnRHa), are now known to undergo marked changes in the pituitary gland according to the state of reproductive function [9]. During the rat estrous cycle, there is a 2-fold increase in pituitary GnRH receptors at proestrus, followed by a fall around the time of the LH surge. Conversely, GnRH receptors are decreased during lactation, when gonadotropin secretion is reduced due to decreased release to GnRH from the hypothalamus, and after prolactin treatment. These changes, with the enhancing effect of castration on pituitary receptors and its prevention by maneuvers that block GnRH secretion or action, have indicated that pituitary GnRH receptors are regulated by the prevailing rate of GnRH secretion [10].

The ability of GnRH receptors to undergo both up- and down-regulation could modulate pituitary function and responsiveness in various reproductive states. In particular, the down-regulatory mechanism could be relevant to the anti-fertility effect of long-term treatment with GnRH agonists. When the *in vitro* effects of GnRH and its analogs were analyzed by addition to cultured rat pituitary cells for a period of 6 h, there was a significant increase in GnRH receptors at low concentration of GnRH agonists, and a decrease at higher concentrations [11]. These concentration-dependent effects of GnRH on its binding sites may be responsible for the changes in GnRH receptors that occur during the estrous cycle. In contrast, GnRH antagonists do not cause down-regulation of GnRH receptors, and prevent the regulatory effect of the agonist ligands on both LH secretion and GnRH receptors (Fig. 2). When the kinetics of action of GnRH upon its receptors were examined by incubation with pituitary cells for up to 10 h, there was an initial loss of sites during the first 1–2 h, followed by a return to the control level and then by an increase in receptors. The early phase of receptor loss or down-regulation was dependent upon activation of receptor sites by the hormonal ligand,

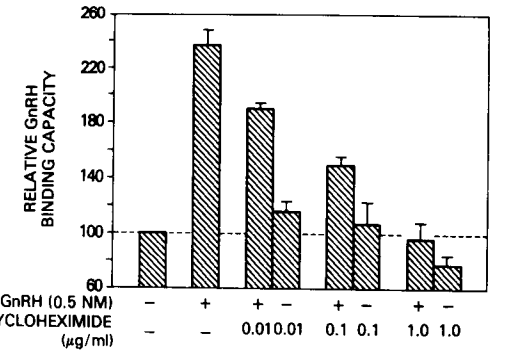

Fig. 2. *In vitro* regulation of GnRH receptors in cultured rat pituitary cells incubated with 10^{-10} M GnRH for 10 h. The increase in GnRH receptors is inhibited by addition of increasing concentrations of the antagonist analog, [D-Phe^2, D-Pro^3-D-Phe^6]GnRH, which alone has no effect on GnRH receptors.

and was not observed after cell stimulation by a depolarizing concentration of potassium. Also, there was no loss of sites after exposure to low affinity GnRH antagonists that effectively block GnRH receptors when present at sufficiently high concentrations, but do not cause occlusion of sites by residual occupancy during the subsequent radioligand-receptor assay. The competition by such an antagonist, [D-Phe^2, D-Pro^3, D-Phe^6]GnRH, for binding to particulate pituitary receptors, and its inhibitory actions on LH release and GnRH receptor regulation in cultured pituitary cells, are shown in Fig. 3. In cultured gonadotrophs, phorbol esters [13] and cyclic AMP analogs [14] also influence the concentration of GnRH receptors, suggesting that a phosphorylation event could be involved in the regulation of receptor turnover in the gonadotroph. The delayed return and up-regulation of GnRH receptors following their initial phase of depletion is prevented by inhibitors of RNA and protein synthesis [12], indicating that new protein synthesis is required for the replenishment of receptors or other proteins that are involved in the recovery process (Fig. 4).

The early loss of GnRH receptors after exposure to agonists results from internalization of the GnRH–receptor complexes, as shown by morphological analysis of the localization and turnover of GnRH receptors. Such studies have included the use of fluorescent and radioiodinated GnRH analogs as probes to follow the binding and disposition of the ligand–receptor complex. In cultured rat pituitary cells, rhodamine-labeled [D-Lys^6]GnRH showed progressive clustering and clumping from 10 to 20 min, consistent with processing and uptake of the receptor-bound ligand [15]. Further, electron microscopy–autoradiographic studies showed initial localization of ^{125}I-labeled GnRH agonists at the gonadotroph plasma membrane, followed by their rapid appearance within the cell in association with endocytic vesicles and other organelles [16]. Internalization of GnRH agonists occurred via receptor-mediated endocytosis, as in many other ligand-stimulated cells. After binding to specific receptors, which are often located on microvilli, GnRH agonists were rapidly taken up through an endocytic mechanism which may be followed by either degradation of the hormone–receptor complex or recycling of binding sites to the plasma membrane. In the gonadotroph, much of the internalized GnRH was delivered to lysosomes, and there is probably little or no recycling of receptors to the cell surface. In contrast to the rapid internalization of GnRH agonists, a potent GnRH antagonist analog remained bound at the cell surface for a much longer period (Fig. 5), a finding consistent with the absence of receptor regulation in cells exposed to GnRH antagonists [11, 12]. The effects of such prolonged binding of the high-affinity antagonist to cultured pituitary cells, with continued occlusion of GnRH receptors, is in contrast to the eventual recovery of sites after exposure to GnRH agonists, and is illustrated in Fig. 6.

The marked difference in rates of internalization of GnRH agonist and antagonist derivatives was also evident in studies with gold-conjugated analogs, which showed rapid incorporation of agonist-coated particles into multi-vesicular bodies and secondary

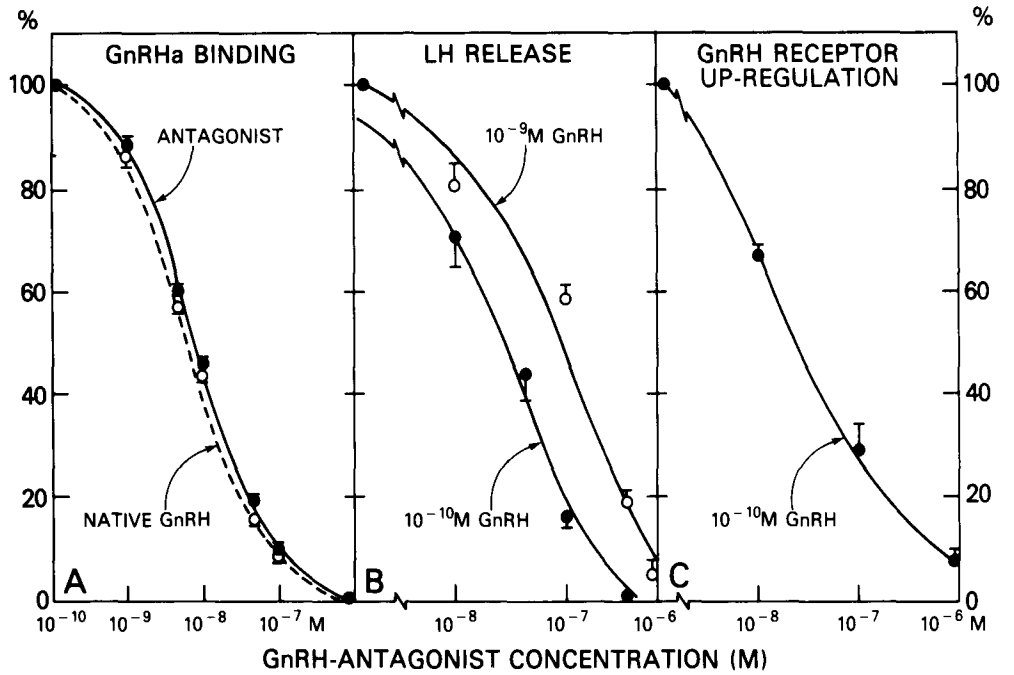

Fig. 3. Inhibitory effects of a GnRH antagonist on GnRH receptor binding, LH release and GnRH receptor regulation. The binding affinity of the [D-Phe^2, D-Pro^3, D-Phe^6]GnRH antagonist is similar to that of native GnRh (1.5×10^8 M^{-1}), and the analog causes dose-dependent blockade of GnRH-induced LH release as well as of the up-regulation of GnRH receptor sites.

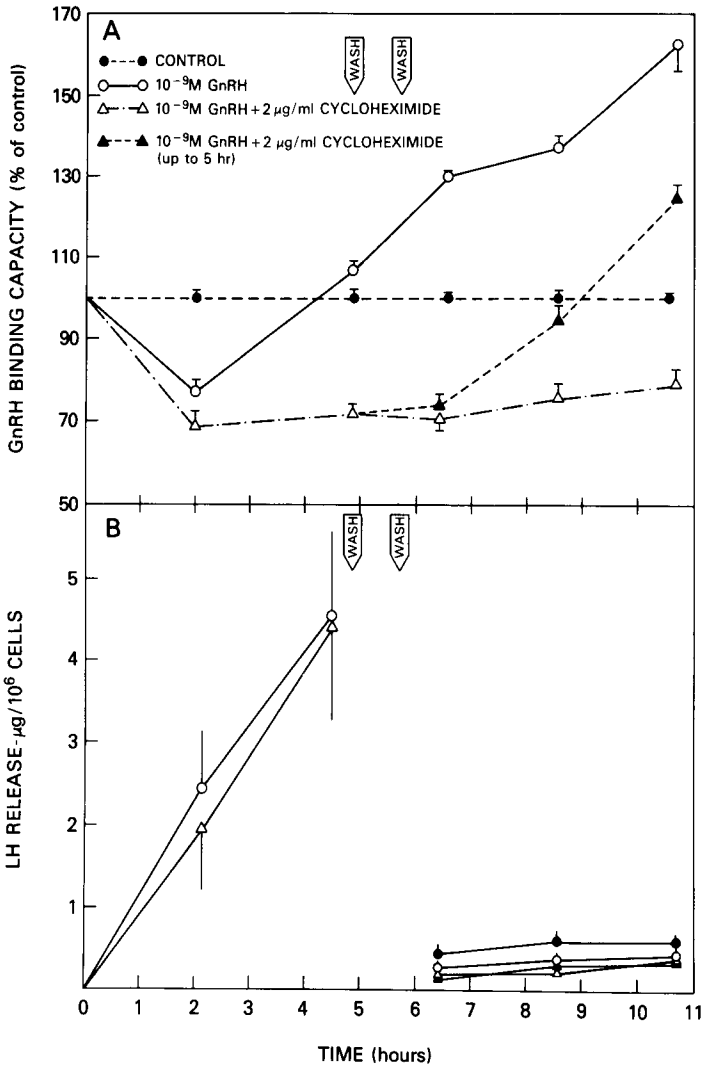

Fig. 4. Time-course of GnRH receptor regulation (above) and LH-release (below) in pituitary cells exposed to GnRH in the absence (○) or presence (△) of cycloheximide (2 μg/ml). GnRH causes a characteristic biphasic change in receptor number, with initial receptor loss followed by delayed up-regulation of receptors. Cycloheximide prevents the late increase in GnRH receptors, which does not occur unless the drug is removed by washing the cells (▲).

lysosomes, whereas the antagonist-coated beads remained at the cell surface for much longer periods [18]. These findings imply that endocytosis of the agonist–receptor complex is stimulated by receptor activation, whereas the non-activating antagonist ligand undergoes much slower cellular processing that is probably more related to plasma-membrane protein turnover than to ligand-stimulated receptor-mediated endocytosis [17]. The ability of GnRH super-agonists to desensitize the pituitary gland, and to suppress gonadotropin release when employed for contraception or treatment of precocious puberty and certain steroid-dependent tumors, is related to the marked loss of receptors and activation responses that follow chronic exposure to the agonist ligand. In contrast, potent GnRH antagonists showed prolonged binding to the gonadotrophs without true receptor loss, and provide an alternative means of achieving sustained blockade of gonadotropin secretion. The contrasting effects of desensitization by a GnRH superagonist analog, and receptor blockade by a potent GnRH antagonist, are evident during studies on the effects of such peptides upon LH release in perifused pituitary cells (Fig. 7).

PHYSICO-CHEMICAL PROPERTIES OF THE GnRH RECEPTOR

Pituitary GnRH receptors have been characterized in several species as specific binding sites with high affinity for GnRH and its potent agonist or antagonist analogs. Similar receptors are present in the rat testis and ovary, where GnRH exerts direct actions that are predominantly inhibitory in nature. The binding properties and physiological regulation of GnRH receptors have been studied in considerable detail, but relatively little is known about their chemical and physical structure. Recently, the application

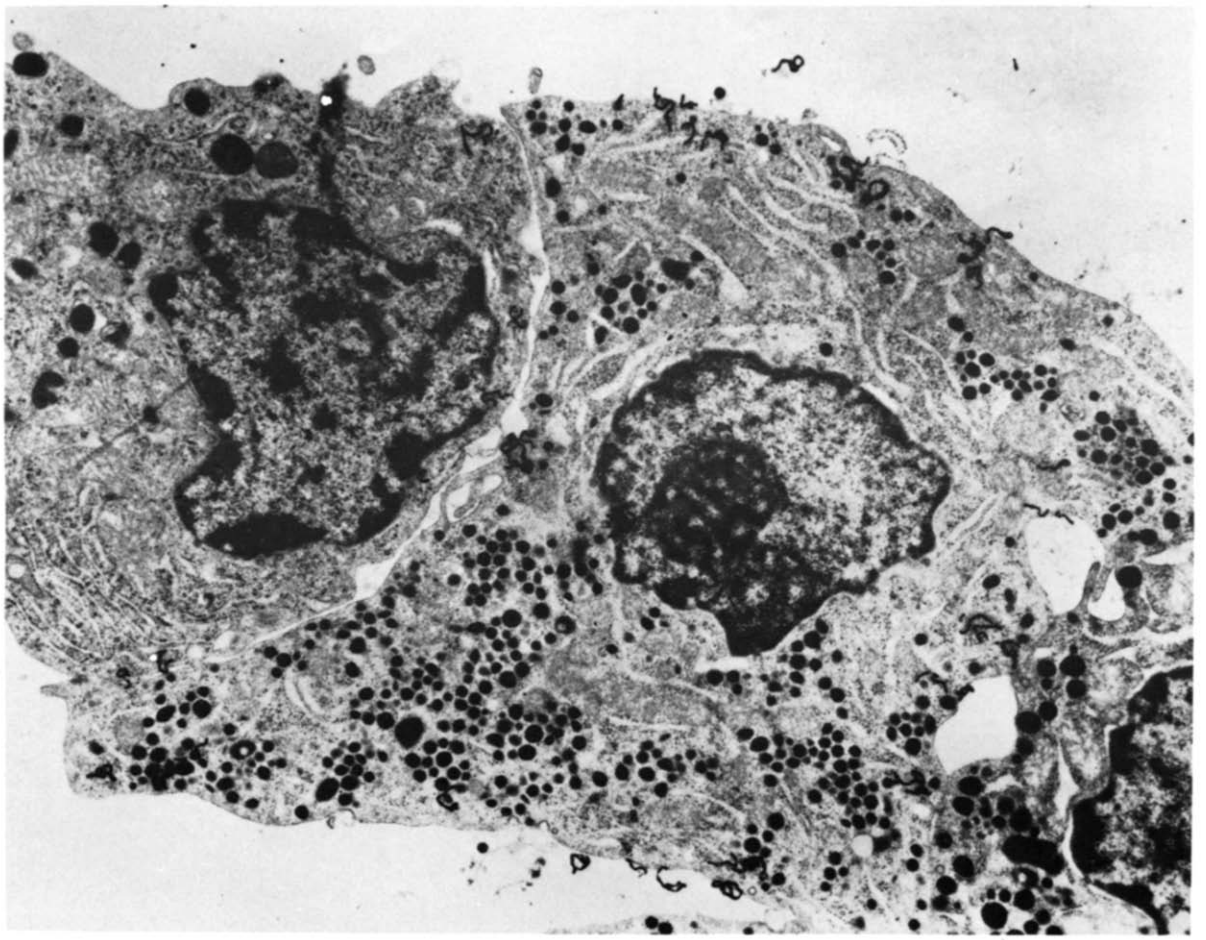

Fig. 5. Electron microscopy autoradiograph of bound ^{125}I-labeled GnRH antagonist on the surface of a cultured gonadotroph following incubation for 2 h. The gonadotroph bears numerous silver grains that are predominantely located at the plasma membrane level, and is flanked by lactotrophs that are devoid of labeling.

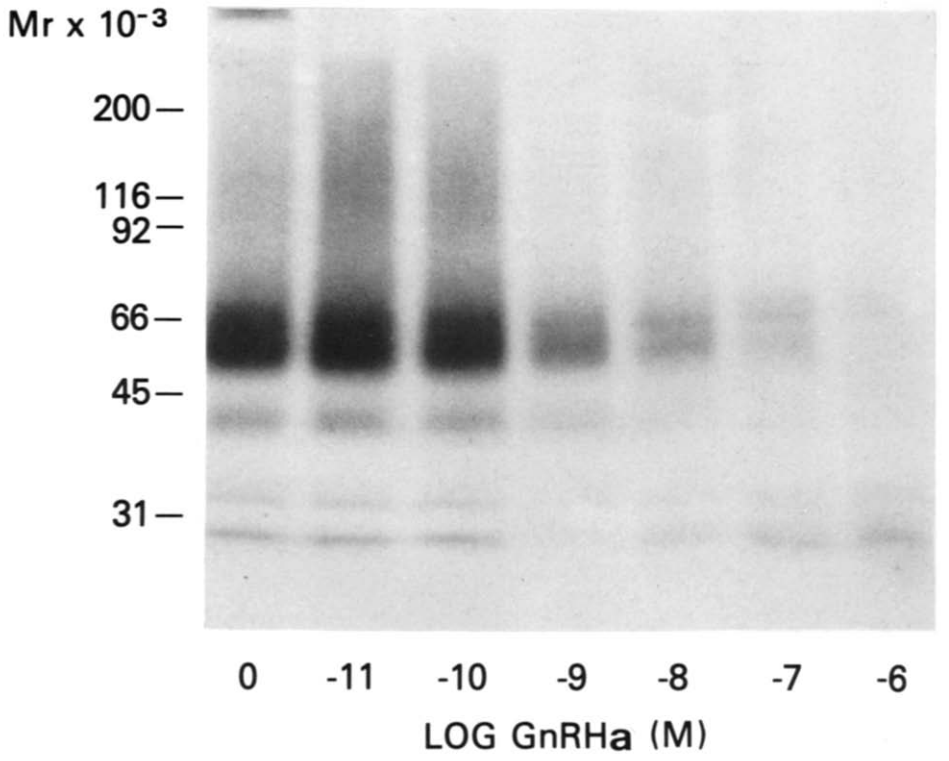

Fig. 9. SDS-gel electrophoresis of photolabeled GnRH receptors of pituitary gonadotrophs. The cells were purified by elutriation and cultured for 2 days, then homogenized and photolabeled with [^{125}I]HSAB-GnRHa in the presence of increasing agonist concentrations as indicated below each lane.

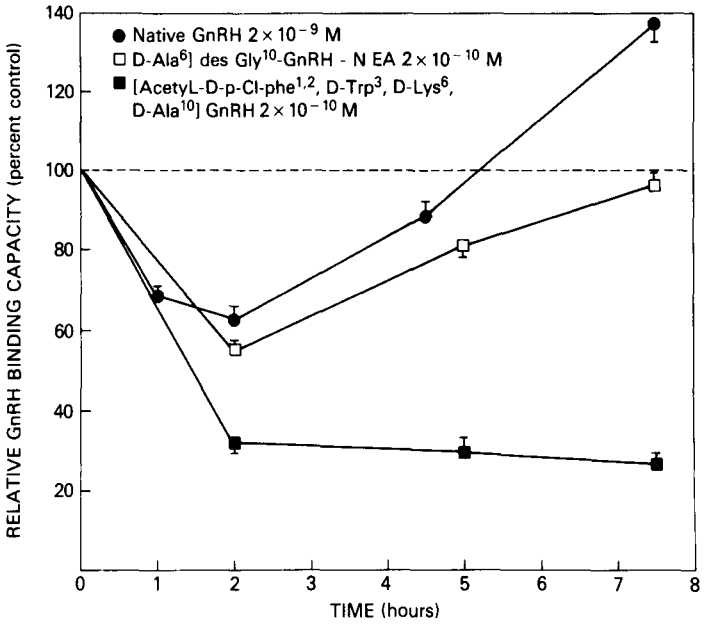

Fig. 6. Time-course of GnRH receptor regulation in pituitary cells exposed to GnRH (●), a GnRH super-agonist analog (□), and a highly potent GnRH antagonist (■).

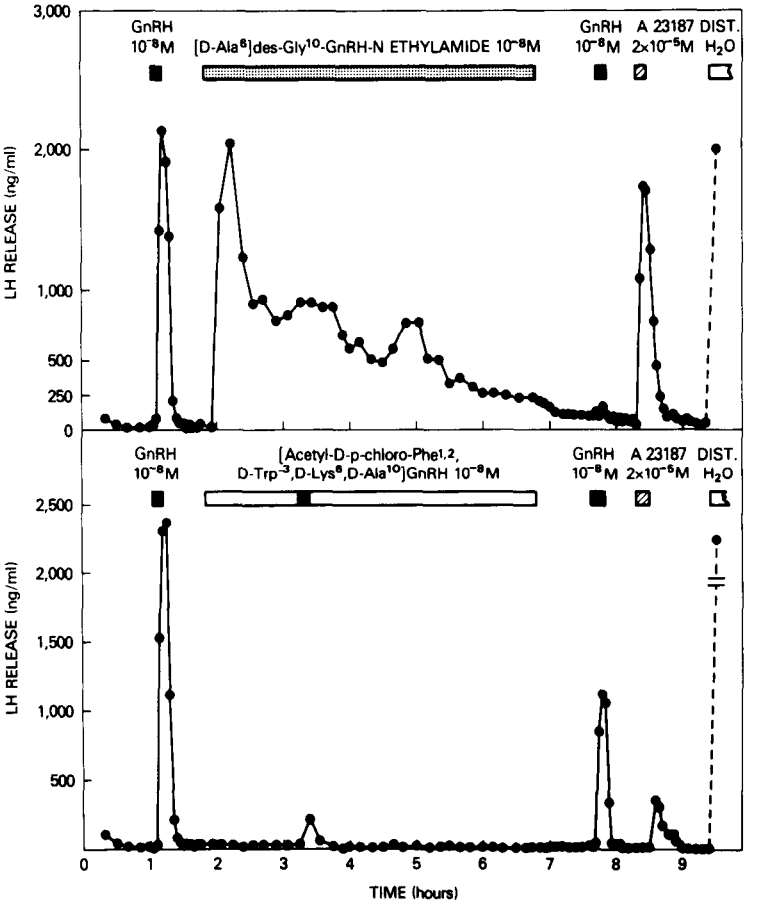

Fig. 7. Inhibitory effects of a GnRH superagonist analog (above) and a highly potent antagonist analog (below) on LH release in perifused pituitary cells. Note the progressive desensitizing action of the agonist analog with complete loss of the subsequent response to GnRH. In contrast, the antagonist completely blocks the LH response to GnRH given during its infusion, but only partially inhibits the response to a subsequent pulse of GnRH.

of photoaffinity labeling and detergent solubilization to the GnRH receptor has begun to provide information about the physical properties of the pituitary and gonadal binding sites. Initial studies with a photoreactive GnRH derivative followed by SDS gel electrophoresis revealed one receptor component in the pituitary and two in the ovary [19]. Formerly, attempts to solubilize and characterize the GnRH holoreceptor under non-denaturing conditions were impeded by the fact that most detergents are poorly effective in extracting the free GnRH receptor in its active form. However, the zwitterionic detergent, CHAPS, has been shown to solubilize pituitary GnRH receptors with retention of ligand binding activity [20] and is currently the agent of choice for extraction of the free receptor sites. The physicochemical properties of the pituitary GnRH receptor have been analyzed after solubilization of the membrane receptors by CHAPS, by binding studies with radioiodinated GnRH superagonist and antagonist analogs, and by covalent labeling with a radioiodinated photoreactive GnRH agonist.

The solubilized GnRH receptors extracted from rat pituitary glands with 5 mM CHAPS retained the high ligand affinity and specificity of the membrane-bound sites. The soluble sites were also relatively more stable than the particulate receptors, and showed higher binding of the potent GnRH antagonist, ^{125}I-[Ac-D-*p*-Cl-Phe^{1,2}, D-Trp^{3}, D-Lys^{6}, D-Ala^{10}]GnRH than of radioiodinated GnRH agonists. When analyzed by gel filtration under non-denaturing conditions, both free and prelabeled GnRH receptors behaved as high molecular weight species with mol. wt of about 700,000, probably representing an aggregated form of the receptor. However, the CHAPS-solubilized receptor exhibited a lower molecular weight on sucrose density gradient centrifugation [21], migrating with sedimentation coefficient of about 10S (Fig. 8). The latter value, in conjunction with estimates of molecular size based on gel filtration of the Triton-solubilized hormone–receptor complex, indicates that the MW of the GNRH holoreceptor is about 250,000 under non-denaturing conditions.

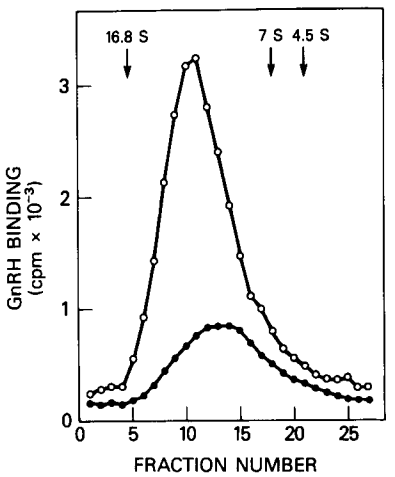

Fig. 8. Sucrose density gradient centrifugation of the CHAPS-solubilized pituitary GnRH receptor, which migrates with a sedimentation constant of about 10S.

To analyze the binding subunits of the GnRH holoreceptor and to compare the binding sites in different species, pituitary and gonadal GnRH receptors were analyzed after photoaffinity labeling using ^{125}I-[N^{ε}-azidobenzoyl-D-Lys^{6}]des-Gly^{10}-GnRH-*N*-ethylamide. This radiolabeled photoreactive probe for the GnRH receptor was prepared by derivatization of radioiodinated [D-Lys^{6}]des-Gly^{10}-GnRH-*N*-ethylamide with the heterobifunctional photolabile reagent, *N*-hydroxysuccinimidyl-4-azido-benzoate (HSAB). The high-affinity photoreactive analog was employed for radiolabeling and characterization of pituitary GnRH receptors in rat, rabbit, mouse, sheep, and cow adenohypophyses, and gonadal GnRH receptors in the rat ovary and testis [21]. In rat, rabbit, and mouse pituitary glands, analysis of the GnRH receptor–ligand complex by SDS polyacrylamide gel electrophoresis and autoradiography showed two labeled components, both of which were displaced by unlabeled GnRH agonist and antagonist analogs. The larger receptor component was a relatively broad band with molecular weights in rat, rabbit, and mouse of 59,000 ± 1900, 62,000 ± 700, and 60,000 ± 800, respectively. In the rat pituitary gland, the larger component was composed of 63,000 and 52,000 mol. wt components, of which the latter was more heavily labeled and was predominant in purified pituitary gonadotrophs (Fig. 9). The mol. wt of the smaller components were 40,000 ± 1600, 43,000 ± 1200 and 41,000 ± 1000, respectively. In bovine and ovine pituitary glands, the photolabeled GnRH receptor was a single band with mol. wt of 42,000 ± 1200 and 39,000 ± 500, respectively. In the rat ovary and testis, photolabeled GnRH receptors were similar to those in the rat pituitary gland, with two distinct components of mol. wt 53,000 ± 1000 and 42,000 ± 1000. These findings demonstrate that the pituitary receptors which mediate similar physiological actions of GnRH different species possess broadly similar structural properties, with minor variations between species. It is also evident that the divergent actions of GnRH in different tissues of the same species, as in the rat pituitary and gonads, are expressed through receptors of similar structure.

In the human placenta, which contains low-affinity GnRH binding sites that interact with GnRH agonist and antagonist analogs, photoaffinity labeling and SDS gel analysis has revealed a single component of Mr 54,000. Although the placental sites differ from the pituitary GnRH receptors in several respects, their ability to bind GnRH antagonists, and the similarity of their binding subunit to that of the gonadotroph, suggest that they represent a placental variant of the GnRH receptor. It is likely that such sites mediate the actions of locally-formed GnRH or related peptides within the placenta.

MECHANISMS OF ACTION OF GnRH

The secretory response to GnRH is highly calcium-dependent, and the acute phase of gonadotropin release does not appear to involve cyclic AMP-mediated processes. In these respects, the gonadotroph shares common features with many other secretory cells in which the mechanisms involved in the calcium-mediated response pathway are now beginning to be defined. GnRH has been shown to stimulate phospholipid metabolism in the gonadotroph, with increased phosphatidylinositol (PI) turnover [22], formation of phosphatidic acid [23], and release of arachidonic acid (AA) [5]. The products formed after phosphoinositide (PI, DPI, or TPI) hydrolysis by phospholipase C include diacylglycerol (DG), inositol phosphates, and phosphatidic acid. In addition, numerous oxygenated metabolites including leukotrienes are produced from arachidonic acid after its release by phospholipase A_2 or diglyceride lipase. Recent evidence has suggested that several of these phospholipid metabolites are putative intermediate in the action of GnRH upon LH release. The abilities of arachidonic acid or its metabolites [5] and of phosphatidic acid [23] to stimulate the release of gonadotropins have been shown by previous studies in cultured rat pituitary cells.

More recently, the potential importance of inositol triphosphate (IP_3) as a mediator of ligand-induced calcium mobilization has been emphasized [24]. The extent to which IP_3 acts as a general mediator of peptide hormone action is not yet known, but its involvement in the action of TRH has been demonstrated in mammotropic tumor cells [25]. In purified rat gonadotrophs, stimulation of LH release by GnRH was associated with prominent increases in inositol monophosphate formation (Fig. 10), consistent with substantial breakdown of phosphatidylinositides by phospholipase C. A smaller increase in inositol biphosphate (from DPI) was also observed, but only minor and transient changes in the inositol triphosphate fraction were evident during analysis by ion exchange chromatography. However, early increases in IP_3 were more readily detected by HPLC analysis of the phosphorylated inositol products. The detection of polyphosphorylated inositides and their metabolites depends upon the optimization of conditions necessary to reveal TPI turnover in individual cell types, and evaluation of their relevance to GnRH action will require the use of suitable methods for the resolution and assay of IP_3 isomers that may be selectively involved in calcium mobilization.

In addition to the early role of calcium mobilization by endogenous cellular ionophores such as PA and IP_3, it appears very likely that other mechanisms which do not depend on changes in cytosolic calcium are involved in the secretory response of the gonadotroph to stimulation by GnRH and its agonist analogs. Probably the most important of these additional mediators is the diacylglycerol formed during the action of phospholipase C on phosphoinositides.

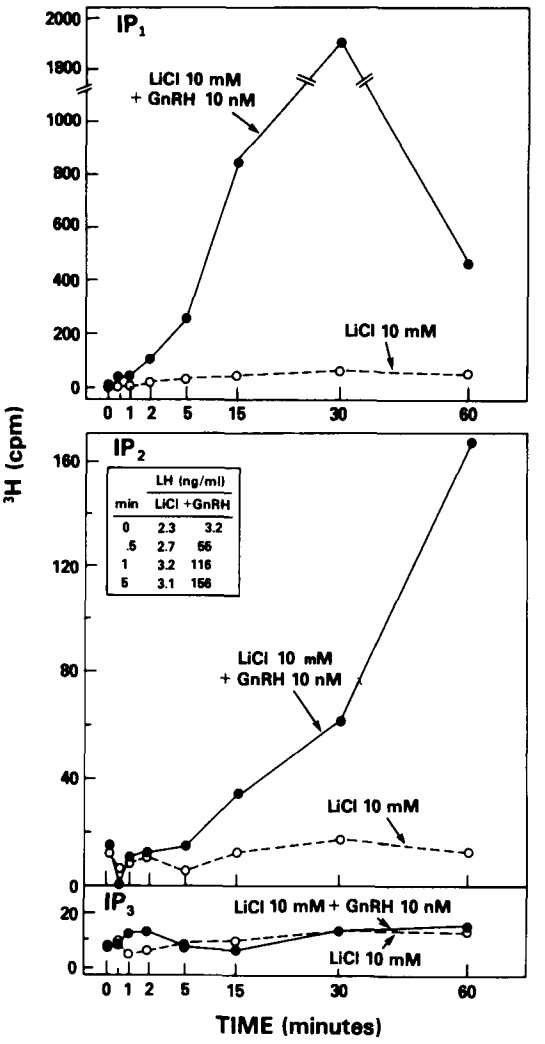

Fig. 10. Formation of inositol phosphates and release of LH in cultured pituitary gonadotrophs during stimulation by GnRH. Gonadotroph-enriched cells were prelabeled with [^{3}H]inositol and then stimulated by 10 nM GnRH in the presence of 10 mM LiCL to inhibit inositol-1-phosphatase. The time course of formation of inositol phosphates (IP_1, IP_2, and IP_3) separated by ion exchange chromatography is shown in comparison with the early LH response to GnRH (inset).

Diacylglycerol is a potent activator of the calcium- and phospholipid-dependent enzyme, protein kinase C [4], that is now recognized to be involved in many ligand-stimulated cell responses. This enzyme is normally present as an inactive form, and becomes activated during ligand-stimulated production of diacylglycerol, which binds to the enzyme and increases its affinity for calcium. Phorbol esters also stimulate the activity of protein kinase C, in both intact cells and subcellular fractions, by binding to the enzyme and/or its phospholipid environment. In certain tissues, activation of protein kinase C by phorbol esters has been accompanied by its redistribution from the cytosolic fraction to membrane-associated sites.

The marked calcium-dependence of the secretory response to GnRH, and the prominent phospholipid

Table 1. Activation of protein kinase C in cytosol of anterior pituitary by calcium, phosphatidylserine (PS), 1,2-diolein and TPA

Assay composition	Protein kinase activity (pmol/min/mg protein)
Cytosol + reaction mixture	200 ± 19
+ Ca^{2+}	470 ± 54
+ Ca^{2+} + PS	580 ± 30
+ Ca^{2+} + diolein	590 ± 53
+ Ca^{2+} + PS + diolein	960 ± 54
+ Ca^{2+} + PS + TPA	990 ± 96

Protein kinase C was assayed by measuring the incorporation of ^{32}P into histone H_1 from [γ-^{32}P]ATP. The reaction mixture contained 20 mM Tris–HCl (pH 7.5), 5 mM Mg nitrate, 50 μg H_1 histone, and 10 μM ATP (1–2 × 10^6 cpm). Also, phosphatidylserine (5 μg), 1–2 diolein (0.2 μg), Ca^{2+} (0.5 mM), and TPA (1 μM) were added as indicated. Data are the mean ± SE of triplicate assays.

turnover in GnRH-stimulated pituitary cells, suggest the involvement of protein kinase C in the processes leading to gonadotropin release. Therefore, the activity of the enzyme in the rat pituitary gland was analyzed, and its role in GnRH action was evaluated in studies on the stimulatory effects of TPA and GnRH on LH secretion and protein kinase C. These responses, and the effects of the protein kinase C inhibitor, retinal, on enzyme activation and gonadotropin release, were analyzed in cultured rat pituitary cells and purified gonadotrophs [26]. The presence of protein kinase C in rat pituitary gland, and its distribution between the cytosolic and membrane fractions of anterior pituitary homogenates, is shown in Fig. 11. In anterior pituitary cytosol, phosphorylation of exogenous histone substrate was increased in the presence of Ca^{2+} and was further

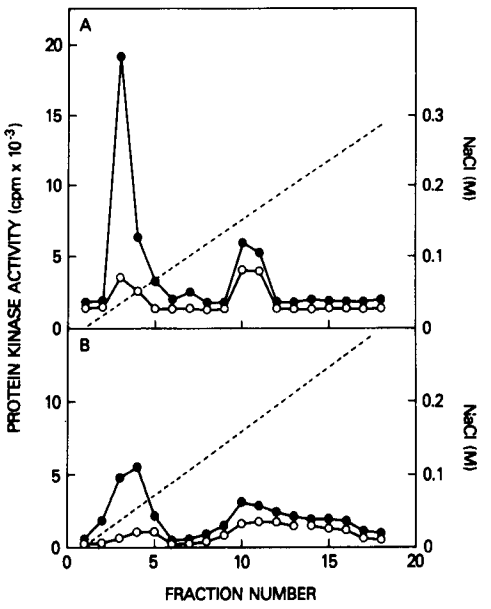

Fig. 11. Protein kinase C activity in rat pituitary cytosol (above) and detergent-extracted membranes (below) during anion exchange chromatography on DE52-cellulose [27]. Enzyme activity was assayed in aliquots of each fraction in the absence (○) and presence (●) of 0.5 mM Ca^{2+}, 5 μg phosphatidylserine, and 0.2 μg diolein.

Table 2. Stimulation of LH production in cultured rat pituitary cells by GnRH, TPA and synthetic diglycerides

Stimulus	LH Production
Control—no additions	3.6 ± 0.8
Gonadotropin-releasing hormone (10^{-7} M)	23.6 ± 3.1
Tetradecanoylphorbol acetate (10^{-7} M)	12.5 ± 0.6
1-oleoyl, 2-acetyl glycerol (10^{-4} M)	4.7 ± 0.5
Dioctanoyl glycerol (10^{-4} M)	7.2 ± 1.7

LH production is given as ng/500,000 cells/3 h, and data are the mean ± SE of results from triplicate incubations.

enhanced by addition of phosphatidylserine (PS) and 1,2-diolein. Also, TPA was found to fully activate kinase C in pituitary cytosol in the presence of Ca^{2+} and PS (Table 1), and to stimulate LH secretion with about 50% of the efficacy of GnRH. In contrast, retinal inhibited the activation of cytosolic protein kinase C by Ca^{2+} and diolein when added at concentrations greater than 10^{-7} M. In cultured pituitary cells stimulated with a maximal dose of GnRH (10 nM), addition of retinal also reduced GnRH-induced LH release in a dose-dependent manner. The maximum impairment of LH release (by almost 50%) was observed at 10^{-4} M retinal, a concentration at which kinase C activity was also maximally inhibited. The ability of TPA to activate pituitary kinase C and to stimulate LH secretion, and the converse effects of retinal on kinase C and GnRH-induced LH release, suggest that protein kinase C participates in the action of GnRH upon LH secre-

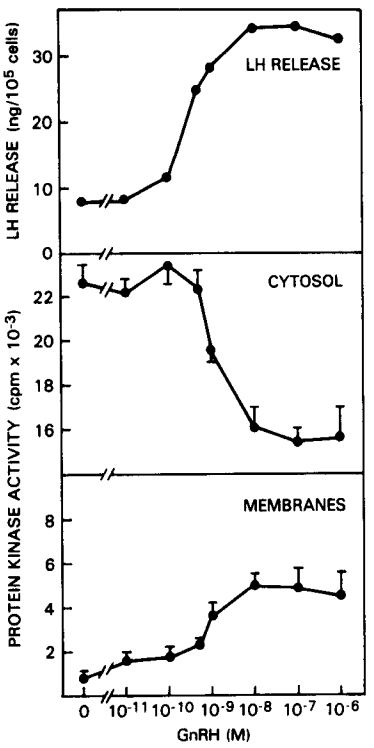

Fig. 12. Concentration-dependent effects of GnRH on LH release and distribution of protein kinase C between cytosolic and membrane fractions in cultured rat gonadotrophs.

tion. Further evidence for the involvement of protein kinase C in GnRH action was provided by the demonstration that not only phorbol esters, but also addition of synthetic diglycerides such as 1-oleoyl, 2-acetyl glycerol and dioctanoyl glycerol, stimulate the release of LH when added to cultured pituitary cells (Table 2).

This mechanism was further evaluated by analysis of the distribution of protein kinase C activity between cytosol and membrane fractions in cells treated with GnRH [27]. In pituitary cells fractionated by centrifugal elutriation, the greatest change in protein kinase C activity during GnRH stimulation was correlated with the maximal enrichment of gonadotrophs. Stimulation of LH release in gonadotroph-enriched cells by GnRH was accompanied by a rapid and dose-dependent decrease in cytosolic protein kinase C activity, and by a significant increase in protein kinase C associated with the membrane fraction (Fig. 12). The ability of GnRH to cause rapid translocation of cytosolic protein kinase C to a membrane-associated form is consistent with the activation of protein kinase C as an early event in the stimulation of LH secretion by GnRH.

The actions of GnRH on LH release in cultured rat pituitary cells are inhibited by certain calcium channel blockers and calmodulin antagonists, and are reproduced by high concentrations of A23187 [28]. As noted above, the ability of TPA to stimulate LH release at low concentrations (0.1 nM), and with up to 50% of the efficacy of 10 nM GnRHa, suggests that protein kinase C is also involved in LH release.

To pursue the possibility that phorbol esters cause receptor activation, their effects on GnRH receptor sites were analyzed in cultured rat anterior pituitary cells. In addition to causing a dose-related stimulation of LH secretion, TPA reduced the binding of GnRH to rat pituitary cells. In each of these effects, TPA was the most potent agent among several phorbol esters including phorbol, 12,13-dibutyrate, phorbol 12,13-diacetate, 4α-phorbol and 4α-phorbol 12,13-didecanoate. Scatchard analysis indicated that the TPA-induced reduction in GnRH binding was due to a decrease in the number of GnRH receptors, without any change in the affinity of the receptors for GnRH. These observations suggest that the actions of phorbol esters are at least partially expressed through protein kinase C-dependent phosphorylation of the GnRH receptor or its regulatory proteins.

Arachidonic acid (AA) metabolites of the 5-lipoxygenase and monooxygenase pathways have also been implicated as potential mediators of GnRH-induced LH secretion from pituitary gonadotrophs [29, 30]. In order to characterize these metabolites during conditions of altered LH secretion, the products released form ^{3}H[AA] pre-labeled rat gonadotrophs during 3 h culture were analyzed by reverse-phase high-performance liquid chromatography. At least 15 readily measurable ^{3}H[AA] metabolites were formed, including several major non-polar products eluting between the monohydroxylated AAs of the lipoxygenase pathway (5-, 12- and 15-HETEs) and AA itself, possibly representing epoxyeicosatrienoic acids of the monoxygenase pathway. At least 8 dihydroxy derivatives and leukotrienes of intermediate polarity were formed from AA via lipoxygenase or monooxygenase activity, in addition to several cyclooxygenase products including PGE_2. Stimulation of LH release by GnRH, phorbol esters, and the Ca^{2+} ionophore A23187, was in general associated with increased production of ^{3}H[AA] metabolites. However, GnRH-induced LH secretion and its enhancement by cyclooxygenase inhibitors were accompanied by both quantitative and qualitative changes (including some decreases) among unidentified ^{3}H[AA] products of intermediate polarity. The LH secretory response to 10 nM GnRH was attenuated by the lipoxygenase inhibitor nordihydroguaiaretic acid (40 μM, and by the general AA metabolism inhibitor eicosatetraynoic acid (40 μM), but was augmented by the cytochrome P-450 (monooxygenase) inhibitor, SKF 525A. Thus, gonadotrophs biosynthesize 5-lipoxygenase and monooxygenase products, as well as several AA metabolites of intermediate polarity which undergo changes during GnRH-stimulation of LH secretion, and which are potential modulators of GnRH action. Although leukotrienes are the most highly active products of the 5-lipoxygenase pathway, in pituitary cells the stimulatory actions of leukotrienes B_4 and C_4 on LH release were relatively small. Also, the leukotriene antagonist FPL 55712 exerted a partial inhibitory effect on GnRH-induced LH release. Therefore, while AA and 5-HETE consistently stimulate LH release, and epoxy derivatives of AA have been implicated in the release process [30], the quantitative contribution of AA and its oxygenated metabolites to GnRH-induced gonadotropin secretion has yet to be established.

Based on the findings described above, it appears likely that the calcium-dependent activation of gonadotropin-secretion by GnRH involves not one but several inter-related mechanisms. While there is now growing evidence for a long-term role of cyclic AMP in regulating the responsiveness of gonadotrophs to GnRH stimulation [31], the immediate effects of GnRH do not appear to involve cyclic AMP-dependent processes. The acute secretory episodes elicited by pulses of GnRH include a rapid initial release of LH that peaks within 1 min, followed by a secondary phase over the ensuing several minutes [32]. It is possible that the early rapid component of this biphasic response is related to mechanisms involving calcium mobilization and arachidonate metabolites, while the subsequent phase is related to activation and redistribution of protein kinase C in the gonadotroph. Arachidonic acid, which is rapidly produced during GnRH action and can stimulate LH release [5], may initiate secretion by promoting fusion and exocytosis of secretion granules adjacent to the plasma membrane. The sub-

sequent, prolonged phase of hormone secretion, in a manner analogous to the combined actions of calcium-calmodulin and kinase C pathways in platelets [4] and other tissues [33], could be related to the sustained redistribution and activation of protein kinase C during GnRH action. Further analysis of the individual and coordinated effects of phospholipid turnover, calcium-calmodulin and protein kinase C, arachidonic acid, and cyclic AMP, should clarify the mechanisms responsible for physiological activation of gonadotropin secretion, as well as the steps at which refractoriness of the gonadotroph occurs during agonist-induced desensitization of pituitary function.

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