GGH_3 CELLS AS A MODEL FOR GnRH RECEPTOR ACTION AND G-PROTEIN COUPLING IN THE GONADOTROPE

by

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CERTIFICATE OF APPROVAL

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ABBREVIATIONS

ADP: adenosine diphosphate

ATP: adenosine triphosphate

BSA: bovine serum albumin

cDNA: complementary deoxyribonucleic acid

CTP: cytosine triphosphate

cAMP: cyclic adenosine monophosphate

cGMP: cyclic guanosine monophosphate

CTX: cholera toxin

D600: methoxyverapamil

DAG: diacylglycerol

DMEM: dulbecco's minimum essential medium

EDTA: (ethylenedinitrilo)-tetraacetic acid

FSH: follicle stimulatin hormone

GAP: GTPase activating protein

GnRH: gonadotropin releasing hormone

GTP: guanosine triphosphate

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IP_3: 1,4,5-inositol triphosphate

LH: luteinizing hormone

MIX: 3-isobutyl-1-methyl xanthine

PCR: polymerase chain reaction

PIP_2: phosphatidylinositol 4,5-bisphosphate

PLC: phospholipase C

PKC: protein kinase C

PTH: parathyroid hormone

PTX: pertussis toxin

rER: rough endoplasmic reticulum

RGS: regulators of G-protein signaling

RIA: radio immunoassay

SII: secretograninII

TMS: transmembrane spanning

TRH: thyrotropin releasing hormone

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PREFACE

In accordance with the guidelines set forth by the Graduate Program of the School of Medicine, Oregon Health Sciences University, Portland, Oregon, I have prepared my dissertation, consisting of a general introduction, five chapters of original data and a final chapter consisting of a discussion introducing a model for the findings in this study. References are listed separately in alphabetical order, and follow the format of *Endocrinology*.

Chapters two, three, and four contain data, figures and text as they appear in original papers that have been published previously (Stanislaus et al., 1994, 1995 and 1997). Chapter six contain data, figures and text as they would appear in an original paper that is currently in press (Stanislaus et al., 1998). Chapters five consist of an original manuscript that has been submitted for publication. Chapter seven represents an original manuscript that is been prepared for publication as a review.

ABSTRACT

The first part of this study describes the characterization of a lactotrope derived cell line stably expressing the GnRH receptor (GGH_3). These cells release prolactin and produce the intracellular second messengers inositol phosphate and cAMP in a dose- and time-dependent manner in response to GnRH or GnRH agonist. Over-expression of different G-proteins in GGH_3 cells showed that the ability of the GnRH receptor to couple multiple G-proteins is not specific to the gonadotrope.

The main focus of this thesis is to identify G-proteins that couple to the GnRH receptor in the rat gonadotropes. Studies here show that the GnRH receptor is able to couple to $G_{q/11}$, G_i and G_s proteins in the rat gonadotrope. These studies utilized agonist bound receptor activation of G-protein palmitoylation to identify the proteins regulated by the cognate receptor. Furthermore, studies with mice lacking G_q or G_{11} gene show that these two proteins are able to compensate functionally for each other.

The ability of the GnRH receptor to couple multiple G-proteins potentially enables it to regulate multiple signal transduction pathways. This would be a putative mechanism for the gonadotrope to produce multiple responses to GnRH.

CHAPTER ONE

INTRODUCTION

How one hormone acting through a single class of receptors stimulates multiple responses is an active area of research in endocrinology, and especially in the field of biology dealing with gonadotropin releasing hormone action (GnRH). GnRH stimulates luteinizing hormone (LH) and follicle stimulating hormone (FSH) release, secretogranin release, gonadotropin subunit synthesis, and GnRH receptor synthesis in the gonadotrope (Conn et al., 1995). Studies have shown that different GnRH pulse frequencies can cause the gonadotrope to respond differently to GnRH by changing the GnRH receptor number (Marshall and Griffin, 1993). However there are no studies to show how GnRH receptor number can affect the response to GnRH in the gonadotrope. A possible mechanism is that GnRH receptor may activate multiple signal transduction pathways by activating multiple G-proteins, although no clearly defined studies have been done to show GnRH receptor coupling to multiple G-proteins. This thesis is an attempt to address this dearth in our understanding of GnRH receptor/ G-protein coupling.

In chapter one a brief overview of the current state of knowledge about GnRH, GnRH receptors and G-proteins is given. This is in no way a comprehensive undertaking, but focuses on how these three factors may play a role in GnRH action in the pituitary. Chapters two and three describe the characterization of a model cell line for the study of GnRH receptor action. This cell line has been used to investigate GnRH receptor/G-protein interactions, GnRH receptor microaggregation, receptor effects on signal transduction. Chapters four through six describe the studies that show GnRH receptor coupling to multiple G-proteins. In chapter five knockout models are used to illustrate how G_q and G_{11} proteins can functionally compensate for each other in GnRH action. Finally, chapter seven serves as the discussion section where the observations in this thesis are used to introduce a model to illustrate how GnRH may produce multiple

physiological responses in the gonadotrope. In this chapter a dynamic model is proposed to explain how GnRH receptor density, GnRH pulse frequency, and multiple G-proteins interact to produce multiple responses in the gonadotrope.

1.Gonadotropin Releasing Hormone

GnRH provides the humoral link between the neural and the endocrine systems. Since the isolation and characterization of this hormone, analogs of GnRH have been used clinically to treat precocious puberty, endometriosis, polycystic ovarian disease, and steroid dependent neoplasia, such as prostate cancer and breast cancer. In addition, GnRH and its analogs have been successfully used to enhance reproductive efficiency in animal husbandry (Conn and Crowley, 1991).

1.1Synthesis and Distribution

GnRH is a decapeptide, which is synthesized and then stored in the arcuate nucleus region of the hypothalamus (for a review see Jennes and Conn, 1994). The neurons responsible for GnRH action exhibit several unusual characteristics. They originate from the olfactory placode, a structure external to the brain, and during early gestation migrate along the olfactory tract to their ultimate location in the arcuate nucleus area of the hypothalamus (Schwanzel-Fukuda et al., 1992). Defects in this migration may account for some cases of GnRH deficiency associated with the absence of olfactory bulb and tract, also known as the Kallmann's syndrome (Schwanzel-Fukuda et al., 1992).

GnRH is synthesized in the arcuate nucleus and is transported to and released from the median eminence into the hypothalamic-hypophyseal portal system. This hormone is released in a pulsatile manner that can be regulated by external signals, such as gonadal steroid hormones (Marshall and Griffin, 1993). In response to GnRH in the portal circulation, gonadotropes in the anterior pituitary release LH and FSH into the

peripheral circulation. The pulsatile nature of GnRH release results in the pulsatile release of LH and FSH. Gonadotropins in the systemic circulation regulate steroidogenesis and gamete maturation in gonadal tissues; LH stimulates ovulation and corpus luteum formation in females and androgen secretion in males, whereas FSH stimulates growth and maturation of ovarian follicles in females and spermatogenesis in males.

1.2 Amino acid structure

A single gene located in the short arm of chromosome 8 encodes GnRH in the human (Yang-Feng et al., 1986). GnRH is synthesized as a 92 amino acid precursor molecule which includes the ten amino acid GnRH peptide, preceded by a 23 amino acid signal peptide (Seeburg and Adelman, 1984). The linear sequence of mammalian GnRH is: pyroGlu^{1}-His^{2}-Trp^{3}-Ser^{4}-Tyr^{5}-Gly^{6}-Leu^{7}-Arg^{8}-Pro^{9}-Gly-amide^{10}.

The half life of GnRH in the circulation is less than ten minutes (Barron et al., 1982). Therefore for clinical utilization of GnRH, various analogs with greater resistance to enzymatic degradation, and greater binding affinity at the receptor, have been developed. The native GnRH molecule can undergo a major conformational change from a fully extended form to a highly folded form. The least energy conformation of the folded form is made structurally possible by a type II β turn at Gly^{6}-Leu^{7} position of the GnRH molecule (Coy et al., 1979). The folded structure is apparently stabilized by hydrogen bond formation between the pyrrolidone carbonyl residue (position one) and the glycineamide group (position 10; Coy et al., 1979). The structurally constrained folded form of GnRH is thought to be the conformation recognized by the GnRH receptor (Karten and Rivier, 1986). Incorporation of D-amino acids with large hydrophobic side chains produce structurally constrained GnRH analogs that attain a thermodynamically favorable folded structure (Karten and Rivier, 1986). In addition, the presence of D-

amino acids make these analogs less susceptible for enzymatic degradation (Karten and Rivier, 1986). Furthermore, the introduction of Pro^9-alkylamides, while not affecting the potency, increases the duration of the analog by virtue of greater resistance to enzymatic degradation (Fujino et al., 1972). Using these methods, over 2000 analogs of GnRH have been synthesized (Karten and Rivier, 1986). Some of these analogs with clinical importance include [D-Ser(tBu)^6-Pro^9-NHEt]GnRH (Buserelin, Hoescht; Coy et al., 1974), and [3-(2-napthyl)Ala^6]GnRH (Nafarelin, Syntex).

2. GnRH Receptor

2.1 GnRH receptor cDNA

Using a PCR based homology cloning strategy, the GnRH receptor cDNA was cloned from the αT3-1 murine gonadotrope cell line (Tsutsumi et al., 1992). Since then mammalian and non-mammalian GnRH receptor cDNA has been identified in human, rat, sheep, cow, pig and catfish (for review, see Sealfon et al., 1997). More than 85% of the overall amino acid sequence of the GnRH receptor is conserved in the six mammalian species so far cloned (Sealfon et al., 1997). The cow, sheep and mammalian GnRH receptors are 328 amino acids long, whereas, the murine and rat receptors are 327 amino acids long.

2.2 Localization of GnRH receptor

The primary site of action for GnRH is in the gonadotropes in the anterior pituitary. However, additional binding sites for GnRH have been identified in other locations in the brain and also in peripheral tissues. In the brain the major targets for GnRH are the amygdala and the hippocampus (Conn et al., 1995). In the amygdala, the medial, lateral and cortical nuclei are labeled moderately with iodinated GnRH analogs, whereas in the hippocampus the strata oriens and radiatum of areas CA1-4 show labeling

with iodinated GnRH analogs (Conn et al., 1995). In the peripheral tissues GnRH binding sites have been observed in the gonads of rats and humans, but not in ovine, bovine or porcine ovaries (Braden and Conn, 1991). In addition investigators have identified GnRH binding sites in human breast carcinomas, and in adrenal membranes (Eidne et al., 1985; Eidne et al., 1985b).

2.3 Physical and Chemical Characteristics of GnRH receptor

Studies show that GnRH binds to a single class of specific and saturable binding sites in pituitary plasma membranes (Clayton and Catt, 1981; Marian et al., 1981; Perrin et al., 1983). The molecular weight of the GnRH receptor ranges from 50,000 to 700,000 Da, depending on the conditions used (Braden and Conn, 1991). Zwitterionic detergent solubilization with CHAPS, followed by nondenaturing sizing gel exclusion studies have indicated a 60,000-150,000 Da range for the GnRH receptor (Iwashita et al., 1988; Ogier et al., 1987). Whereas, photoaffinity agonist (^{125}I-Tyr^{5}-azidobenzoyl-D-Lys^{6}-GnRH) labeling of GnRH receptor, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography indicated an apparent molecular weight range of 46,000-60,000 (Hazum, 1981; Janovick et al., 1993). It is still unclear whether this photoaffinity labeled GnRH receptor is the holoreceptor, or a ligand binding portion of the receptor.

Biochemical studies of the GnRH receptor have indicated that it is a sialic acid containing glycoprotein (Sealfon et al., 1997). The cow, sheep, pig, rodent and human GnRH receptor sequences have two potential sites for N-linked glycosylation at the N-terminus, where as the rodent sequence has an additional site on the first extracellular loop of the GnRH receptor (Sealfon et al., 1997). Mutagenesis studies have indicated that only the two putative glycosylation sites at the N-terminus may be glycosylated in the rodent GnRH receptor (Sealfon et al., 1997). Glycosylation of the GnRH receptor

may play a potential role in producing a range of apparent molecular weights for the GnRH receptor. Glycosylation of the GnRH receptor may improve membrane expression of the receptor by decreasing the rate of degradation, but does not affect the affinity of the agonist for the receptor (Sealfon et al., 1997).

Addition of GTP or a metabolically stable analog (guanylimidodiphosphate) to permeabilized pituitary cells stimulated a time- and concentration dependent increase in inositol phosphate accumulation and LH release, indicating that GnRH receptor may couple to G-proteins (Andrews and Conn, 1986). Further evidence for G-protein coupling to the GnRH receptor came from studies by Perrin and co-workers (1989). These studies showed that the affinity of GnRH agonists for the receptor is decreased in the presence of guanine nucleotides, a characteristic common to other G-protein coupled receptors.

Studies of the primary sequence of all G-protein coupled receptors show that they have an extracellular amino terminal and an intracellular carboxy terminal. Direct structural information for this class of receptors is available for only two G-protein coupled receptor proteins: the bacteriorhodopsin receptor and the frog rhodopsin receptor (for review see Pardo et al., 1992). The data from these studies are consistent with a seven membrane spanning domains for G-protein coupled receptors, and show that the transmembrane spanning domains are comprised of predominantly hydrophobic amino acids in an α-helical conformation arranged around a hydrophilic core (Sealfon et al., 1997). Since the first cloning of the GnRH receptor, sequence and hydropathy analyses have shown the characteristic seven stretches of predominantly hydrophobic amino acids. Due to these hydrophobic regions the GnRH receptor can adopt a serpentine, seven transmembrane spanning conformation in the plasma membrane, the characteristic common to other G-protein coupled receptors (Tsutsumi et al., 1992).

2.4 Signal transduction pathways associated with the GnRH receptor

As mentioned earlier, GnRH receptor action is mediated through G-protein coupled signal transduction pathways. In chapters four through seven the G-proteins involved in GnRH receptor action and the signal transduction pathways modulated by these proteins are discussed in detail. Therefore, only a brief discussion on signal transduction pathways would be given here. However, the role of calcium as a signal molecule in GnRH action will be discussed in more detail as it is not discussed elsewhere, and is important for GnRH stimulated LH release.

GnRH stimulates dose dependent inositol phosphate production in dispersed rat pituitary cell cultures (Andrews and Conn, 1986). GnRH stimulation also results in the activation of protein kinase C, suggesting the involvement of diacylglycerol. Inositol phosphates and diacylglycerol are the hydrolytic products of phosphoinositol 4,5-bis phosphate (PIP_2; Downes and Wusterman, 1983; Kishimoto et al., 1980).

In rat pituitary cell cultures, GnRH stimulation does not result in detectable increase in cyclic AMP (Conn et al., 1979). Although, treatment with pharmacological agents that lead to increases in intracellular cyclic AMP levels result in enhanced GnRH stimulated LH release (Janovick and Conn, 1993).

For some actions of GnRH, ionic calcium (Ca^{2+}) is required. Removal of extracellular calcium inhibits both hypothalamic extract and depolarization stimulated LH release from pituitary cell cultures (Samli and Geschwind, 1968). Moreover, pharmacological agents that increase the intracellular calcium concentration, such as the ionophores A23187 and X5371A release LH with efficacies similar to GnRH (Conn et al., 1995).

GnRH receptor stimulation in gonadotropes activates specific plasma membrane channels. Patch clamp studies have indicated that GnRH does not depolarize the gonadotrope, although it activates a specific calcium channel (Mason and Waring, 1968).

Furthermore, GnRH does not need to contact the calcium channel for activation, indicating that a second messenger system maybe involved in mediating GnRH action (Mason and Waring, 1968). Calcium channel involvement is further demonstrated by specific calcium channel blockers (verapamil or D600), which block GnRH stimulated LH release in rat pituitary cell cultures (Conn et al., 1983).

GnRH receptor associated calcium channels play an important role in homologous desensitization of the gonadotrope to GnRH. Homologous desensitization is thought to develop in two phases. Initially, loss of responsiveness to GnRH is due to receptor loss, and subsequently it is maintained due to a loss of functional activity of calcium ion channel (Conn et al., 1987).

A potential intracellular mediator of calcium signal in the gonadotrope is calmodulin, a ubiquitous calcium binding protein (Conn et al., 1995). In the pituitary, calmodulin redistributes from a soluble fraction to a particulate fraction in response to GnRH administration into rats (Conn et al., 1981). A role for calmodulin action in the gonadotrope is further supported by inhibitors of calmodulin action, such as pimozide. Pimozide treatment antagonizes GnRH stimulated LH release in the rat pituitary (Conn et al., 1987b).

3. G-Proteins

The receptors for many hormones (such as gonadotropins, catecholamines, and glucagon), odorants and light mediate their action through a group of proteins that bind GTP (guanyl triphosphates). These proteins, also known as heterotrimeric G-proteins, couple the receptors to a variety of enzymes and ion channels.

3.1 General features

Heterotrimeric G-proteins are comprised of three subunits: α-subunits (molecular weight 39-52 kD), β-subunits (35-37 kD) and γ-subunits (6-10 kD; Ulloa-Aguirre and Conn, 1997). The individual subunits by themselves are highly diverse. Molecular cloning has revealed the existence of at least fifteen different α-subunit genes in mouse and human (Wilkie et al., 1992), five different β-subunits and eight different γ-subunits (Ulloa-Aguirre and Conn, 1997). G-protein α-subunits can be categorized under four major classes based on their function: G_s, G_i, $G_{q/11}$ and G_{12} (Table 1; Wilkie et al., 1992).

3.2 Regulatory cycle

Although G-proteins are made up of three polypeptide subunits, the β and γ subunits are tightly associated with each other and therefore can be regarded as a functional monomer. Figure 1 illustrates the cycle of G-protein activation. The α-subunit when bound to GDP (α-GDP) is in an inactive conformation and is able to bind the βγ-subunit to form an inactive heterotrimer. GDP liganded α-subunits can associate with the receptor, although the association is greatly enhanced in the presence of βγ-subunits. When a chemical or mechanical signal stimulates the receptor, the receptor becomes activated and undergoes a conformational change. This conformational change is transduced to the GDP-liganded heterotrimeric G-protein, which is associated with the receptor, and it in turn changes its conformation. The change in the conformation of the G-protein decreases its affinity to GDP, so that GDP comes off the active site. Because the intracellular concentration of GTP is so much greater than GDP, the GDP that comes off the active site is replaced by GTP (Neer, 1995). Binding of GTP to the α-subunit changes its conformation to the active state, and results in the dissociation of the α-subunit from the receptor and the βγ-subunits. The α-subunit has GTPase activity that

hydrolyses GTP to GDP. Therefore the active state lasts till GTP is hydrolyzed, then the α-subunit re-associates with a free βγ-subunit to form a GDP liganded heterotrimer, and is able to resume the activation cycle (Neer, 1995; Gilman, 1987). Both the active α- and βγ-subunits can each stimulate effectors down stream, and re-association turns off the signaling activity of the subunits.

The duration of the signal initiated by G-proteins depends on the rate of hydrolysis of GTP by the α-subunit (Gilman, 1987). Many factors control GTPase activity of the α-subunit. These include down stream effectors such as PLCβ (Berstein et al., 1992), regulators of G-protein signaling (RGS proteins; Watson et al., 1996) and lipid moieties on G-protein (Iri et al., 1997). Moreover, the intrinsic GTPase activity of the α-subunit differs among the different subunits (Neer, 1995). Therefore the end result is that the duration and the amplitude of the response to an extracellular stimuli is dependent on the GTPase activity of G-proteins, the effectors and other regulatory proteins.

3.3 G_α subunits: structure and signal transduction.

As alluded to earlier, α-subunits consists of at least 15 genes and at least 20 different G-protein α-subunits, including alternatively spliced isoforms (Neer, 1995). The functional classes mentioned earlier are based on the activity of the α-subunit (Table 1). The α-subunit is responsible for the GTPase activity of the heteromeric G-protein, although the associated βγ-subunits and other posttranslational modification associated with the α-subunit may have a modulatory effect on the GTPase activity (Clapham and Neer, 1993).

The α-subunit consists of two domains: one, a GTP binding domain that contains the guanine nucleotide binding pocket, and sites for binding receptors, effectors and βγ-subunits; second a helical domain whose function is not clear to date (Neer, 1995). The first 25 amino acids of the α-subunit are essential for βγ-subunit binding. The βγ-subunit

binding surface also includes a helix (α_2-helix) of the α-subunit whose position changes with the GDP- or GTP-liganded state of the subunit (Lambright, et al., 1994). Therefore the α_2-helix may play a critical role in the receptor activated dissociation of the trimeric G-protein. The effector binding domain partially overlaps the βγ-subunit binding domain on the α-subunit, therefore making it unlikely that both the βγ-subunit and the effector bind the α-subunit simultaneously (Conklin and Bourne, 1993). The extreme C-terminus of the α-subunit interacts with the receptor and has an important role in defining the specificity of the receptor G-protein interactions (Conklin et al., 1993), although it is not the only region with determinants of specificity. For example, G_{oA} and G_{oB} interact with different receptors, despite being identical at their C-terminus (Kleuss et al., 1991).

The α-subunits of the G_s class of proteins are involved in stimulating adenylyl cyclase activity and regulating calcium channels (Neer, 1995). Activation of adenylyl cyclases by the α-subunits stimulate these enzymes to hydrolyze ATP to cAMP (Gilman, 1987). The α-subunits of the G_i class are predominantly associated with inhibiting adenylyl cyclase activity, but more recently have been shown to regulate K^+ and Ca^{2+} channels and activate cGMP phosphodiesterase (Neer, 1995). The α-subunits of $G_{q/11}$ class of proteins activate phospholipase Cβ to hydrolyze phosphoinositol 4,5 bisphosphate to inositol 1,3,5 triphosphate and diacylglycerol (Simon et al., 1991). Finally, the α-subunits of $G_{12/13}$ class of proteins regulate the N^+/H^+ exchange and also may have a role in regulating cell growth (Neer, 1995; Ulloa-Aguirre and Conn, 1997). More recently studies have shown that the α-subunit of G_{12} proteins activate a signal transduction pathway that includes the small monomeric GTP binding proteins Ras and Rac (Collins, et al., 1996). It is evident that heterotrimeric G-proteins are able to relay signals arising outside the cell with a high degree of specificity by activating distinct cellular components. The versatility of G-proteins is further enhanced by the ability of the βγ-subunits to perform a role in the signal transduction pathways.

3.4 $G_{\beta\gamma}$-subunits: structure and signal transduction

The βγ-subunits form a globular structure with very tight interactions among its different parts (Neer, 1995). The β and γ subunits bind tightly to each other and can only be separated by denaturants, and as a result these two subunits function as a monomer.

All β-subunits are made up of a N-terminal amphipathic helix connected to eight repeating segments of amino acid sequences (Neer, 1995; Simon et al., 1991). Each repeating segment has a class of repeating sequences, containing the amino acids tryptophan and aspartic acid (WD repeats), that are found in proteins that mediate not only signal transduction but also cell division, transcription, processing of pre-mRNA, cytoskeletal assembly and vesicle fusion (Neer, 1995).

The γ-subunits are predicted to be largely α-helical (Lupas et al., 1992). This subunit is prenylated at its C-terminus. Lipidation of the γ-subunit is important for maintaining the signaling characteristics of the βγ-subunit and for membrane attachment (Simon et al., 1991). A 14 amino acid stretch in γ-subunit interacts with the N-terminus of the β-subunit through a disulfide linkage. Although there are many combinations of β and γ-subunits possible, not all occur in nature. For example, β_1 subunit can form a dimer with both γ_1 and γ_2, but β_2 subunit is able to form a dimer only with γ_2 (Neer, 1995). The selectivity of these interactions between the two subunits is determined by the 14 amino acid stretch of the γ-subunit and the WD repeats of the β-subunit (Neer, 1995). The α-subunit interacts with both the β- and the γ-subunits.

Initially the βγ-subunit complex was thought to play a passive role in signal transduction mediated by heterotrimeric G-proteins. However, it is now clear that βγ-subunit plays an active role in signal transduction. One of the main functions of the βγ-subunit is to assemble macromolecular complexes at the cell membrane. For example, this subunit helps to form the ternary complex by bringing the α-subunit and membrane receptors together. In addition, the formation of complexes that include receptors and

specific receptor kinases, such as the β-adrenergic receptor kinase, is facilitated by the ability of βγ-subunit to bind these kinases (Clapham and Neer, 1993).

The βγ-subunit also plays a more direct role in receptor mediated signal transduction by activating many effectors. The βγ-subunit can control the activity of the K^+ channel, independently of the α-subunits (Clapham and Neer, 1993). Moreover, this subunit can stimulate arachidonic acid production by stimulating phospholipase A_2 activity, which enables arachidonic acid to act as a second messenger to stimulate other physiological effects (Jelsema and Axelrod, 1987). In the yeast mating response pathway the βγ-subunit has a central role, and the α-subunit acts as a negative regulator of βγ (Leberer et al., 1992). The βγ-subunit also plays a role in stimulating the production of cAMP from adenylyl cyclase subtype II and IV (Tang and Gilman, 1991), and inhibiting the G_sα-stimulated cAMP production from adenylyl cyclase I by sequestering the G_sα in an inactive trimer. Certain combinations of the βγ-subunit are also able to stimulate PLCβ to produce inositol triphosphates and diacylglycerols (second messengers; Clapham and Neer, 1993). Above mentioned functions of the βγ-subunits suggest that these subunits play an active role in signal transduction by coordinating protein-protein interactions and modulating the activity of various effectors.

3.5 Lipid moieties associated with G-protein

G-proteins as a group are not hydrophobic (Ulloa-Aguirre and Conn, 1997). Lipid modification is crucial in targeting G-proteins to cell membranes (Casey, 1994). Three main types of lipids modify G-proteins. They include palmitoyl, myristoyl and prenyl groups (Casey, 1994).

Palmitoylation (C16:0) of G-proteins is a post-translational event, occurring through a labile thioester bond to a N-terminus cysteine residue (Mumby et al., 1994).

Members of the G_s, $G_{q/11}$ and G_i (except G_t) classes are palmitoylated (Mumby et al., 1994).

Myristoylation (C14:0) of G-proteins is co-translational. Myristoylation occurs at the N-terminal glycine residues (Casey, 1994). The G_i class of G-proteins are myristoylated whereas both the G_s and $G_{q/11}$ proteins are not (Mumby, et al., 1994). Myristoylation is essential for membrane attachment of some G-proteins (Jones et al., 1990). Myristoylation of the β-subunit is necessary for its membrane localization (Neer, 1995).

Prenylation involves the attachment of either a 15-carbon farnesyl or 20-carbon geranylgeranyl isoprenoid to conserved cysteine residues (Casey, 1994). The extent of prenylation on γ-subunits differs among the different subunits (Neer, 1995). Prenylation of the γ-subunits is not necessary for βγ dimer formation but is necessary for membrane attachment and βγ-subunit mediated signal transduction.

4. Scope and aims of the thesis

Previous studies involving GnRH receptor have been hampered by the lack of suitable cell lines. Studies in this thesis characterized four cell lines stably expressing the GnRH receptor (chapters two and three). The aim of these studies was to characterize a non-gonadotrope derived cell line stably expressing the GnRH receptor that has the ability to couple different signal transduction pathways.

The main focus of this thesis is to examine the G-proteins involved in GnRH receptor action in the gonadotrope. Studies up to now had focused on using toxins and second messenger assays to broadly identify potential classes of G-proteins that may be involved in GnRH action. The studies described in this thesis used in vitro labeling with ^{3}H-palmitic acid to identify specific G-proteins that are activated by the GnRH receptor. As will be discussed later (chapters four and five) incorporation of palmitic acid into G-

proteins is dependent on the activation state of the protein. Specific antibodies were used to identify specific G-proteins. It is outside the scope of this thesis to identify all the G-proteins that are activated by the GnRH receptor in the gonadotrope. However, data from toxin and second messenger studies were used to narrow the field of investigation to those G-proteins that may play a role in GnRH action.

5.Contributions

Many of the work described in this thesis were done in collaboration with other scientists. In this section I would like to acknowledge their contributions.

Chapter 2: Drs. Ursala Kaiser and William W. Chin were responsible for making the stable transfections of the GnRH receptor in GH_3 cells. Dr Lothar Jennes was instrumental in performing electronmicroscopy studies on GGH_3 cells.

Chapter3: Vivek Arora was a summer college intern working with me, and Dr. Wageh Awara was a visiting professor who initially looked at the time course of cholera toxin stimulation in GGH_3 cells.

Chapter 4: Jo Ann Janovick and Shaun Brothers did the radioimmuno assays for $G_{q/11}\alpha$ protein in GGH_3 cells.

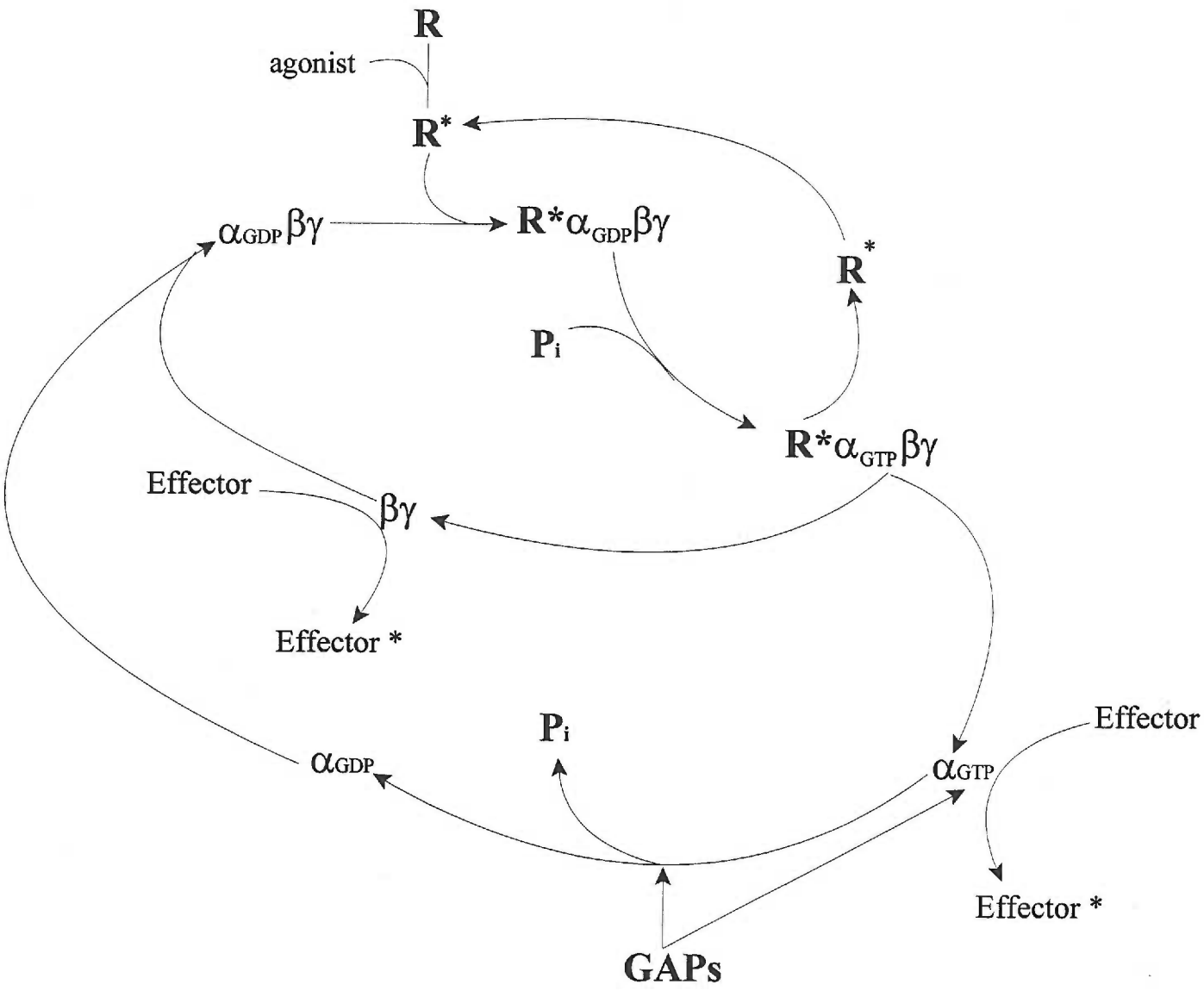
Chapter 5: Shelly Ponder was a summer high school intern working with me. Dr. Tae Ji was helpful in obtaining different G-protein plasmids, and discussing the data.

Chapter 6: Drs. Tom Wilkie and Stefan Offermanns made the G_{11} and G_q knockout mice, respectively.

Chapter 7: Dr. J.H. Pinter and Jo Ann Janovick were helpful in discussing the models described in this chapter.

In many of these studies ms. Jo Ann Janovick either repeated or performed assays described in this thesis.

Figure 1-1. GTP regulatory cycle of Gα subunits. R: receptor; R*: agonist bound receptor; GDP: guanosine diphosphate; GTP: guanosine triphosphate; GAPs: GTPase activating proteins; Effector*: activated effector.


R
agonist
R*
$\alpha_{GDP}\beta\gamma$
$R^*\alpha_{GDP}\beta\gamma$
R*
P_i
$R^*\alpha_{GTP}\beta\gamma$
Effector
$\beta\gamma$
Effector *
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Effector
α_{GDP}
α_{GTP}
Effector *
GAPs

Table 1-1: Classes of G_α Subunits, their members and identified functions

Class	Members	Functions
α_s	α_s, α_{olf}	Stimulate Adenylyl Cyclase, regulate Ca^{2+} channels
α_i	α_{i1-3}, α_o,α_{t1-2}, α_{gust}, α_z	Inhibit Adenylyl Cyclase, regulate K^+ and Ca^{2+} channels, activate cGMP phosphodiesterase
α_q	α_q, α_{11}, α_{14}, α_{15}, α_{16}	Activate PLCβ
α_{12}	α_{12}, α_{13}	Regulate Na^+/H^+ exchange

CHAPTER TWO

FUNCTIONAL AND MORPHOLOGICAL CHARACTERIZATION OF FOUR CELL LINES DERIVED FROM GH_3 CELLS STABLY TRANSFECTED WITH GnRH RECEPTOR cDNA

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Abstract

Four cell lines, stably transfected with rat GnRH receptor cDNA, have been prepared from the lactotropic GH_3 cell line. All four lines (as well as the parent line and a line transfected with the vector DNA) show extensive rosettes of circular polyribosomes, characteristic of high protein synthetic activity, although secretory granules are virtually absent; the rough endoplasmic reticulum (rER) cisternae were short and straight. Instances were observed in which the ER reaches to the plasma membrane, suggesting a possible non-granular secretory route. All four lines (but not the parent or a control transfected line) expressed GnRH receptors which are down-regulated (1-5 h, depending on the cell line) following exposure to 10 nM GnRH; receptors then recover (2-7 h). This pattern is reminiscent of the GnRH receptor in the primary gonadotrope cell cultures. All cell lines released prolactin (4-96 h) in response to a GnRH agonist (D-tBuSer^6-desGly^{10}-Pro^9 ethylamide-GnRH), an event which was inhibited by all three major classes of Ca^{2+} ion channel antagonists (methoxyverapamil, 1,4 dihydropyridines, and diltiazem); in contrast, GnRH-stimulated LH release from pituitary derived primary cultures is only inhibited by methoxyverapamil. One line became refractory to GnRH analog stimulation after 24 h, although the other three released prolactin vigorously up to the longest time point examined (96 h). All four lines responded substantially more robustly to 1 μg/ml Buserelin than to 1 μg/ml TRH. All four lines produced IP metabolites and released immunoassayable cyclic AMP (24 h) in response to treatment with Buserelin. These cell lines are good models for understanding the mechanisms by which the GnRH receptor is coupled to second messenger systems, and for comparing these mechanisms with TRH receptor coupling in the same cell.

Introduction

The GnRH receptor has been cloned from mouse (Tsutsumi et al., 1992; Reinhart 1992; Eidne et al., 1992), rat (Eidne et al., 1992; Kaiser et al., 1992; Perrin et al., 1993) and human (Kakar et al., 1992) sources. The sequences of the receptor from these sources are substantially homologous, and all lack the intracellular C-terminal extension usually associated with 7-transmembrane sequence (7-TMS) receptors. Because this region is associated with specific biological functions, including desensitization and down-regulation (Liggett et al., 1993), of some of the 7-TMS receptors, we sought to express the GnRH receptor in a lactotrope-derived cell line in order to determine whether stimulus and secretion coupling, down-regulation and desensitization are preserved. The four cell lines discussed here show different physiological characteristics with regard to desensitization and coupling to second messenger systems, thereby providing good comparative models for the study of the GnRH receptor and the functions which it moderates.

Materials and Methods

Transfection of GH_3 Cells

Four clonal GH_3 cell lines containing the rat GnRH receptor sequence (GGH_31', GGH_32', GGH_36', and GGH_312' cells, collectively referred to as GGH_3 cells; Kaiser et al., 1994) were used in these studies. GH_3 cells were stably transfected with the rat GnRH receptor as previously reported (Kaiser et al., 1994). Briefly cells were maintained in a monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 C in humidified 5% CO_2 and 95% air. Cells were suspended in 0.4 ml of Dulbecco's phosphate buffered saline plus glucose containing 10 μg of pcDNA1-GnRHR expression vector and 0.5 μg pSVneo (which carries the neomycin-resistance gene), and electroporated with a single electrical pulse at 220 V from a total capacitance of 1000 microfarads, using an Invitrogen (San Diego, CA) Electroporator II apparatus. After electroporation, cells were plated in serum containing media. Forty eight hours after transfection, cells were grown in the presence of 600 μg /ml G418 (Gibco). Neomycin-resistent cell clones were selected, expanded, and tested for binding of GnRH and GnRH responsiveness. The transfected cells were maintained in an atmosphere of 5% CO_2 at 37 C in DMEM (obtained from the UI Diabetes and Endocrine Research Center, supported by DK 25295) containing 10% fetal calf serum (HyClone Laboratories, Inc., Logan, UT) and 20 μg/ml gentamicin (Sigma, St. Louis, MO).

Electron microscopy

Cells were fixed in 4% glutaraldehyde in phosphate buffered saline, followed by incubation in 1% osmium tetroxide and dehydration through a series of ethanols with increasing concentrations. Cells were embedded in Epon 812, sectioned, counterstained with uranyl acetate and lead citrate, and examined and photographed with a Hitachi H 7000 electron microscope. At least ten sections were examined from each of the four

transfected cell lines as well as the parent line (GH_3) and a line transfected with vector DNA.

Quantitation of prolactin and cyclic AMP

Cells were cultured in DMEM/10% FCS containing 20 μg/ml gentamicin (Sigma) until nearly confluent in 162 cm^2 T-flasks (Costar, Cambridge, MA). Cells were scraped and replated for 24-48 h in 24-well culture plates. Prior to use in prolactin and cyclic AMP release studies, the adherent cells were washed twice in DMEM containing 0.1% bovine serum albumin (Irvine Scientific, Santa Ana, CA) and 20 μg/ml gentamicin (DMEM/BSA). Release was measured following incubation in a 1 ml volume containing the indicated secretogogues or test substances. The treatment solutions were changed after 48 h as indicated. Prolactin release was measured by RIA using materials obtained from the Hormone Distribution Program of the National Pituitary Agency, NIDDK. Prolactin was radioiodinated by standard procedures (Hunter and Greenwood, 1962). Data shown are the mean of triplicate assay wells. The SEM was typically <10% of the mean. Intra- and interassay variances were 5% and 7%, respectively.

Cyclic AMP production was measured after 24 h. The transfected cells were stimulated with Buserelin (10^{-12} to 10^{-6} g/ml when measuring dose-response relations) in DMEM/0.1% BSA containing 0.2 mM methylisobutylxanthine (MIX) to prevent degradation of cyclic AMP. After stimulating the GGH_3 cells, the samples were collected in tubes containing sufficient theophylline for a final concentration of 1 mM. The samples were heated (95 C) for 5 min to destroy phosphodiesterase activity. RIA of cyclic AMP was performed by a modification of the method of Steiner et al. (1972), with the addition of the acetylation step described by Harper and Brooker (1975). Cyclic AMP antiserum C-1B (prepared in our laboratory; Andrews et al., 1986) was used at a titer of 1:5,100. This

antiserum showed less than 0.1% cross reaction with cyclic GMP, 2':3'-cyclic AMP, 5'-cyclic AMP, 3'-cyclic AMP, ADP, GDP, ATP, CTP, MIX, or theophylline.

Measurement of inositol phosphate production

All four GGH_3 cell lines (Kaiser et al., 1994), were suspended and plated in DMEM/10% FCS/20 μg/ml gentamicin for 24 h at 37 C, 5% CO_2. Cells were washed twice with DMEM/0.1% BSA/20 μg/ml gentamicin, then incubated in 0.5 ml DMEM (inositol free) containing 4 μCi/ml [^{3}H]-inositol for 18 h at 37 C. After the pre-loading period, cells were washed twice with DMEM (inositol free) containing 5 mM LiCl, and incubated at 37 C with DMEM/LiCl (inositol free) containing the indicated treatments for the indicated times. The treatment solutions were removed and 1 ml 0.1 M formic acid was added to each well. Cells were frozen and thawed to disrupt cell membranes. IP accumulation was determined by Dowex anion exchange chromatography and liquid scintillation spectroscopy as previously described (Huckle and Conn, 1987; McArdle et al., 1987). Data shown are the mean of triplicate assay wells.

Down-regulation of the GnRH receptor

All four cell lines were separately suspended in DMEM/ 10% FCS/ 20 μg/ml gentamicin then plated in 6-well culture plates for 24 h at 37 C, 5% CO_2. Cells were washed twice with 37 C DMEM/ 0.1% BSA, treated with 10 nM GnRH (a desensitizing dose) or medium alone for the indicated times, and washed 3 times (4 ml/well) with 23 C DMEM/BSA to remove excess GnRH. The medium was decanted and replaced with 2 ml/ well of 0.4 μCi/ml ^{125}I-Buserelin (^{125}I-iodoTyr^5-D-tBuSer^6-desGly^{10}-Pro^{9}ethylamide-GnRH, Hoechst, which was labeled as previously reported; Marian and Conn, 1980). Binding was assessed after 30 min (23 C). Nonspecific binding was determined in the presence of 10 μM unlabeled GnRH. Binding was terminated by decanting the

radioligand-containing medium and placing the cells on ice. Cells were washed twice with ice cold DMEM/BSA. Cells were then collected by scraping in 1 ml DMEM/BSA containing 2.5 mM EGTA (4 C) twice. The cell lysate was layered over 2 ml 0.3 M sucrose in DMEM and the pellet collected by centrifugation (10 min, 2,000 x g, 4 C). The radioactivity of each pellet was determined using a Beckman 5500 gamma counter.

Results

The general morphology of the stably transfected cell lines is indistinguishable from either the vector-transfected cells or the parent GH_3 cells. The cell nuclei are usually round with 2 or 3 prominent nucleoli, a relatively small marginal heterochromatin display is present and most of the nuclei are occupied by euchromatin (figs. 1A, 1B). The cells contain a large number of rosettes of circular polyribosomes and several short, sometimes curved, stretches of rough endoplasmic reticulum (rER) (figs. 1C, 1D, 1F). The cisternae of the rER are not arranged in parallel stacks but instead they occur as individual structures throughout the cytoplasm without any obvious preferential orientation. Sometimes, the rER cisternae are seen in juxtaposition to the plasma membrane (fig. 1F, arrowhead). Several Golgi stacks are present in the perinuclear region of the cytoplasm and many small, sometimes electron dense, granules with a diameter of up to 100 nm can be seen next to the Golgi cisternae (fig. 1E). In general, larger electron dense granules are rare while secondary lysosomes, identified by their heterogeneous content, are a common feature of the transfected and parent cell line.

Figure 2 shows the homologous down-regulation and recovery of the GnRH receptor expressed in the four cell lines, after being continuously exposed to 10 nM GnRH. Down-regulation of the GnRH receptors in the GGH_31' cells took more time (5 h after treatment) than the other three cell lines. While GGH_32', GGH_36' and GGH_312' cells took 2 h to reach maximal down regulation; GGH_31' cells took 5 h. Parent or the control-transfected cell lines did not show measurable GnRH receptor (data not shown).

Prolactin release from GGH_31', GGH_32', GGH_36' and GGH_312' was coupled to the occupancy of the GnRH receptor by the GnRH agonist Buserelin in a time- and dose-dependent manner (figs. 3 and 4). MIX, a phosphodiesterase inhibitor, potentiated the action of Buserelin stimulated prolactin release in GGH_31' and GGH_32' cells (figs. 4A & 4B). The stimulated release of prolactin was linear for 96 h (duration of the experiment)

in three cell lines ($GGH_3$1', $GGH_3$2' and $GGH_3$12'; figs. 3A, 3B and 3D). $GGH_3$6' cells showed a decrease in the rate of prolactin release to the homologous agonist after 24 h (fig. 3C). TRH, a secretogogue in primary lactotropes and GH_3 cells, was a relatively poor secretogogue in all four cell lines examined. TRH stimulated modest prolactin release in $GGH_3$2' cell line, which was additive with Buserelin-stimulated prolactin release.

Similar to primary cell cultures prepared from weanling female rats (Huckle and Conn, 1987; Andrews and Conn, 1986), all four cell lines showed dose dependent IP release (fig. 5).

Unlike primary cultures prepared from weanling female rats (Conn et al., 1979), Buserelin (GnRH analog) stimulated the dose dependent cyclic AMP release from all four cell lines (fig. 6). Near maximal levels of cyclic AMP release was seen with agonist concentrations of 10^{-12} g/ml for $GGH_3$1', $GGH_3$2', and $GGH_3$6' cells. As expected, inhibition of phosphodiesterase activity by MIX was necessary to see measurable release of cyclic AMP. In the absence of MIX the accumulation of measurable amounts of cyclic AMP at basal levels in all four cell lines were low. TRH stimulated cyclic AMP release only in $GGH_3$2' cells.

The possibility of cross talk between protein kinase C (PKC) and cyclic AMP production was examined by stimulating PKC activity with phorbol myristyl acetate (PMA) and assaying for cyclic AMP release in the presence of MIX (0.2mM). This resulted in no dose dependent release of cyclic AMP from any of the four cell lines over the range of 0 to 100 μg/ml PMA, indicating that PKC activation does not increase cyclic AMP accumulation (fig. 7). This makes it unlikely that the action of Buserelin on cyclic AMP accumulation is mediated by PKC.

To examine the dependence of the GnRH-agonist stimulated prolactin release on extracellular Ca^{2+}, GGH_3 cells were treated with drugs from the three major classes of

Ca^{2+}-channel antagonists: diltiazem, nifedipine and methoxyverapamil (D600). GnRH agonist-stimulated prolactin release was seen to be sensitive to all three major classes of Ca^{2+}-channel inhibitors (fig. 8). A 10^{-4} M concentration of Ca^{2+} channel inhibitors was effective in ablating the response to Buserelin. A trypan blue exclusion assay showed that only a 10^{-4} M dose of D600 (24 h) were toxic to GGH_312' cells with a lethality rate approaching 50%. No apparent cell death was observed in the other cell lines or with other drugs at 24 h.

Discussion

In this study, four GH_3 derived cell lines stably transfected with rat GnRH receptor cDNA were characterized. All four cell lines showed similar morphologies as studied with electron microscope including rosettes of 4-5 ribosomes (circular polyribosomes), and virtual absence of secretory granules. These morphological features indicate high protein synthetic activity and regulation of prolactin release at the level of translation or transcription. The close proximity of the rough ER with the plasma membrane may suggest a potential path of release for the synthesized prolactin from the cell. No major differences from the parent (GH_3) line were observed.

All four cell lines showed biphasic regulation of GnRH receptor, similar to that reported in primary cells (Conn et al., 1984). The time-course of down-regulation is similar in GGH_32', GGH_36', GGH_312', and primary cells (approximately 2 h). However, GGH_31' cells require longer time to down-regulate (approximately 5 h). The ability of the GnRH receptor to down-regulate in lactotropes, which do not normally express this receptor, suggests that down-regulation does not require cell specific components other than the receptor itself. The GnRH receptor does not have the long intracellular C-terminal region characteristic of 7-TMS receptors. This region is believed to be required for down-regulation and desensitization for some receptors (Liggett et al., 1993), although it is apparently not required for these actions in these transfected GnRH receptors.

The prolactin response of the GGH_36' cell line to Buserelin was desensitized after 24 h of continuous exposure to 1 μg/ml Buserelin. GGH_31', GGH_32', and GGH_312' cells showed no measurable desensitization in response to the continuous presence of the agonist, and continued to release prolactin at a steady rate even after 96 h.

Down-regulation and recovery of the GnRH receptors in GGH_36' cells occurs before the appearance of homologous desensitization. Therefore the loss of receptors

does not likely account for the observed desensitization in these cells. The ability to maintain prolactin release in the presence of receptor down-regulation suggests the presence of "spare" receptors in the GGH_3 cells.

Surprisingly, TRH, a secretogogue in lactotropes (Sheward et al., 1983), was a poor secretogogue in all four GGH_3 cell lines. This may be due to the uncoupling of the TRH stimulated prolactin release or the absence of receptors for TRH. In binding studies not shown here (Kaiser, unpublished), all four GGH_3 cell lines showed measurable TRH receptors. However only one clonal cell line, namely GGH_32', responded substantively with prolactin release to TRH, which was additive with Buserelin, indicating that the GnRH agonist induced prolactin release and TRH induced prolactin release are mediated through two distinct mechanisms in this cell line or that the TRH receptor is not well coupled to its response effector system. Although we have not tested individual cells for the presence of both TRH and GnRH receptors, the GGH_32' cells were derived from a single clone.

The role of cyclic AMP in the pituitary lactotrope cultures is not clear. It is known that some neuropeptides that enhance prolactin release do not increase pituitary cyclic AMP levels (Lamberts and Macleod, 1990). At the same time, cyclic AMP or its analogues can increase prolactin release (Delbeke et al., 1984), but addition of dopamine even in the presence of high levels of cyclic AMP abolish prolactin release (Delbeke and Dannies, 1985; Delbeke et al., 1986). Cyclic AMP has been suggested to increase the responsiveness of the intracellular Ca^{2+} system in the lactotropes (Delbeke and Dannies, 1985). In GGH_3 cells cyclic AMP production increases in a dose dependent manner in response to Buserelin, and in GGH_31' cells, this cyclic nucleotide fulfills the role of a second messenger (Kuphal et al., 1994); i.e., GnRH stimulates cyclic AMP release, cyclic AMP analogs stimulate increased prolactin release and phosphodiesterase inhibitors potentiate GnRH action. Furthermore we have shown that PKC is not involved in cross

talk between IP and cyclic AMP second messenger systems, and MIX potentiated the action of Buserelin in GGH_31' and GGH_32' cells. Indicating that the Buserelin stimulated increase in cyclic AMP may be due to the coupling of the GnRH receptor with cyclic AMP dependent second messenger pathway. These results suggest that the GnRH receptor in GGH_3 cells may use at least one of the pathways utilized by the pituitary prolactin releasing mechanisms, and a distinct mechanism is not necessary for its function. As in primary cultures of lactotropes (Lamberts and Macleod, 1990), TRH has minimal effect on cyclic AMP release from GGH_3 cells.

GGH_3 cells show a dose-dependent release of inositol phosphates when stimulated with Buserelin. In GH_3 cells, TRH stimulates the hydrolysis of phosphoinositol 4,5, bisphosphate by phospholipase C, leading to increased levels of inositol phosphates and diacylglycerols (Kolesnick and Gershengorn, 1984). These effects of the TRH receptor are mediated by a guanine nucleotide binding protein (Lucas et al., 1985; Hsieh and Martin, 1992; Aragay et al., 1992). The GnRH receptor in GGH_3 cells appear to be coupled to a similar mechanism as is the TRH receptor in GH_3 cells. The TRH receptor appears to be uncoupled from its signal transduction mechanism in all but one cell line (GGH_32'; Janovick and Conn, 1994). Interestingly this cell line shows additive release of prolactin when stimulated with Buserelin and TRH.

In primary lactotropes and clonal GH_3 cell lines, Ca^{2+} plays an important role in the biphasic release of prolactin (Lamberts and Macleod, 1990). In these cells both internal (for the transient peak) and external (for the plateau phase) Ca^{2+} are used in regulating the release of prolactin, and Ca^{2+} channel blockers can inhibit the plateau phase of prolactin release (Lamberts and Macleod, 1990). LH release from pituitary cells in response to GnRH, veratridine and maitotoxin can be blocked by the Ca^{2+} channel antagonist D600 (Conn et al., 1987), whereas 1,4 dihydropyridines and diltiazem have no antagonistic action on GnRH stimulated LH release in primary pituitary cell cultures

from female weanling rats (Conn et al., 1983; Conn et al., 1983c). Although other investigators have reported studies of 1,4 dihydropyridines inhibiting LH release from pituitaries derived from adult rats (Chang et al., 1986). In contrast, all three Ca^{2+} channel inhibitors blocked Buserelin stimulated prolactin release from GGH_3 cells.

The rat GnRH receptor in primary pituitary cells is linked to multiple G-protein-coupled second messenger systems (Barnes and Conn, 1993; Hawes et al., 1993; Hawes and Conn, 1992). In a similar manner, the GnRH receptor in GGH_3 cells may be coupled to G-proteins that are involved in the inositol phosphate and cyclic AMP second messenger systems. This suggests that the GnRH receptor can recruit available G-protein-coupled second messenger systems to mediate its cellular functions.

Figure 2-1. Electron micrographs of GGH_3 cells showing vector-transfected (figs. 1A, 1C), and vector and insert transfected cells (figs. 1B, 1D, 1E, 1F). No differences are apparent in the morphological features of the different cell lines as judged by the appearances of the nuclei, Golgi apparatus, mitochondria, smooth and rough endoplasmic reticula, polysomes and occasional small secretory granules. Note the close proximity of a rough endoplasmic reticulum cisterna to the plasma membrane (fig. 1F, arrow head). Scale bars: figs. 1A, 1B = 2 μm; figs 1C, 1F = 0.5 μm; figs. 1D, 1E = 1 μm.

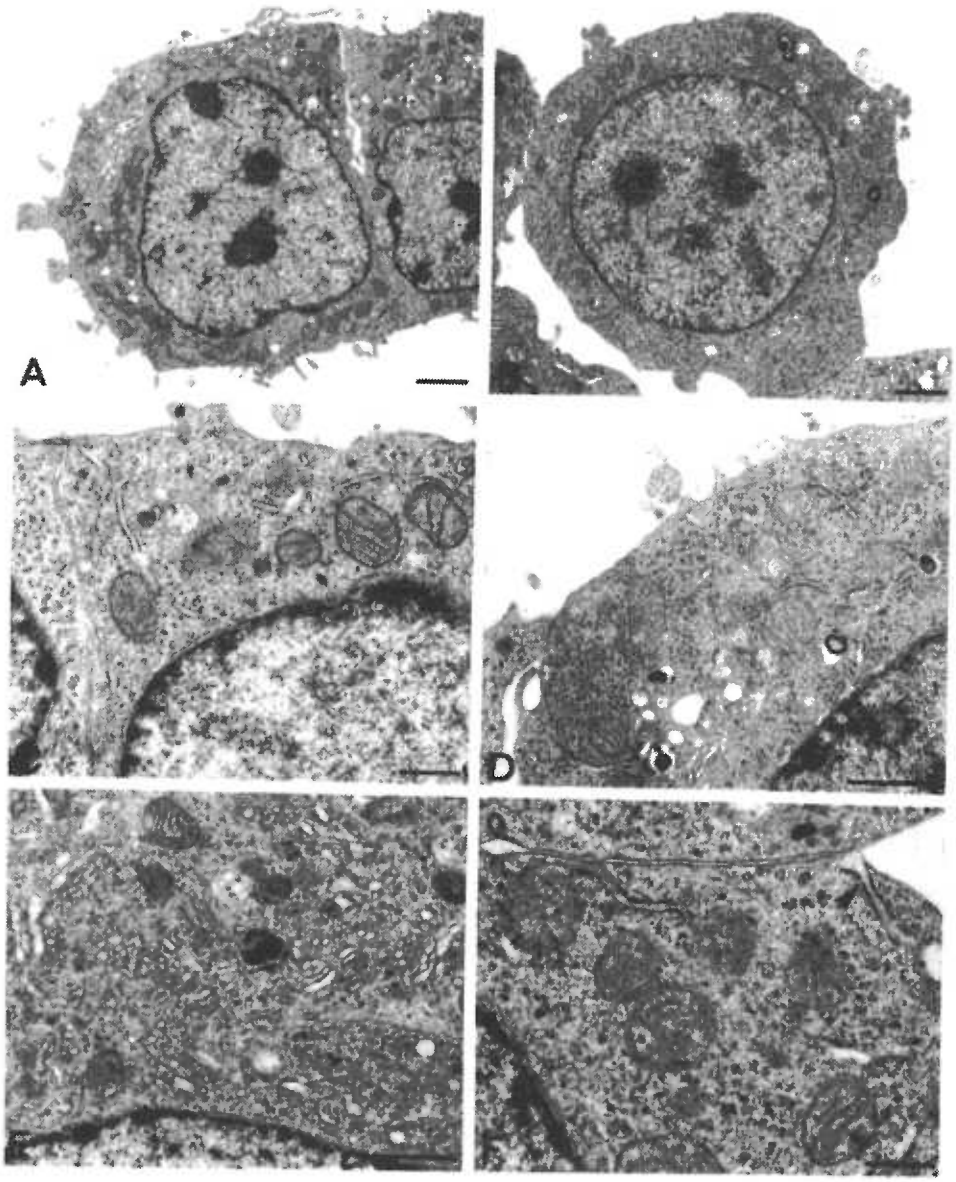

A
D

Figure 2-2. Down-regulation and up-regulation of the GnRH receptor in GGH_31', GGH_32', GGH_36' and GGH_312' cells. Cells were maintained and treated as described in Methods. Data shown are the mean of triplicate treatments, and the error bars shows the SEM. Each experiment was repeated at least three times with similar results.

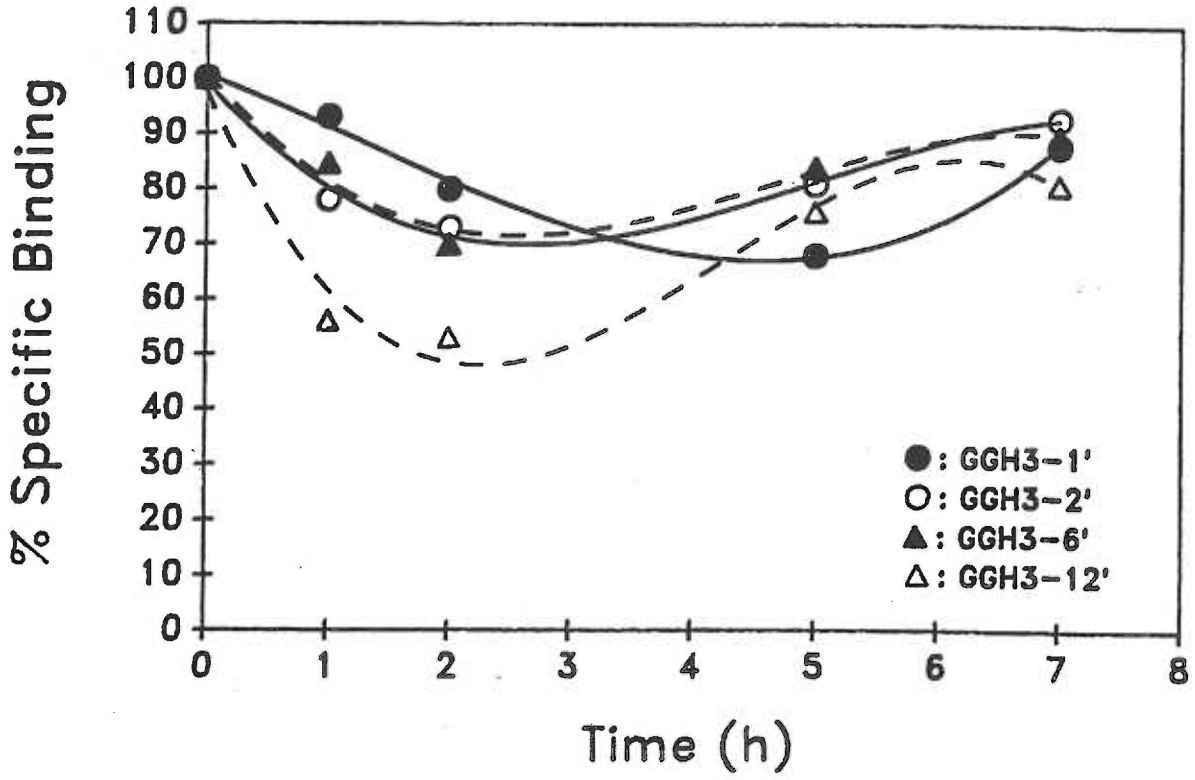

% Specific Binding
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
Time (h)
●: GGH3-1'
○: GGH3-2'
▲: GGH3-6'
△: GGH3-12'

Figure 2-3. Time-course of prolactin release in response to various secretogogues in $GGH_3$1' (A), $GGH_3$2' (B), $GGH_3$6' (C) and $GGH_3$12' (D) cells respectively. GGH_3 cells were incubated with the vehicle, Buserelin (0.01 μg/ml), TRH (1 μg/ml) or Buserelin (0.01 μg/ml) and TRH (1 μg/ml) for 24, 48, 72 and 96 h. The treatment solutions were replaced after 48 h. Prolactin release was determined by RIA. Data shown are the mean of triplicate treatments, and the error bars shows the SEM. Each experiment was repeated at least three times with similar results.

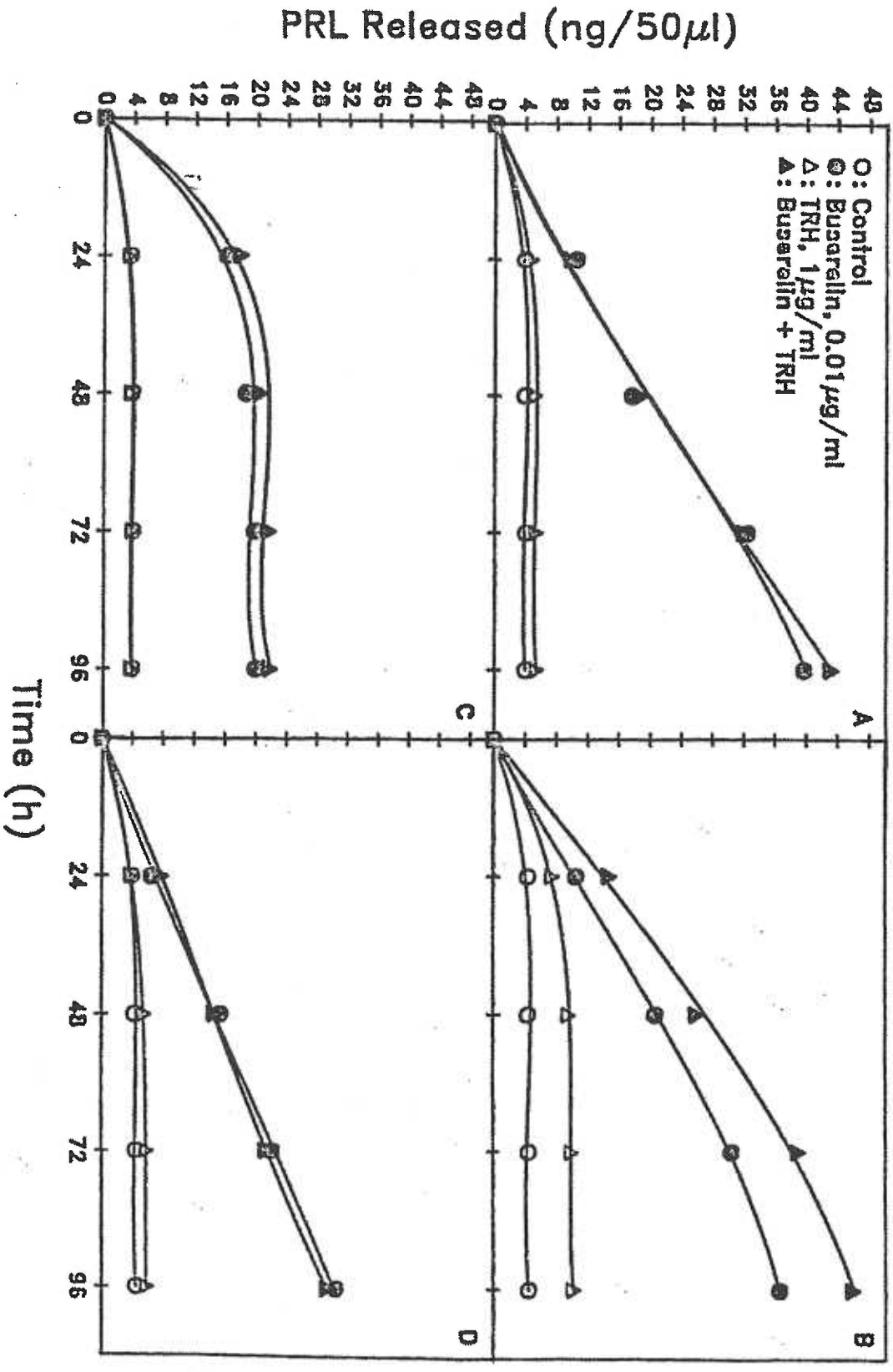

PRL Released (ng/50μl)
Time (h)
O: Control
●: Buserelin, 0.01μg/ml
△: TRH, 1μg/ml
▲: Buserelin + TRH
A
B
C
D

Figure 2-4. Dose-response curves for Buserelin stimulated prolactin release in GGH_31' (A), GGH_32' (B), GGH_36' (C) and GGH_312' (D) cells respectively. Cells were treated with the indicated concentrations of Buserelin, Buserelin and MIX (0.2 mM), Buserelin and TRH (1 μg/ml) and Buserelin, TRH (1 μg/ml) and MIX for 24 h. Prolactin release was determined by RIA. Data shown are the mean of triplicate treatments, and the error bars shows the SEM. Each experiment was repeated at least three times with similar results.

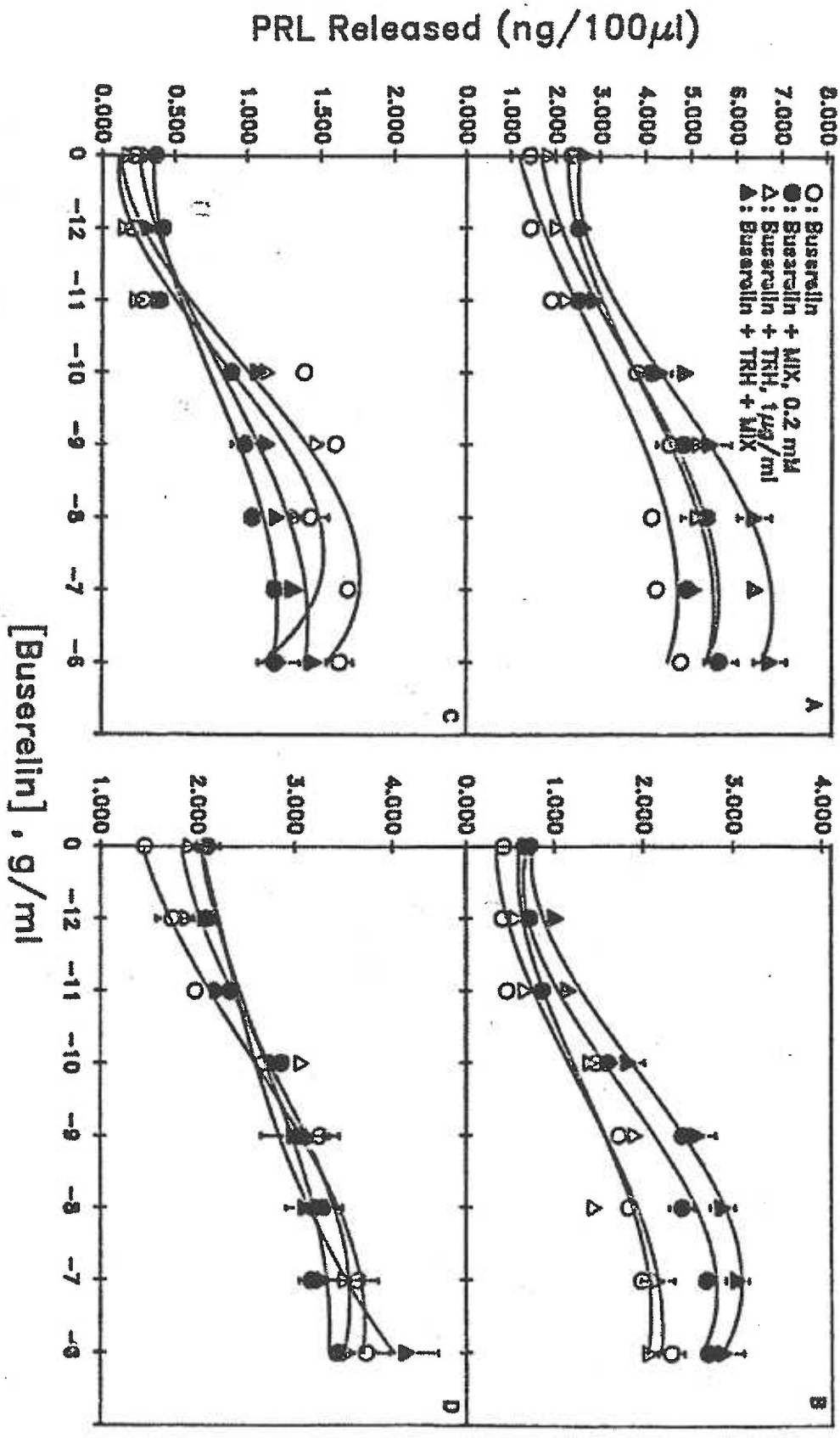

PRL Released (ng/100μl)
[Buserelin] , g/ml
○: Buserelin
●: Buserelin + MIX, 0.2 mM
△: Buserelin + TRH, 1μg/ml
▲: Buserelin + TRH + MIX
A
B
C
D

Figure 2-5. Dose-response curves for Buserelin stimulated IP production in GGH_31', GGH_32', GGH_36' and GGH_312' cells respectively. Cells were treated with the indicated concentrations of Buserelin for 2 h. Total IP production was determined as described in Methods. Data shown are the mean of triplicate treatments, and the error bars shows the SEM. Each experiment was repeated at least three times with similar results.

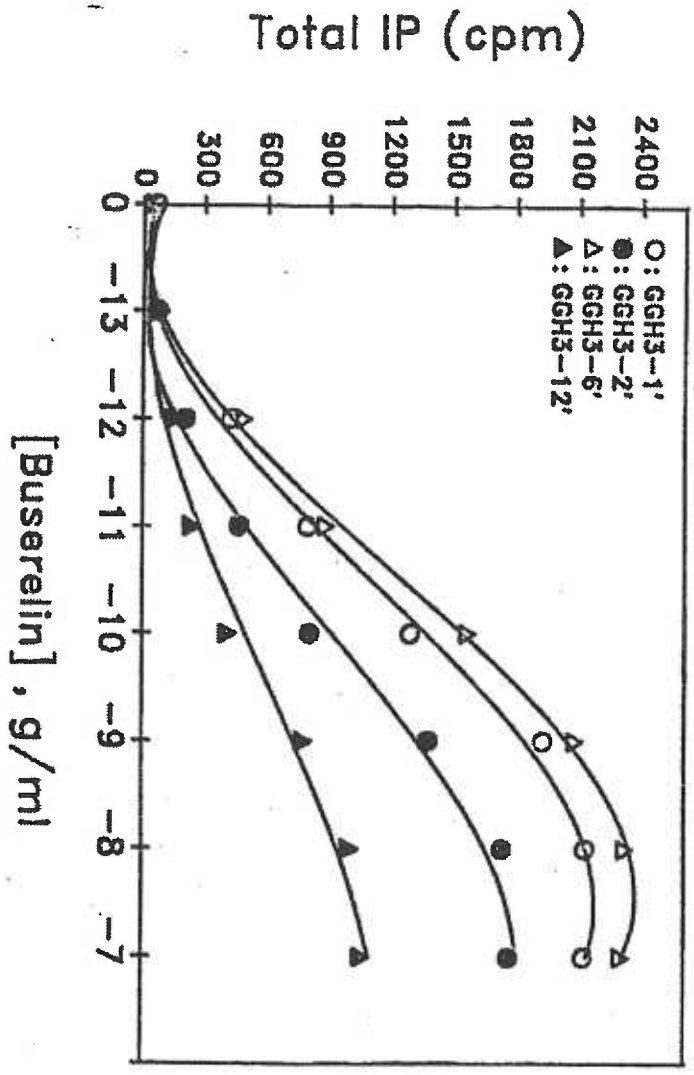

Total IP (cpm)
2400
2100
1800
1500
1200
900
600
300
0
0
-13
-12
-11
-10
-9
-8
-7
[Buserelin], g/ml
○: GGH3-1'
●: GGH3-2'
△: GGH3-6'
▲: GGH3-12'

Figure 2-6. Dose-response curves for Buserelin stimulated cyclic AMP release in GGH_31' (A), GGH_32' (B), GGH_36' (C) and GGH_312' (D) cells respectively. Cells were treated with the indicated concentrations of Buserelin, Buserelin and MIX (0.2 mM), Buserelin and TRH (1 μg/ml) and Buserelin, TRH (1 μg/ml) and MIX for 24 h. cyclic AMP release was determined by RIA. Data shown are the mean of triplicate treatments, and the error bars shows the SEM. Each experiment was repeated at least three times with similar results.

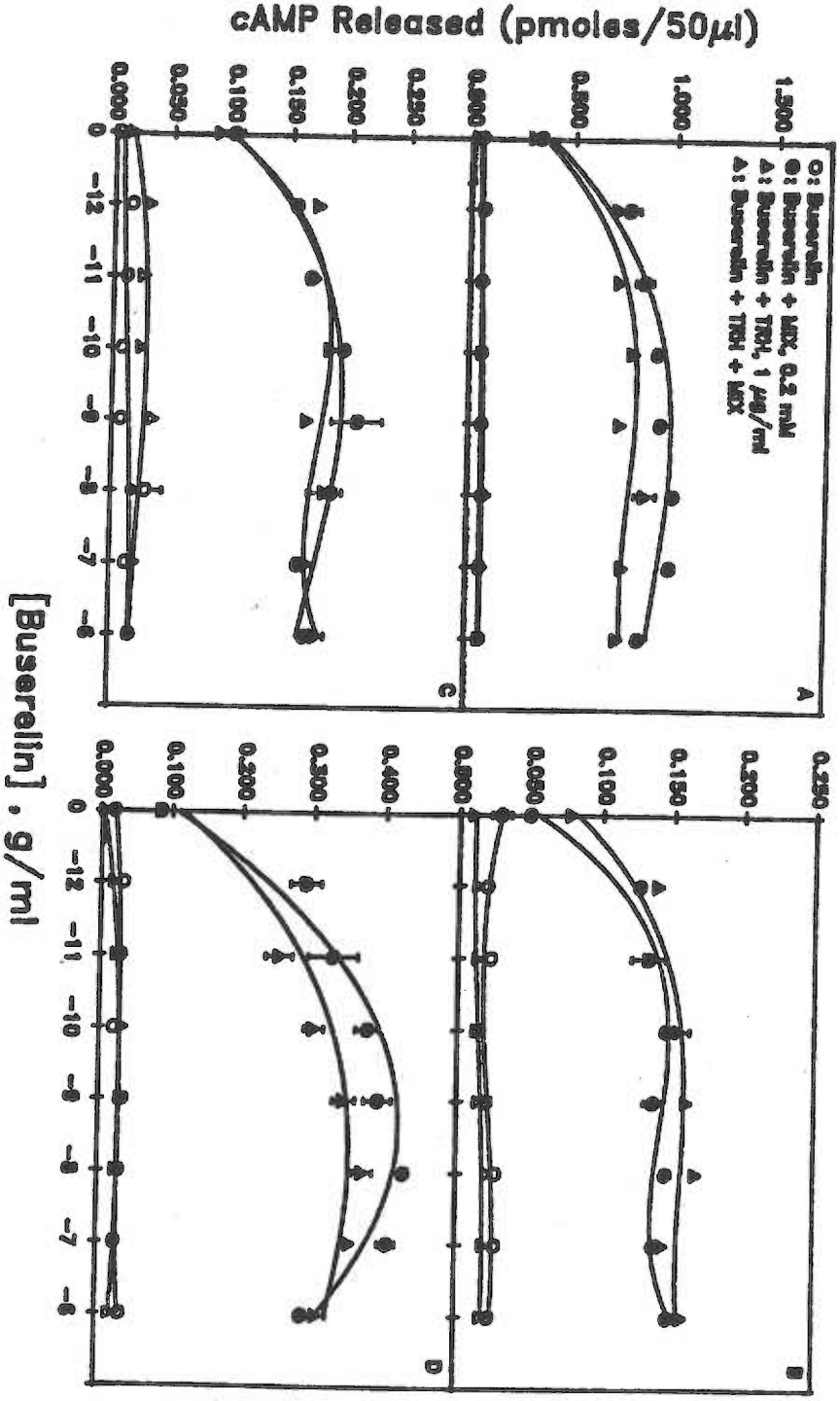

cAMP Released (pmoles/50μl)
[Buserelin] · g/ml
○: Buserelin
●: Buserelin + MIX, 0.2 mM
△: Buserelin + TRH, 1 μg/ml
▲: Buserelin + TRH + MIX
A
B
C
D

Figure 2-7. Effect of phorbol myristyl acetate (PMA) on cyclic AMP accumulation in GGH_31', GGH_32', GGH_36' and GGH_312' cells. Cells were stimulated with the indicated concentrations of PMA for 24 h in the presence of MIX (0.2 mM). Cyclic AMP release was determined by RIA. Data shown are the mean of triplicate treatments, and the error bars shows the SEM. Each experiment was repeated at least three times with similar results.

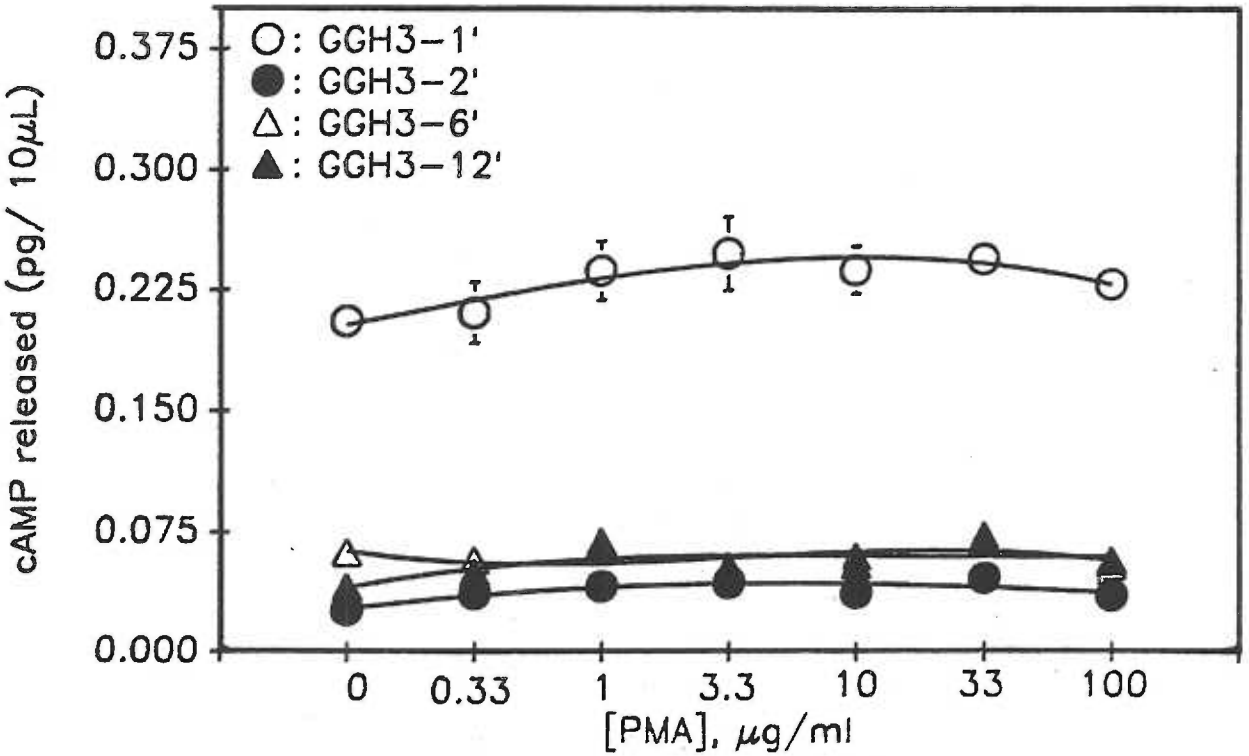
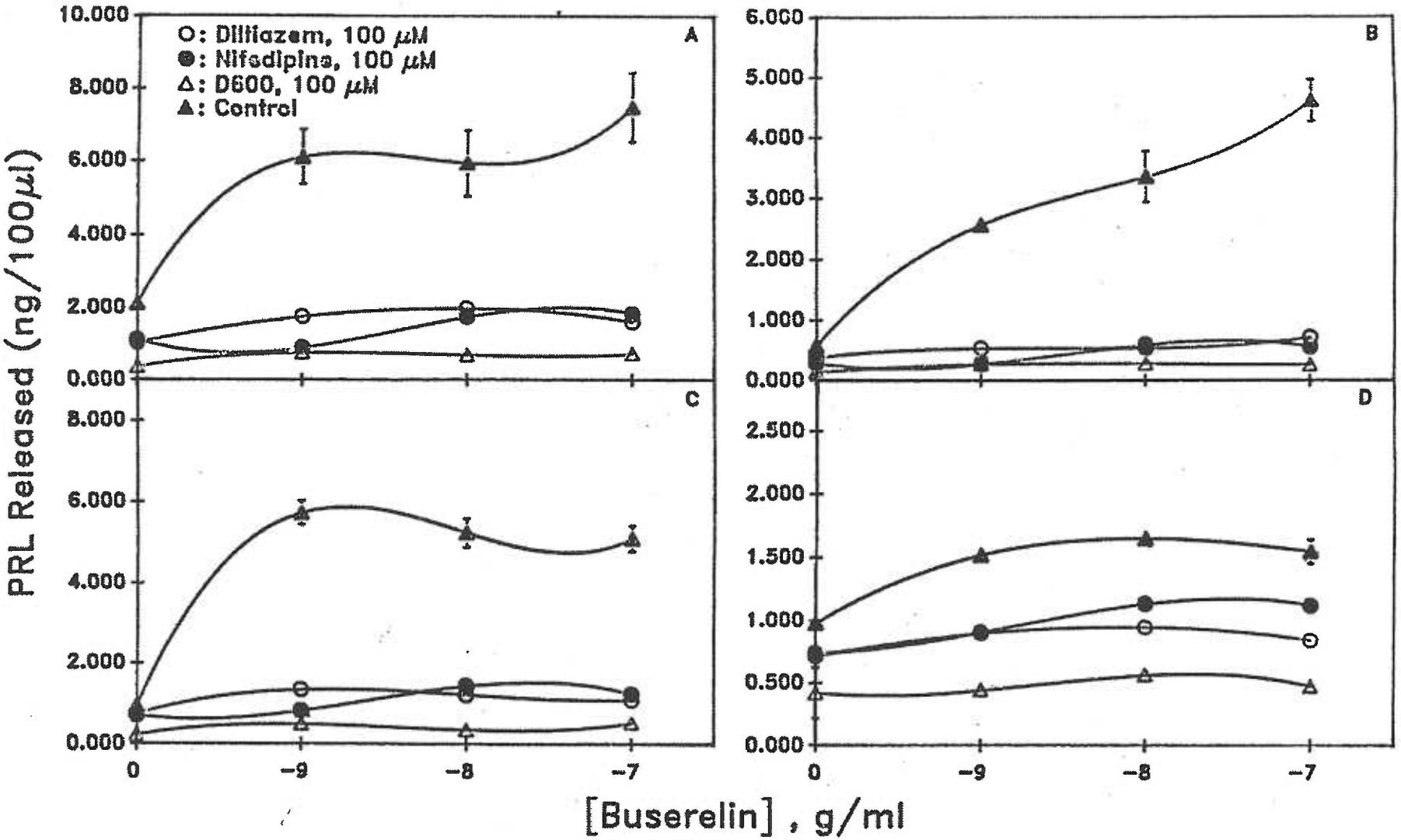

○: GGH3−1'
●: GGH3−2'
△: GGH3−6'
▲: GGH3−12'
cAMP released (pg/ 10μL)
0.375
0.300
0.225
0.150
0.075
0.000
0
0.33
1
3.3
10
33
100
[PMA], μg/ml

Figure 2-8. Effect of inhibition of Ca^{2+} ion channels on Buserelin stimulated prolactin release in GGH_31' (A), GGH_32' (B), GGH_36' (C) and GGH_312' (D) cells respectively. Cells were treated with diltiazem (100 μM), nifedipine (100 μM) or D600 (100 μM) and the indicated concentrations of Buserelin, or the vehicle with the same Ca^{2+}-channel antagonists and concentrations. Cells were treated for 24 h. Prolactin release was determined by RIA. Data shown are the mean of triplicate treatments, and the error bars shows the SEM. Each experiment was repeated at least three times with similar results.


○: Diltiazem, 100 μM
●: Nifedipine, 100 μM
△: D600, 100 μM
▲: Control
A
B
C
D
PRL Released (ng/100μl)
[Buserelin] , g/ml
10.000
8.000
6.000
4.000
2.000
0.000
5.000
3.000
1.000
2.500
1.500
1.000
0.500
0
-9
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-7

CHAPTER THREE

BIPHASIC ACTION OF CYCLIC AMP IN GnRH ANALOG-STIMULATED HORMONE RELEASE FROM GH_3 CELLS STABLY TRANSFECTED WITH GnRH RECEPTOR cDNA

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Abstract

GH_3 cells are a prolactin-secreting adenoma cell line derived from pituitary lactotropes. These cells have been stably transfected with rat GnRH receptor cDNA to produce four cell lines: GGH_31', GGH_32', GGH_36', and GGH_312'. In response to either GnRH or to Buserelin (a metabolically stable GnRH agonist), these cell lines synthesize prolactin in a cyclic AMP dependent manner. Only GGH_36' cells desensitize in response to persistent treatment with 10^{-7} g/ml Buserelin. GGH_31', GGH_32' and GGH_312' cells, however, can be made refractory to Buserelin stimulation by raising cyclic AMP levels either by addition of dibutryl cyclic AMP to the medium or by treatment with cholera toxin. In GGH_3 cells, low levels of cyclic AMP fulfill the requirements for a second messenger, while higher levels appear to mediate the development of desensitization. The observation that, in GGH_36' cells, cyclic AMP production persists after the onset of desensitization is consistent with the view that the mechanism responsible for desensitization is distal to the production of cyclic AMP. Moreover, the absence of any significant difference in the amount of cyclic AMP produced per cell in GGH_32', GGH_36' or GGH_312' cells suggests that elevated cyclic AMP production per cell does not explain the development of desensitization in GGH_36' cells. We suggest that Buserelin-stimulated prolactin synthesis in GGH_36' cells is mediated by a different cyclic AMP dependent protein kinase pool(s) compared to non-desensitizing GGH_3 cells. Such a PKA pool(s) may be more susceptible to degradation via cyclic AMP mediated mechanisms than the protein kinase pools mediating the Buserelin response in non-desensitizing GGH_3 cells. A similar mechanism has been reported in other systems.

Introduction

Four GH_3 (lactotrope) derived cell lines (GGH_31', GGH_32', GGH_36' and GGH_312') have been obtained by stable transfection with the rat GnRH receptor cDNA (Kaiser et al., 1994). These cells express a GnRH receptor similar in binding affinity and specificity to the GnRH receptor found in the gonadotrope (Kuphal et al., 1994). GnRH and its analogs stimulate prolactin release in GGH_3 cells in a dose- and time-dependent manner. All four cell lines show biphasic regulation (down-regulation and recovery) of the GnRH receptor in response to persistent (1-4 h) occupancy of the receptor by an agonist (Stanislaus et al., 1994). Responsiveness to Buserelin in GGH_36' cell line is diminished after 24 h exposure (Stanislaus et al., 1994). In contrast, neither GGH_31', GGH_32' nor GGH_312' cells desensitize in response to prolonged Buserelin stimulation. Desensitization in GGH_36' cells is not due to receptor down-regulation, since receptors recover from down-regulation by 4 h. Although all four GGH_3 cells contain the identical sequence of GnRH receptor cDNA, they respond differently to Buserelin. This study was performed to identify the basis for Buserelin evoked desensitization in GGH_36' cells.

Materials and Methods

Transfection of GH_3 cells

Four stable cell lines of GH_3-derived cells expressing the rat GnRH receptor cDNA (GGH_3) were prepared by electroporation (Kaiser et al., 1994). Neither GH_3 cells (parent line) nor a transfection control (pSVneo and pcDNA1 vector) produced elevated prolactin synthesis or release in response to GnRH agonist. Likewise, none had measurable GnRH receptors (Kaiser et al., 1994). The transfected cells were maintained in an atmosphere of 5% CO_2 at 37 C in DMEM (GIBCO, Grand Island, NY) containing 10% fetal calf serum (HyClone Laboratories, Inc., Logan, UT) and 20 μg/ml gentamicin (Gemini Bio-products, Calabassas, CA).

Quantitation of prolactin and cyclic AMP

Cells were cultured in DMEM/10% FCS containing 20 μg/ml gentamicin until nearly confluent in 162 cm^2 T-flasks (Costar, Cambridge, MA). Cells were scraped and replated for 24 h in 24- or 48-well culture plates (Costar). Prior to use in prolactin and cyclic AMP release studies, the adherent cells were washed twice in DMEM containing 0.1% bovine serum albumin (Irvine Scientific, Santa Ana, CA) and 20 μg/ml gentamicin. Release was measured following incubation in a 1 ml volume for 24-well or 0.5 ml volume for 48-well culture plates containing the indicated secretogogues or test substances. The treatment solutions were changed after 48 h, as indicated, when experiments required time points up to 96 h. At the time treatment solutions were changed, cells were washed three times with DMEM/BSA/gentamicin. Prolactin release was measured by RIA using materials obtained from the Hormone Distribution Program of the National Pituitary Agency, NIDDK. Prolactin was radioiodinated by standard procedures (Hunter and Greenwood, 1962). Cell number was assessed by a mini-diphenylamine assay for DNA quantitation (Burton, 1956), estimating 6 pg DNA/ cell.

Cyclic AMP production was measured at the indicated times. The transfected cells were stimulated with Buserelin (10^{-13} to 10^{-7} g/ml in dose-response studies and 10^{-7} g/ml in time-course studies) in DMEM/0.1% BSA/20 μg/ml gentamicin containing 0.2 mM methyl isobutyl xanthine (MIX) to prevent degradation of cyclic AMP by phosphodiesterases. After stimulating GGH_3 cells, the samples (incubation medium) were collected in tubes containing sufficient theophylline for a final concentration of 1 mM. The samples were heated (95° C) for 5 min to destroy phosphodiesterase activity. Intracellular cyclic AMP was measured by first washing the wells with medium then solubilizing the cells with DMEM/0.1% Triton X-100/1 mM theophylline. The solutions were heated for 5 minutes at 95 C. Samples were frozen prior to a cyclic AMP RIA. RIA of cyclic AMP was performed by a modification of the method of Steiner et al., (1972), with the addition of the acetylation step described by Harper and Brooker (1975). Cyclic AMP antiserum C-1B (prepared in our laboratory; Andrews et al., 1986) was used at a titer of 1:5,100. This antiserum showed less than 0.1% cross reactivity with cyclic GMP, 2':3'-cyclic AMP, 5'-cyclic AMP, 3'-cyclic AMP, ADP, GDP, ATP, CTP, MIX, or theophylline.

Statistics

Data shown are the mean of triplicate assay wells and presented as the mean ± SEM of the replicates in each experiment. The SEM was typically <10% of the mean. Intra- and interassay variances were 5% and 7%, respectively. The data were analyzed by one-way analyses of variance, followed by Student's modified *t*-test with the Bonferroni correction for multiple comparisons between means (Wallenstein et al., 1980). Each experiment was repeated three or more times to ensure the reproducibility of the findings.

Results

Buserelin (10^{-7} g/ml, in the presence of 0.2 mM methyl isobutyl xanthine, MIX) stimulated cyclic AMP release in a dose- (10^{-13} to 10^{-7} g/ml) and time-dependent (up to 96 h) manner in all four GGH_3 cell lines examined (figures 1 and 2). In the absence of MIX, cyclic AMP accumulation was not measurable, likely due to its degradation by phosphodiesterases. Buserelin-stimulated cyclic AMP release at a constant rate up to the last time point examined (96 h, figure 2).

In all four cell lines a maximally stimulatory dose of dBcAMP (5 mM) provoked prolactin synthesis in a time-dependent manner (figure 3). Maximal prolactin synthesis in response to dBcAMP was less than that produced in response to Buserelin. All experiments were conducted in the presence of MIX (0.2 mM) to inhibit phosphodiesterase mediated cyclic AMP degradation.

MIX (0.2 mM) potentiated prolactin release in response to Buserelin in all four GGH_3 cell lines (figure 4). Buserelin-stimulated prolactin release at high doses (10^{-8} and 10^{-7} g/ml) was not additive with MIX, suggesting that both agents act by enhancing the accumulation of cyclic AMP.

Cholera toxin (CTX, 5 μg/ml) treatment, which stimulates the production of cyclic AMP via constitutive activation of G_q, stimulated prolactin synthesis poorly (figure 5). Buserelin-stimulated prolactin synthesis in the non-desensitizing cell lines was inhibited by treating these cells at 48 h with CTX alone (5 μg/ml, figure 5) or CTX (5 μg/ml) in the presence of Buserelin (10^{-7} g/ml). Buserelin evoked desensitization was not reversed in GGH_36' cells with the same treatments (figure 5). Figure 6 shows that treating GGH_3 cells with CTX (5 μg/ml), after a 48 h Buserelin treatment, increased the rate of cyclic AMP release above that of Buserelin (10^{-7} g/ml) stimulated levels in all four cell lines. Notably, Buserelin-stimulated cyclic AMP release was not ablated with the onset of desensitization in GGH_36' cells (figure 6). The addition of CTX at 48 h

increased the cyclic AMP production above Buserelin-stimulated levels, and coincided with the inhibition of prolactin synthesis in GGH_31', GGH_32' and GGH_312' cells. The steady increase in cyclic AMP also suggested that GGH_3 cells were actively producing cyclic AMP, and that CTX was not affecting cell viability to the extent that it inhibited cyclic AMP synthesis. Furthermore, CTX (5 μg/ml or 0.1 μg/ml) stimulated more cyclic AMP production than Buserelin (10^{-7} g/ml) at all time points examined (figures 6 and 7). At 180 min, CTX-stimulated cyclic AMP levels were greater than two fold that of Buserelin-stimulated levels in all four cell lines (figure 7).

Increasing the cyclic AMP concentration by treating GGH_3 cells with dBcAMP (10 mM) attenuated Buserelin-stimulated prolactin synthesis. Dibutryl cyclic AMP (10 mM) stimulated prolactin synthesis poorly or not at all (figure 8). Furthermore, in GGH_31', GGH_32' and GGH_312' cells after a 48 h Buserelin (10^{-7} g/ml) stimulation, 10 mM dBcAMP alone or dBcAMP (10 mM) in the presence of Buserelin (10^{-7} g/ml) reduced prolactin synthesis from levels when Buserelin alone was present (figure 8). Buserelin-evoked desensitization in GGH_36' cannot be reversed with the addition of dBcAMP at 48 h (figure 8). The attenuated response to dBcAMP was not the sole result of the dibutryl moiety: dibutryl cGMP in the presence of Buserelin (10^{-7} g/ml) failed to inhibit or reduce prolactin synthesis from the levels obtained in response to Buserelin alone (data not shown).

CTX mediated increase in cyclic AMP production or treatment with dBcAMP produced an inhibitory effect on prolactin synthesis in GGH_31', GGH_32' and GGH_312' (the non-desensitizing) cell lines. This inhibitory effect was similar to Buserelin-evoked desensitization in GGH_36' cells. Therefore, the basis for Buserelin-evoked desensitization in GGH_36' cells was either over-production of cyclic AMP compared to non-desensitizing GGH_3 cells, or an event distal to cyclic AMP production. Thus, the amount of cyclic AMP released per 10^6 GGH_3 cells was determined.

Table 1 shows cyclic AMP produced in 10^6 GGH_3 cells treated with Buserelin (10^{-7} g/ml). After 24 h of Buserelin stimulation there was no significant difference between GGH_36' (351 $\pm$ 70 pmoles/10^6 cells) and GGH_32' (387.4 $\pm$ 106.3 pmoles/10^6 cells) cells with respect to the total amount of cyclic AMP release. After 48 h of Buserelin stimulation, there was no significant difference between GGH_36' (967 $\pm$ 60 pmoles/10^6 cells) and GGH_312' (1030.8 $\pm$ 219.5 pmoles/10^6 cells) cells with respect to the total amount of cyclic AMP release. Neither GGH_32' nor GGH_312' cells became desensitized to Buserelin; and furthermore, both cell lines produced more cyclic AMP than GGH_36' cells.

Discussion

In the present study, we show that Buserelin stimulates dose- and time-dependent prolactin synthesis in GGH_3 cells via a cyclic AMP dependent pathway. Cyclic AMP analog, dBcAMP, stimulates prolactin synthesis, and the phosphodiesterase inhibitor, methyl isobutyl xanthine, increases prolactin synthesis at sub-maximal doses of Buserelin. These observations indicate that cyclic AMP satisfy the criteria (Sutherland et al., 1968) of a second messenger in Buserelin-stimulated prolactin synthesis.

Cyclic AMP production persists in the presence of Buserelin, even in GGH_36' cells in which the prolactin response desensitizes. Although treatment with CTX causes rapid increases in cyclic AMP release, it does not result in prolactin synthesis comparable to that of Buserelin-stimulated levels. When Buserelin stimulated prolactin production is first detected (180 min), CTX-stimulated cyclic AMP levels are two-fold more than that of Buserelin-stimulated levels. Moreover, CTX-stimulated cyclic AMP levels are higher than those of Buserelin treated levels at all time points examined.

Production of cyclic AMP by CTX or by treatment with the cyclic AMP analog, dBcAMP, attenuates Buserelin-stimulated prolactin synthesis in cells which the prolactin response does not desensitize in response to Buserelin (GGH_31', GGH_32' and GGH_312' cells). This observation suggests that the response of the non-desensitizing GGH_3 lines can be made refractory to Buserelin stimulation by raising cyclic AMP levels. The refractoriness to Buserelin stimulation in the presence of dBcAMP is not a consequence of the dibutryl moiety since the addition of dibutryl cGMP does not have any effect on Buserelin-stimulated prolactin synthesis.

The view that the event involved in Buserelin-evoked desensitization in GGH_36' cells is distal to the production of cyclic AMP is supported by several observations: (1) GGH_36' cells do not recover from desensitization after the addition of a cyclic AMP analog or CTX; (2) cyclic AMP production does not attenuate with the onset of

desensitization; (3) there is no significant difference between the amount of cyclic AMP synthesized in GGH_36' cells compared to other GGH_3 cells.

The ability of cyclic AMP to serve both as a second messenger and as an modulator of prolactin production likely involves concentration dependent effects on cyclic AMP-dependent protein kinase (PKA). It is known for example that sustained high levels of cyclic AMP in GH_3 cells cause degradation of catalytic subunits of PKA thereby diminishing its activity (Richardson et al., 1990; Schwoch, 1987; Hougeg et al., 1990). It is reasonable to believe that a similar phenomena could be responsible for desensitization in the presence of sustained levels of cyclic AMP.

The fact that only GGH_36' cells desensitize in response to chronic Buserelin stimulation suggests that an aspect of the PKA dependent pathway mediating prolactin synthesis is inherently different from that of the other three cell lines. It is possible, for example, that Buserelin-stimulated prolactin synthesis in GGH_36' cells may utilize PKA pools that are localized to the cytosol, whereas the other three GGH_3 cell lines may utilize PKA pools compartmentalized to subcellular organelles. Such sub-cellular localization of PKA pools have been reported in other systems (Ndubuka et al., 1993; Scott, 1991). Therefore the cyclic AMP dependent response to a persistent stimulant may depend on the distribution of PKA holoenzymes and their susceptibility to cyclic AMP mediated degradation. As PKA pools localized to the cytosol are more accessible to cyclic AMP than PKA pools in subcellular organelles, their catalytic subunits are more susceptible to cyclic AMP mediated degradation (Spaulding, 1993). Therefore Buserelin stimulation may cause an adequate increase in cyclic AMP and, thus, catalytic subunit degradation in GGH_36' cells. This results in decreased PKA activity which manifest as diminished prolactin synthesis or desensitization. In GGH_31', GGH_32' and GGH_312' cells, cyclic AMP concentration may not reach critical levels to cause the degradation of the catalytic subunit of PKA, however, Buserelin-stimulated prolactin synthesis diminishes when

overwhelmed with cyclic AMP presumably due to catalytic subunit degradation. Although the mechanism of desensitization described above may occur in GGH_36' cells, it is possible that GGH_36' cells may utilize other mechanisms for Buserelin-evoked desensitization.

Although the binding data for the four cell lines show similar k_d values, the sensitivity of the cyclic AMP response in GGH_36' cells is less than the non-desensitizing cells. This may implicate other mechanistic events in the development of desensitization. Furthermore, it should be noted that GGH_36' cells have the highest number of GnRH receptors, although the total cyclic AMP it produces is not significantly different to the non-desensitizing cell lines.

The inhibitory effect of intracellular cyclic AMP may explain why CTX is not as effective as Buserelin in stimulating prolactin synthesis in GGH_3 cells. Although the threshold cyclic AMP concentration capable of inhibiting prolactin production in each cell line is not known, the immediate and profound increase in intracellular cyclic AMP levels when stimulated with CTX may result in degradation of the catalytic subunit of PKA. It is unlikely that CTX stimulates a different pool of cyclic AMP from Buserelin, because CTX will then be unable to attenuate Buserelin-stimulated prolactin synthesis. However, CTX attenuates Buserelin-stimulated prolactin synthesis suggesting that both agents act through a common pool.

The present study shows that although cyclic AMP is a second messenger for GnRH or GnRH agonist stimulated prolactin synthesis in all four GGH_3 cells, it can also produce refractoriness in GGH_3 cell response to GnRH stimulation. Buserelin-evoked desensitization in GGH_36' cells cannot be reversed by dBcAMP or CTX treatment, and they do not produce significantly different amounts of cyclic AMP compared to GGH_32' and GGH_312' cells. Therefore, the event causing the desensitization in GGH_36' cells is distal to the production of cyclic AMP. We propose that distinct cyclic AMP-dependent

protein kinase pool(s), may be differentially susceptible to intracellular cyclic AMP levels, may mediate GnRH or GnRH analog stimulated prolactin synthesis in GGH_36' in contrast to GGH_31', GGH_32' and GGH_312' cells, and this may underlie the differential response to Buserelin with regard to desensitization.

Acknowledgments

We thank Jo Ann Janovick, David Kuphal, Chuanhai Guo and Richard A. Maurer for helpful comments on the manuscript. Also, we would like to thank Linda Wolf for secretarial help with the manuscript.

Figure 3-1. Dose-response curves for Buserelin-stimulated cyclic AMP release in GGH_31', GGH_32', GGH_36' and GGH_312' cells respectively. Cells were treated with the indicated concentrations of Buserelin in the presence or absence of MIX for 24 h before radioimmunoassay of cyclic AMP released to the medium. Data are the mean of triplicate treatments, and the error bars show the SEM. Each experiment was repeated at least three times with similar results.

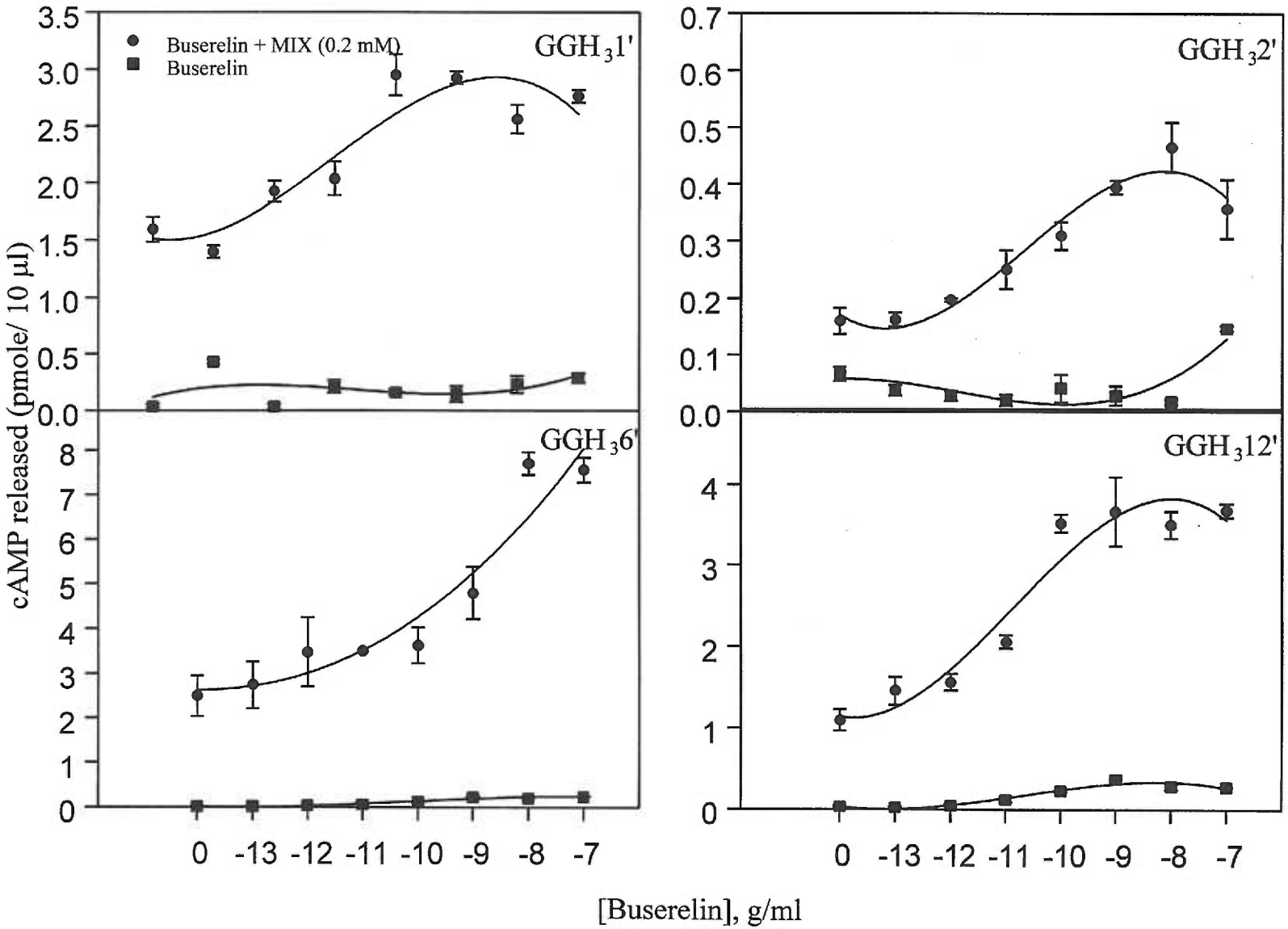
Buserelin + MIX (0.2 mM)
Buserelin
GGH31'
GGH32'
GGH36'
GGH312'
cAMP released (pmole/ 10 µl)
[Buserelin], g/ml
0 -13 -12 -11 -10 -9 -8 -7

Figure 3-2. Time-course of cyclic AMP release in response to the presence or absence of Buserelin (10^{-7} g/ml) in $GGH_3$1', $GGH_3$2', $GGH_3$6'and $GGH_3$12' cells respectively. GGH_3 cells were incubated with the indicated treatments for 3, 24, 48, 72 and 96 h. Cyclic AMP released to the medium was determined by RIA. Data are the mean of triplicate treatments, and the error bars show the SEM. Each experiment was repeated at least three times with similar results.

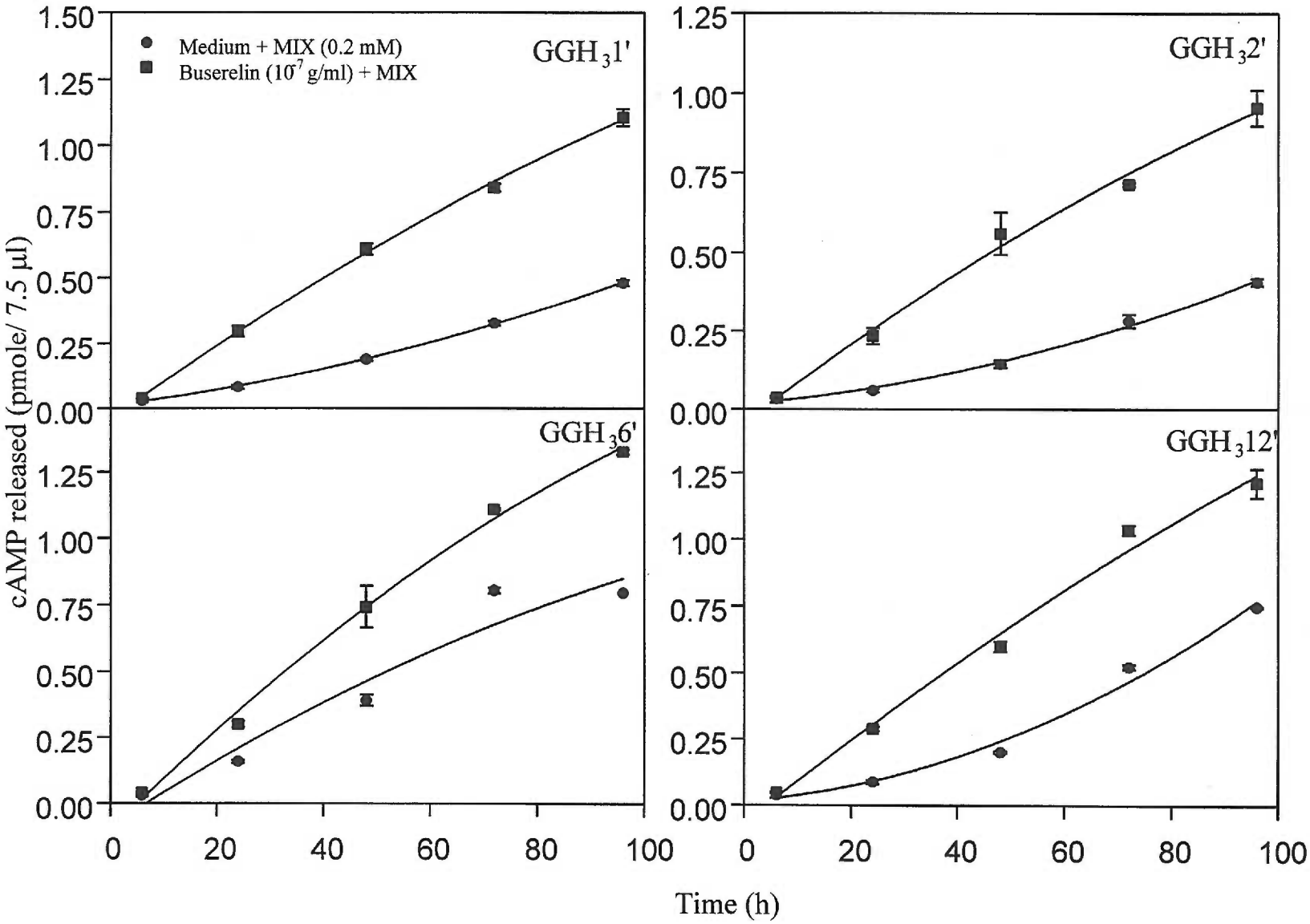
Medium + MIX (0.2 mM)
Buserelin (10^{-7} g/ml) + MIX
GGH 1'
GGH 2'
GGH 6'
GGH 12'
cAMP released (pmole/ 7.5 µl)
Time (h)
0
20
40
60
80
100
0.00
0.25
0.50
0.75
1.00
1.25
1.50

Figure 3-3. Time-course of prolactin release in response to indicated secretagogues in $GGH_3$1', $GGH_3$2', $GGH_3$6' and $GGH_3$12' cells respectively. GGH_3 cells were incubated with vehicle, Buserelin (10^{-7} g/ml) or dBcAMP (5 mM) for 3, 24 and 30 h. Prolactin released to the medium was determined by RIA. Data are the mean of triplicate treatments, and the error bars show the SEM. Each experiment was repeated at least three times with similar results. Asterisks indicate values significantly ($p < 0.025$) different from those in unstimulated cells.

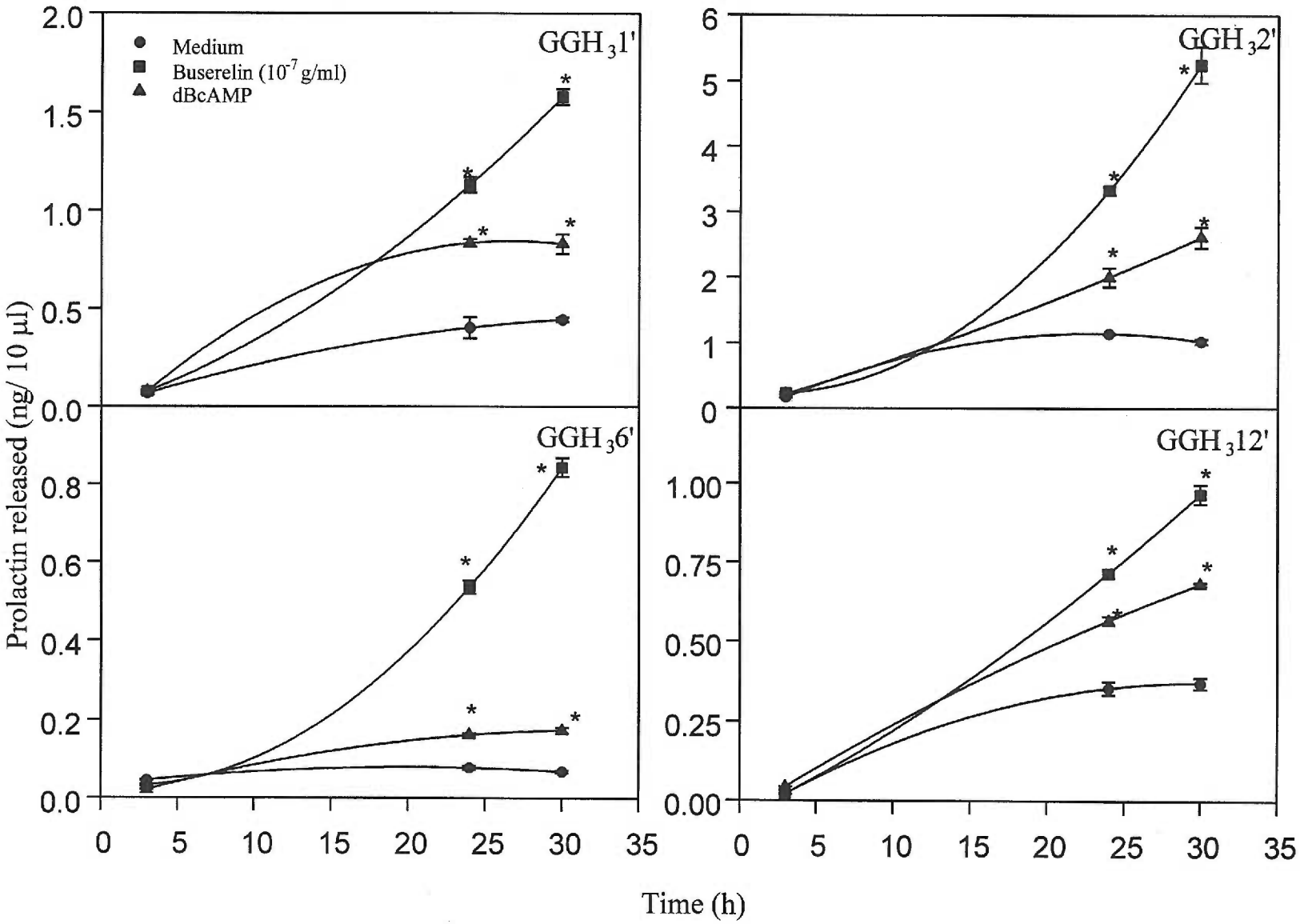
Medium
Buserelin (10^{-7} g/ml)
dBcAMP
GGH 31'
GGH 32'
GGH 36'
GGH 312'
Prolactin released (ng/ 10 µl)
Time (h)

Figure 3-4. Effect of inhibition of cyclic nucleotide degradation on Buserelin-stimulated prolactin release in $GGH_3$1', $GGH_3$2', $GGH_3$6' and $GGH_3$12' cells respectively. GGH_3 cells were incubated with the indicated concentrations of Buserelin in the presence or absence of MIX for 24 h. Prolactin released to the medium was determined by RIA. Data are the mean of triplicate treatments, and the error bars show the SEM. Each experiment was repeated at least three times with similar results. Asterisks indicate Buserelin + MIX stimulated values that are significantly ($p < 0.05$) different from Buserelin-stimulated values.

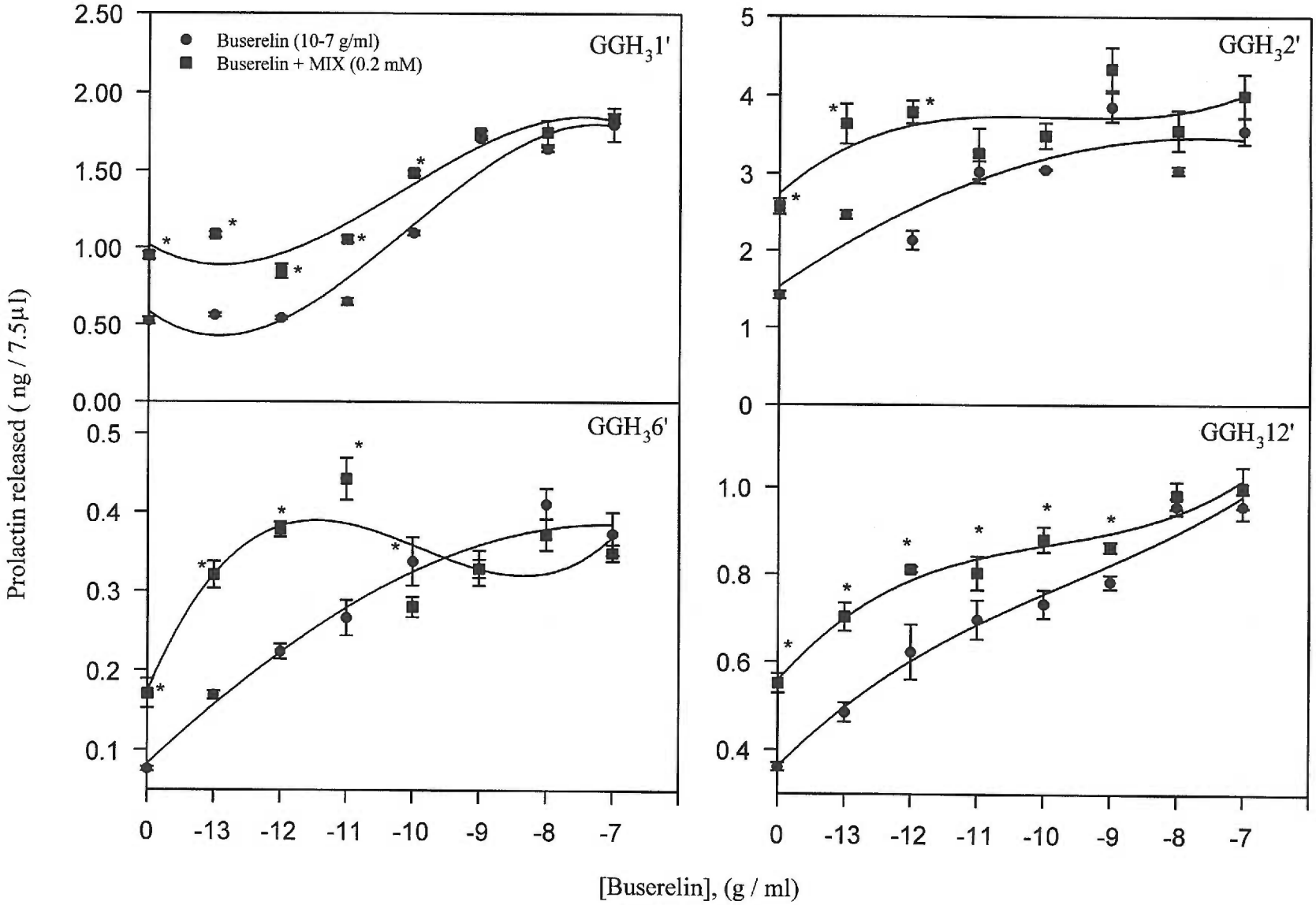
Buserelin (10-7 g/ml)
Buserelin + MIX (0.2 mM)
GGH3 1'
GGH3 2'
GGH3 6'
GGH3 12'
Prolactin released (ng / 7.5µl)
[Buserelin], (g / ml)
2.50
2.00
1.50
1.00
0.50
0.00
5
4
3
2
1
0
0.5
0.4
0.3
0.2
0.1
1.0
0.8
0.6
0.4
0
-13
-12
-11
-10
-9
-8
-7

Figure 3-5. The effect of CTX on Buserelin-stimulated prolactin release in $GGH_3$1', $GGH_3$2', $GGH_3$6' and $GGH_3$12' cells respectively. GGH_3 cells were incubated with vehicle, Buserelin (10^{-7} g/ml), Buserelin (10^{-7} g/ml) in the presence of CTX (5 μg/ml) or CTX (5 μg/ml) alone, for 3, 24, 48, 72 and 96 h. Treatment solutions were replaced ("chd" in graph) after 48 h with the same treatment or Buserelin (10^{-7} g/ml) in the presence of CTX (5 μg/ml) or CTX (5 μg/ml) alone. Prolactin released to the medium was determined by RIA. Data are the mean of triplicate treatments, and the error bars show the SEM. Each experiment was repeated at least three times with similar results. Asterisks indicate CTX or Buserelin + CTX stimulated values significantly different ($p < 0.05$) from Buserelin-stimulated values.

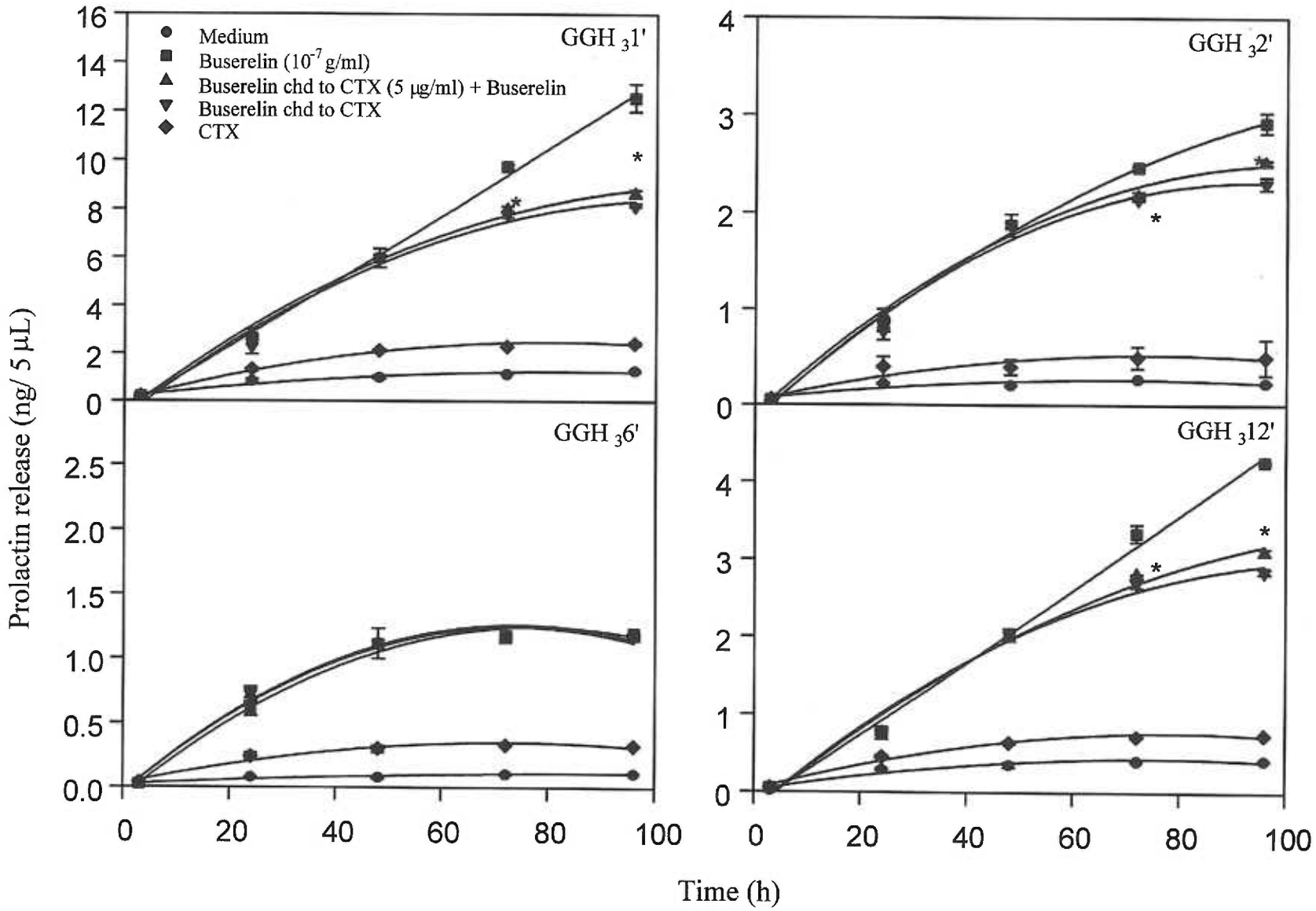
Medium
Buserelin (10^{-7} g/ml)
Buserelin chd to CTX (5 µg/ml) + Buserelin
Buserelin chd to CTX
CTX
GGH 31'
GGH 32'
GGH 36'
GGH 312'
Prolactin release (ng/ 5 µL)
Time (h)

Figure 3-6. The effect of CTX on Buserelin-stimulated cyclic AMP release in $GGH_3$1', $GGH_3$2', $GGH_3$6' and $GGH_3$12' cells respectively. GGH_3 cells were incubated in the presence or absence of Buserelin (10^{-7} g/ml) for 3, 24, 48, 72 and 96 h. Treatment solutions were replaced after 48 h with a fresh batch of the same treatment or CTX (5 μg/ml). All treatment solutions had MIX (0.2 mM). Cyclic AMP released to the medium was determined by RIA. Data are the mean of triplicate treatments, and the error bars show the SEM. Each experiment was repeated at least three times with similar results.

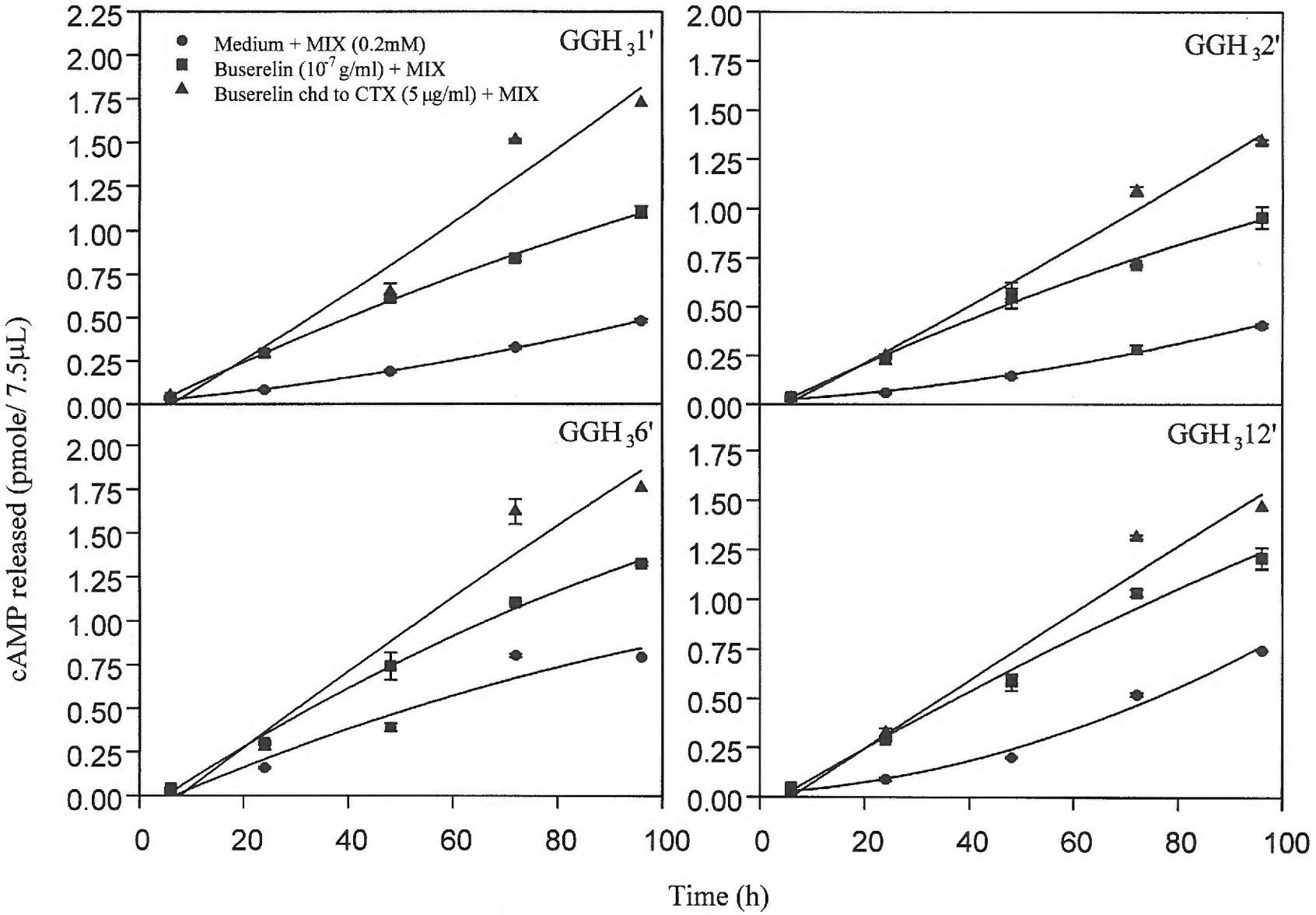
Medium + MIX (0.2mM)
Buserelin (10^{-7} g/ml) + MIX
Buserelin chd to CTX (5 µg/ml) + MIX
GGH 1'
GGH 2'
GGH 6'
GGH 12'
cAMP released (pmole/ 7.5µL)
Time (h)
0
20
40
60
80
100
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
2.25

Figure 3-7. The time-course of cyclic AMP synthesis in response to Buserelin (10^{-7} g/ml) or CTX (0.1 μg/ml) in GGH_31', GGH_32', GGH_36' and GGH_312' cells respectively. Cells were incubated with the indicated secretogogues for 60, 90, 120, 180 and 360 minutes. Cyclic AMP released to the medium was measured by RIA. Data are the mean of triplicate treatments, and the error bars show the SEM. Each experiment was repeated at least three times with similar results.

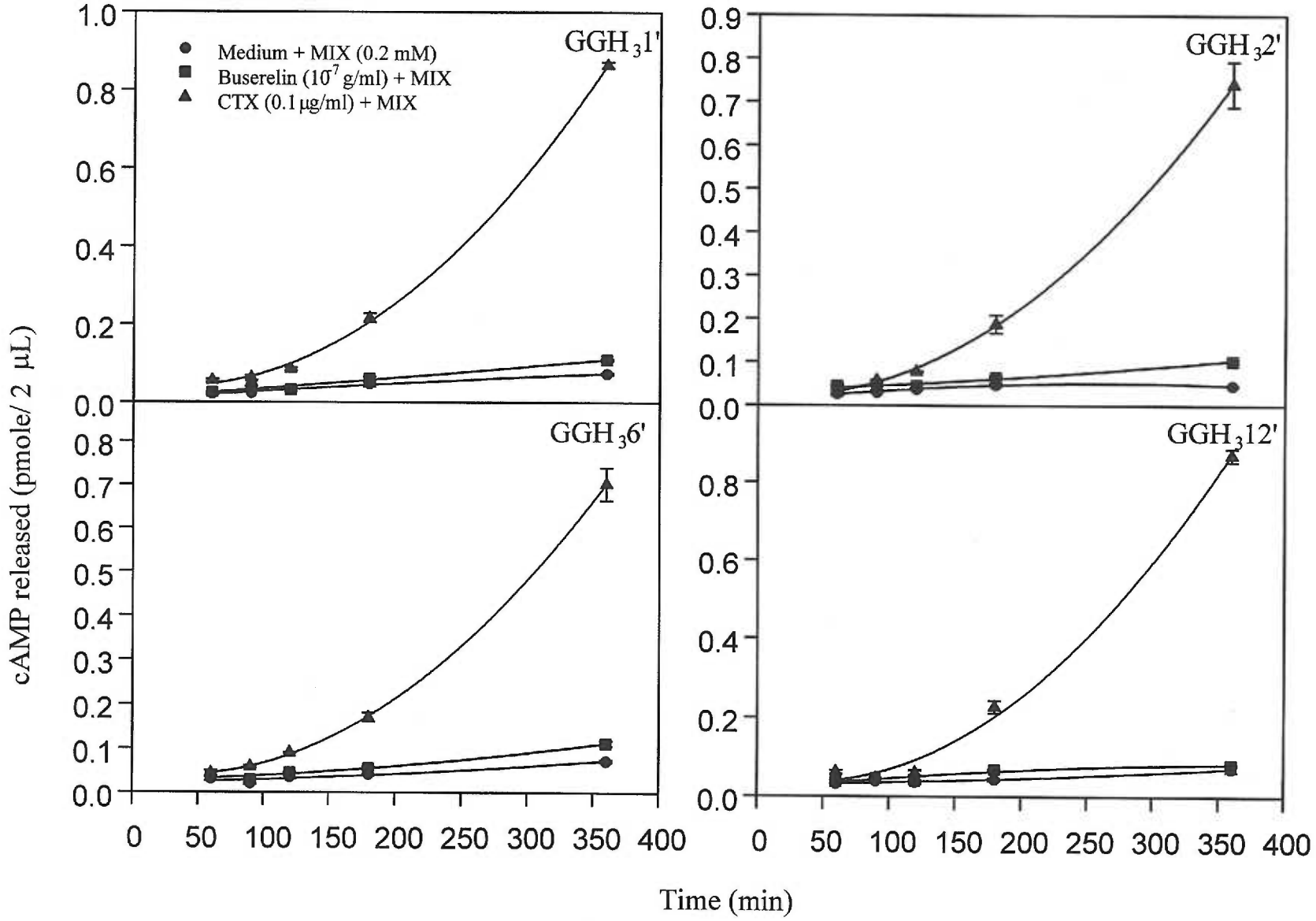
Medium + MIX (0.2 mM)
Buserelin (10⁻⁷ g/ml) + MIX
CTX (0.1 µg/ml) + MIX
GGH 31'
GGH 32'
GGH 36'
GGH 312'
cAMP released (pmole/ 2 µL)
Time (min)

Figure 3-8. The effect of dBcAMP on Buserelin-stimulated prolactin release in $GGH_3$1', $GGH_3$2', $GGH_3$6' and $GGH_3$12' cells respectively. GGH_3 cells were incubated with vehicle, Buserelin (10^{-7} g/ml), Buserelin (10^{-7} g/ml) in the presence of dBcAMP (10 mM) or dBcAMP (10 mM) alone, for 3, 48, 72 and 96 h. Treatment solutions were replaced after 48 h with a fresh batch of the same treatment or dBcAMP (10 mM) in the presence or absence of Buserelin (10^{-7} g/ml). Prolactin released to the medium was determined by RIA. Data are the mean of triplicate treatments, and the error bars show the SEM. Each experiment was repeated at least three times with similar results. Asterisks indicate dBcAMP or Buserelin + dBcAMP stimulated values significantly different ($p < 0.05$) from Buserelin-stimulated values.

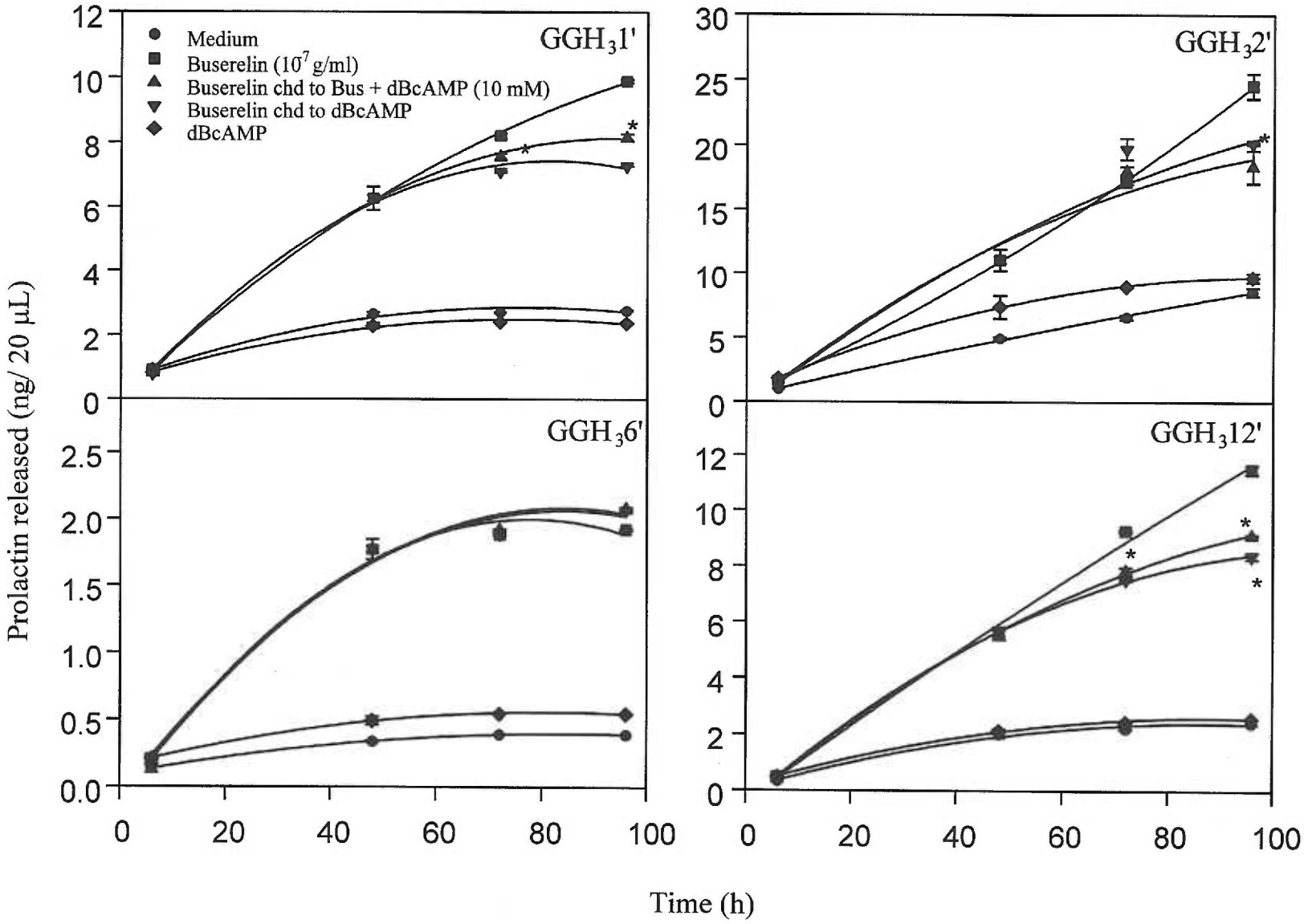
Medium
Buserelin (10^{-7} g/ml)
Buserelin chd to Bus + dBcAMP (10 mM)
Buserelin chd to dBcAMP
dBcAMP
GGH$_3$1'
GGH$_3$2'
GGH$_3$6'
GGH$_3$12'
Prolactin released (ng/ 20 µL)
Time (h)

Table 3-1. Cyclic AMP released in response to Buserelin (10^{-7} g/ml) treatment in GGH_3 cells. Cells were incubated with Buserelin for 24 or 48 h. Cell number and cyclic AMP release was determined as described in methods (n=4).

	Cyclic AMP released (pmole/10^6 cells) in 24h	Cyclic AMP released (pmole/10^6 cells) in 48h
GGH_31'	207.0 ± 54.1	386.1 ± 19.0
GGH_32'	387.4 ± 106.3	697.2 ± 57.6
GGH_36'	351.0 ± 70.0	967.6 ± 60.0
GGH_312'	522.0 ± 134.0	1030.8 ± 219.5

CHAPTER FOUR

REGULATION OF $G_{q/11}\alpha$ BY THE GnRH RECEPTOR

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Abstract

Evidence from use of pertussis and cholera toxins and from NaF suggested the involvement of G-proteins in GnRH regulation of gonadotrope function. We have used three different methods to assess GnRH receptor regulation of $G_{q/11}\alpha$ subunits ($G_{q/11}\alpha$). First, we used GnRH- stimulated palmitoylation of $G_{q/11}\alpha$ to identify their involvement in GnRH receptor mediated signal transduction. Dispersed rat pituitary cell cultures were labeled with [9,10-^{3}H(N)]-palmitic acid, and immunoprecipitated with rabbit polyclonal antiserum made against the C-terminal sequence of $G_{q/11}\alpha$. The immunoprecipitates were resolved by 10% SDS-polyacrylamide gel electrophoresis and quantified. Treatment with GnRH resulted in time-dependent (0-120 min) labeling of $G_{q/11}\alpha$. GnRH (10^{-12}, 10^{-10}, 10^{-8} or 10^{-6} g/ml) for 40 min resulted in dose-dependent labeling of $G_{q/11}\alpha$ compared to controls. Cholera toxin (5 μg/ml; activator of $G_s\alpha$), pertussis toxin (100 ng/ml; inhibitor of $G_i\alpha$ actions) and Antide (50 nM; GnRH antagonist) did not stimulate palmitoylation of $G_{q/11}\alpha$ above basal levels. However, phorbol myristic acid (100 ng/ml; PKC activator) stimulated the palmitoylation of $G_{q/11}\alpha$ above basal levels, but not to the same extent as 10^{-6} g/ml GnRH. Second, we used the ability of the third intracellular loop (3_i) of other seven-transmembrane segment (7-TMS) receptors that couple to specific G-proteins to antagonize GnRH receptor stimulated signal transduction, and therefore act as an intracellular inhibitor. Because the third intracellular loop of α_{1B}-adrenergic receptor ($\alpha_{1B}3_i$) couples to $G_{q/11}\alpha$, it can inhibit $G_{q/11}\alpha$ mediated stimulation of inositol phosphate (IP) turnover by interfering with receptor coupling to $G_{q/11}\alpha$. Transfection (efficiency 5-7%) with $\alpha_{1B}3_i$ cDNA, but not the third intracellular loop of M_1-acetylcholine receptor (which also couples to $G_{q/11}\alpha$), resulted in 10-12% inhibition of maximal GnRH evoked IP turnover, as compared to vector

transfected GnRH-stimulated IP turnover. The third intracellular loop of α_{2A}- adrenergic receptor, M_2-acetylcholine receptor (both couple to $G_i\alpha$) and D_{1A}-receptor (couples to $G_s\alpha$) did not inhibit IP turnover significantly from control values. GnRH stimulated LH release was not affected by the expression of these peptides. Third, we assessed GnRH receptor regulation of $G_{q/11}\alpha$ in a prolactin secreting adenoma cell line (GGH_31') expressing the GnRH-receptor. Stimulation of GGH_31' cells with 0.1 μg/ml Buserelin (a metabolically stable GnRH agonist) resulted in a 15-20% decrease in total $G_{q/11}\alpha$ at 24 h following agonist treatment compared to control levels; this action of the agonist was blocked by GnRH antagonist, Antide (10^{-6} g/ml). Neither Antide (10^{-6} g/ml, 24 h) alone nor phorbol myristic acid (0.33-100 ng/ml, 24 h) mimicked the action of GnRH agonist on loss of $G_{q/11}\alpha$ immunoreactivity. The loss of $G_{q/11}\alpha$ immunoreactivity was not due to an effect of Buserelin on cell doubling times. These studies provide the first direct evidence for regulation of $G_{q/11}\alpha$ by the GnRH receptor in primarily pituitary culture and in GGH_3 cells.

Introduction

The gonadotropin releasing hormone (GnRH) receptor, like other receptors in the 7-transmembrane segment receptor super-family, couples to multiple G-proteins (Hawes et al., 1992; Andrews and Conn, 1986). In dispersed pituitary cell cultures, pertussis toxin (PTX) pre-treatment results in decreased IP turnover compared to medium pretreated levels in response to GnRH (Hawes et al., 1993), suggesting that a PTX sensitive G-protein (such as G_i or G_o) couples the receptor to IP turnover. However, in GGH_312 cell cultures (GH_3 cells stably transfected with the rat GnRH-receptor cDNA), GnRH agonist-evoked IP turnover is insensitive to PTX (Janovick and Conn, 1994), indicating that a PTX insensitive G-protein may be involved in signal transduction and the receptor may be coupled differently in different cells.

$G_{q/11}\alpha$ immune-depletion studies show that in membranes derived from αT3-1 cells, GnRH receptor is coupled to $G_{q/11}\alpha$ (Hsieh and Martin, 1992). Furthermore, prolactin synthesis in GGH_3 cell cultures in response to GnRH is mediated by cyclic AMP, implicating $G_s\alpha$ in this signal transduction pathway (Kuphal et al., 1994), although cyclic AMP does not mediate GnRH-stimulated hormone release from the gonadotrope (Conn et al., 1979). It is evident from these studies that GnRH-receptor is able to couple to different G-proteins in different cell lines. Therefore in order to investigate the G-proteins that couple to the GnRH receptor, it is important to undertake these studies in primary pituitary cultures.

In the gonadotrope, the little that is known about G-proteins involved in GnRH receptor mediated signal transduction has been obtained from toxin studies and from second messenger studies (Hawes et al., 1992; Andrews and Conn, 1986; Hawes et al., 1993). PTX sensitive G-proteins have been implicated in the GnRH receptor/G-protein coupling. Also

the observation that cholera toxin (CTX) pre-treatment enhances GnRH stimulated LH release (Janovick and Conn, 1993) has implicated $G_s\alpha$ in modulation of GnRH action. Such studies provide only indirect evidence to the identity of the G-proteins involved in GnRH-receptor mediated signal transduction. Complicating this further is the observation that both protein kinase C (PKC) and protein kinase A (PKA), regulated by different G-proteins, are capable of regulating IP turnover by phosphorylating phospholipase C (Tsutsumi et al., 1992). Therefore the sole use of IP turnover as a marker for a specific G-protein activation would lead to unclear results. Furthermore, cross talk between CTX sensitive G-protein and PKC (Barnes and Conn, 1993) can further complicate the identification of the G-proteins that couple to the GnRH receptor.

Palmitoylation (i.e. the addition of a 16-carbon fatty acid to a cysteine residue through a thioester link) of G-protein α-subunits is a dynamic process that is regulated following receptor activation. Receptor evoked palmitoylation of $G_{q/11}\alpha$ and $G_s\alpha$ is a well-characterized phenomenon (Degtyarev et al., 1993; Mumby et al., 1994; Wedegaertner et al., 1993) and occurs in a time- and dose-dependent manner (Degtyarev et al., 1993; Mumby et al., 1994). Furthermore, G-proteins that do not associate with a specific receptor do not incorporate [^{3}H]-palmitic acid into their α-subunits when that receptor is stimulated (Degtyarev et al., 1993). In addition, mutationally activated $G_s\alpha$ turns over [^{3}H]-palmitic acid labeling more rapidly than wild type $G_s\alpha$ (Wedegaertner and Bourne, 1994). These studies suggest that activation of G-protein is required for the α-subunit to undergo palmitoylation. In the present study, we used the ability of G-protein coupled receptors to stimulate the palmitoylation of G-protein α-subunits they activate, to identify the moieties that are affected when cells are stimulated with GnRH.

Several reports (Luttrell et al., 1993; Hawes et al., 1994) have shown that the cellular expression of the third intracellular loop of G-protein coupled receptors can inhibit receptor evoked second messenger production. This effect is greatest when the peptide is derived from the same receptor, although expression of heterologous third intracellular loops inhibit receptor stimulated second messenger production to a lesser extent as long as the intracellular loop and the receptor couple to a common G-protein. These studies demonstrate that peptides derived from the third intracellular loop of G-protein coupled receptors produce G-protein specific inhibition of receptor mediated signal transduction (Luttrell et al., 1993). We used the ability of the third intracellular loop to act as an intracellular inhibitor to corroborate our findings from the palmitoylation studies.

To examine further the regulation of G-proteins by the GnRH receptor, we examined receptor evoked down regulation of G-protein α-subunits. Agonist-induced reduction in total cellular G-protein α-subunits have been observed for members of the G-protein family ($G_s\alpha$, $G_i\alpha$, $G_{q/11}\alpha$; Milligan, 1993). This reduction is observed for G-proteins that interact with the activated receptor (Milligan, 1993), and can be used as a marker for receptor regulation of G-proteins. Therefore, we used an RIA developed in our laboratory to assess GnRH agonist-evoked reduction of total cellular $G_{q/11}\alpha$ in a rat pituitary adenoma cell line stably expressing the GnRH receptor. We opted to use a homogeneous cell line instead of a dispersed rat pituitary cell culture, because gonadotropes are only about 20% of cells obtained from a rat pituitary cell dispersion (female weanlings) and changes in total G-protein content may be masked against a relatively high background from non-gonadotrope cells.

In this study we assess evidence to indicate GnRH receptor regulation of $G_{q/11}\alpha$. Evidence for regulation of $G_{q/11}\alpha$ by the receptor is present in rat pituitary cell dispersions and also in a cell line stably expressing the GnRH receptor.

Material and Methods

Materials

Horse and fetal calf sera (Hyclone Laboratories, Logan, UT), bovine serum albumin (Irvine Scientific, Santa Ana, CA). HEPES (United States Biochemical, Cleveland, OH), Collagenase (Worthington Biochemical, Freehold, NJ), formic acid (Mallinkrodt, McGraw Park, IL), ammonium formate, sodium deoxycholate and EDTA (Fisher Scientific, Fairlawn, NJ), Nonidet P-40 (Particle Data Laboratory, Elm Hurst, IL), gentamicin sulfate (Gemini, Bio-products, Calabasas, CA), hyaluronidase, DNAse I and phorbol myristic acid (PMA, Sigma, St. Louis, MO), Antide (Ares-Serono). Other reagents were obtained at the highest grade available from commercial vendors as indicated. The G-protein coupled receptor third intracellular loops expression plasmids- $pRK\alpha_{1B}3_i$, $pRK\alpha_{2A}3_i$, $pRKM_13_i$, $pRKM_23_i$ and $pRKD_{1A}3_i$, were a gift from Dr. R.J. Lefkowitz, Duke University, Durham, NC (Luttrell et al., 1993).

Preparation of pituitary cell cultures

Pituitary cell cultures were prepared as previously described (Conn et al., 1979). Briefly, pituitary glands were removed from 28 day old female Sprague-Dawley rats (B&K Universal Inc, Kent, Washington) and placed in medium 199 (Irvine Scientific, Santa Ana, CA) containing 0.3% (w/v) BSA and 10 mM HEPES, pH 7.4 (M199/BSA). The pituitaries were minced and incubated in sterile M199/BSA containing 0.125% (w/v) collagenase and 0.1% (w/v) hyaluronidase in a 37 C shaking water bath for 15 min. The dissociated cells were filtered through organza cloth, and the remaining tissue was incubated a second time with a similar enzyme solution for another 15 min. The combined cells were collected by

centrifugation (10 min at 200 x g) and resuspended in M199/BSA containing 10% (v/v) horse serum, 2.5% (v/v) fetal calf serum, and 20 μg/ml gentamicin sulfate and filtered through an organza cloth. For palmitoylation studies, cell suspensions were plated at a cell density of $2.5x10^6$ cells/well in six-well culture plates (Costar, Cambridge, MA). Single cell suspensions were obtained for transfection studies by resuspending the cell pellet with M199/BSA/4 mM EDTA containing 0.2% (w/v) collagenase and 0.2% (w/v) hyaluronidase and incubating for 30 min at 37 C, and adding DNAse I (100 μg/ml) for the last 5 min of the incubation. The dissociated cells were filtered through a 10 μm mesh cloth and incubated for another 15 min at 37 C with M199/BSA/4 mM EDTA containing 0.2% collagenase and 0.2% hyaluronidase. The cells were collected by centrifugation (15 min at 200 x g; 4 C) and resuspended in cold M199/BSA containing 10% horse serum, 2.5% fetal calf serum, and 20 :g/ml gentamicin sulfate. The cell suspensions were plated at cell density of $15x10^4$ cells/well in 24-well culture plates (Costar, Cambridge, MA). Cells were maintained for approximately 48 h at 37 C in a water-saturated atmosphere prior to beginning experiments.

Metabolic labeling of G-proteins with [9,10-^{3}H]-palmitic acid and immunoprecipitation.

Pituitary cell cultures were washed twice with M199/BSA (pH 7.4), 2 h before labeling with [9,10-^{3}H]-palmitic acid (specific activity 30-60 Ci/mmol, 0.5 mCi/ml of M199/BSA; DuPont NEN) containing the indicated compounds for the indicated times. Labeling was stopped at the appropriate times by aspirating the labeling medium and washing once with cold Dulbecco's-PBS, the cells were lysed for 1 h on ice with 750 μl of cold RIPA buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5%

(w/v) sodium deoxycholate, 1 mM EDTA and 2.5 mM $MgCl_2$). The insoluble material and nuclei were removed by centrifugation at 12,000 x g (Eppendorf microcentrifuge) for 3 min. Non-specific binding was removed by rocking the cell extract in 1.5 ml Eppendorf tubes containing 75 µl of Protein A-Sepharose 6MB (Pharmacia Biotech), previously coupled to IgG from normal rabbit serum, for 30 min at 4 C. After this step, the cell extract was transferred to new 1.5 ml Eppendorf tubes containing 75 µl of Protein A-Sepharose coupled to our Q7 rabbit polyclonal antibody specific for $G_{q/11}\alpha$ and immunoprecipitated overnight at 4 C. The cell extract was centrifuged gently and the supernate was discarded, the beads were washed three times with 750 µl of cold RIPA buffer. Finally the beads were resuspended in SDS-polyacrylamide gel electrophoresis sample buffer (reducing agents were omitted to prevent the hydrolysis of thioester linked fatty acids) and heated at 100 C for 2 min. The immunoprecipitates were resolved by 10% SDS-PAGE, fixed and prepared for fluorography with Fluoro-Hance (RPI, Mt. Prospect, IL). The gels were exposed to Kodak X-OMAT autoradiography film for approximately 30 days at –70 C. In parallel experiments gels were treated with 1 M hydroxylamine (pH 7.0) after a 15 min fixing period, before fluorography and exposure to autoradiography film (Mumby and Buss, 1990). Treatment with hydroxylamine cleaves the thioester bonds of palmitic acids to G-proteins, and not the amide bonds of myristic acids, indicating palmitate labeling of G-proteins as opposed to myristate labeling (Mumby and Buss, 1990).

Transfection of primary pituitary cell cultures

Transfection of primary cell cultures were done in 24-well plates (Costar). Approximately 48 h after cell dispersion, cells were washed with M199 (pH 7.4), and 0.4 μg of DNA mixed with 2 μl of lipofectamine (GIBCO BRL, Gaithersburg, MD) in 0.25 ml of M199/BSA was added to each well in triplicate. After 5 h at 37 C, 0.25 ml of M199 containing 20% horse serum and 5% fetal calf serum was added to each well. After 24 h from start of transfection, medium was removed and plates prepared for inositol phosphate assays or LH RIA.

Measurement of inositol phosphate accumulation

After removing the transfection medium, plates were washed twice with a balanced salt solution (BSS) containing 0.3% BSA to remove serum and unattached cells. Cellular inositol lipids were labeled with [^{3}H]-myo-inositol (specific activity 30-60 Ci/mmol, 4 μCi/ml; Dupont NEN, Boston, MA) for 18 h. After inositol labeling cells were washed twice with BSS containing 5 mM LiCl (BSS/LiCl), and stimulated for 2 h with the indicated GnRH concentrations prepared in BSS/LiCl. The treatment solutions were removed, and 1 ml of 0.1 M formic acid added to each well. The cells were freeze-thawed once to disrupt the cell membranes, and the total [^{3}H]-inositol phosphates were determined by Dowex anion exchange chromatography and liquid scintillation spectroscopy (Huckle and Conn, 1987).

Quantification of LH release

After removing the transfection medium, cells were washed twice with M199/BSA, and stimulated with the indicated GnRH concentrations for 2 h. The medium was collected from the culture wells. LH released was determined by RIA.

The RIA used a highly purified rat LH for iodination (Hunter and Greenwood, 1962) and a reference preparation (RP3) obtained from the NIDDK (Baltimore, MD). LH antisera (C102) was prepared and characterized as previously described (Smith et al., 1982). Bound and free hormone were determined with immobilized protein A (Gupta and Morton, 1979).

Western blots

SDS-polyacrylamide gels (12% acrylamide) and Western transfers to nitrocellulose paper (Hoefer Scientific Instruments, San Francisco, CA) were performed as previously described (Conn et al., 1992). Polyclonal antisera (Kurose et al., 1993; a gift from Dr. Hitoshi Kurose, University of Tokyo, Tokyo, Japan) made against the third intracellular loop of the α_{2A}-adrenergic receptor was used at 1:500 titer. Color was developed on Western blots using 4-chloro-1-napthol (horseradish peroxidase) color development reagent (Bio-Rad Laboratories, Richmond, CA). Standards were color-stained proteins (rainbow markers, Amersham) with the following molecular weights (including the dye): myosin (200K), phosphorylase (92.5K), BSA (69K), ovalbumin (46K), carbonic anhydrase (30K), trypsin inhibitor (21.5K), and lysozyme (14.3K).

Cell culture and transfection

GGH_31' cells were derived from GH_3 cells stably transfected with the rat GnRH receptor cDNA as previously reported (Kaiser et al., 1994). The GGH_31' cells were maintained in an atmosphere of 5% CO_2 at 37 C in DMEM (Gibco, Grand Island, NY) containing 10% fetal calf serum and 20 μg/ml gentamicin. Cells were grown to confluency in 162-cm^2 T-flasks (Costar), then scraped and plated at a density of 350,000 cells/well in a 24-well culture plate for 36-40 hours at 37 C in 5% CO_2. Cells were washed twice in DMEM, 0.1% BSA, and 20 μg/ml gentamicin and treated with the indicated secretogogues for the indicated times.

Production of polyclonal $G_{q/11}\alpha$ antisera and $G_{q/11}\alpha$ RIA

Antisera was raised in rabbits using the C-terminal sequence ("CTS", QLNLKEYNLV) for the alpha subunit of the $G_{q/11}$ family of guanyl nucleotide-linked proteins coupled to keyhole limpet hemocyanin. This same CTS was radioiodinated to serve as the immunoligand in the radioimmunoassay. Unlabeled CTS was the standard, accordingly a molar correction factor of 0.03 should be used to adjust the values obtained to account for the ratio of the mol wt of the standard (CTS) to that of $G_{q/11}\alpha$. The sensitivity and LLOQ values for this assay were <5 pg/tube at a final antiserum titer of 1:100,000. The RIA was set-up by disproportionation for 12 hours. The cellular $G_{q/11}\alpha$ proteins were measured after solubilization of the cells in 0.1% Triton X-100. Bound and free proteins were separated using the second antibody technique (Gupta and Morton, 1979).

DNA quantitation

$GGH_3$1' cells were plated at 350,000 cells/well in a 24-well plate and maintained at 37 C, 5% CO_2 for 36-40 hours. Cells were treated with medium alone (control) or with the GnRH agonist, Buserelin (Hoescht-Roussel Pharmaceuticals, Somerville, NJ), 0.1 μg/ml for 0, 1, 2, 3, 4, 5, 6, and 24 hours. The supernate was removed and the cells were frozen. The previously frozen cells were scraped, washed and assayed in 0.44 N perchloric acid (PCA). The DNA content per well for each treatment and time point was assessed using the mini-diphenylamine assay (Burton, 1956).

Results

The time course of GnRH-stimulated palmitoylation of $G_{q/11}\alpha$ in pituitary cell cultures is shown in figure 1. Rat pituitary cell cultures were treated with 10^{-6} g/ml GnRH or cell culture medium in the presence of [^{3}H]-palmitate for 0, 20, 40, 60, 90 and 120 min. Immunoprecipitation of $G_{q/11}\alpha$ showed an increase in [^{3}H]-palmitate incorporation with GnRH treatment. The earliest detectable incorporation of the label is measurable at 20 min after the addition of GnRH. GnRH stimulated incorporation of the label is detectable up to 120 min. The basal incorporation of [^{3}H]-palmitate label increases with time, although in the presence of GnRH there is an increased incorporation over basal levels.

The palmitate incorporation to $G_{q/11}\alpha$ was dose dependent with GnRH (figure 2). Pituitary cell cultures were treated with medium, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} g/ml of GnRH in the presence of [^{3}H]-palmitate for 60 min. GnRH concentration of approximately 10^{-9} g/ml produced a half maximal incorporation of palmitate label on $G_{q/11}\alpha$.

To further assess the specificity of GnRH receptor stimulated palmitoylation of $G_{q/11}\alpha$, we examined the ability of cholera toxin (5 μg/ml), pertussis toxin (100 ng/ml), phorbol myristic acid (PMA, a protein kinase C activator; 100 ng/ml), and Antide (GnRH antagonist; 50 nM) to evoke palmitoylation of $G_{q/11}\alpha$ (figure 3). Rat pituitary cell cultures were incubated in the presence of the above agents for 40 min, and $G_{q/11}\alpha$ was immunoprecipitated. Only PMA and GnRH increased the incorporation of [^{3}H]-palmitate label on $G_{q/11}\alpha$ above basal incorporation levels. The level of $G_{q/11}\alpha$ incorporation of palmitate when treated with CTX, PTX and Antide were not significantly different from basal levels.

Gels treated with 1 M hydroxylamine before autoradiography did not show any residual radioactivity (data not shown), indicating that the radiolabel was alkali sensitive, as would be expected from a thioester linked palmitate label.

Transfection of primary cell cultures with the cDNA for the third intracellular loops of the α_{1B}-adrenergic receptor ($\alpha_{1B}3_i$), α_{2A}-adrenergic receptor ($\alpha_{2A}3_i$), M_1-muscarinic receptor (M_13_i), M_2-Muscarinic receptor (M_23_i) and D_{1A}-dopamine receptor ($D_{1A}3_i$) did not inhibit GnRH-stimulated LH release from vector transfected levels (figure 4). Primary cell cultures were transiently transfected using lipofectamine, and GnRH-stimulated LH release was measured by RIA. Transfection efficiency was measured to be 5-7%.

Although $\alpha_{1B}3_i$ did not inhibit GnRH-stimulated LH release, transfection of this loop resulted in approximately 10% inhibition of GnRH stimulated IP turnover as compared to vector transfected values in rat pituitary cell cultures. IP turnover was measured as described in Methods. Transfection of cDNA for $\alpha_{2A}3_i$, M_13_i, M_23_i and $D_{1A}3_i$ resulted in no significant inhibition of GnRH stimulated IP turnover (figure 5).

Cellular expression of $\alpha_{2A}3_i$ was determined by immunoblotting cell lysates with the peptide specific antisera (figure 6). Cell lysates were prepared from rat pituitary cell cultures transiently transfected with the cDNA for $\alpha_{2A}3_i$. A single band was seen at the apparent MW of approximately 21 kDa. This band was absent in cell lysates obtained from pRK5 transfected rat pituitary cell cultures.

The time-course of Buserelin stimulated net loss of $G_{q/11}\alpha$ immunoreactivity is shown in figure 7. GGH_31' cell cultures (GH_3 derived cell line stably expressing the GnRH receptor) were treated for the indicated times with 0.1 μg/ml of Buserelin or medium alone, and the total cellular $G_{q/11}\alpha$ was measured by RIA as described in Methods. Consistent with

a cell doubling time of approximately 1 day, the total amount of $G_{q/11}\alpha$ increased with time both in control cells and in cells treated with 0.1 μg/ml of Buserelin. Although, in cells treated with Buserelin, total levels of $G_{q/11}\alpha$ were consistently 15-20% less than in control cells at 24 h. The reduction in total cellular $G_{q/11}\alpha$ after Buserelin treatment was first observed at 6 h.

The loss of $G_{q/11}\alpha$ immunoreactivity was dose-dependent with Buserelin (figure 8). GGH_31' cells were treated with the indicated doses of Buserelin for 24 h and the total cellular $G_{q/11}\alpha$ immunoreactivity was assayed by RIA. Buserelin concentration of 10^{-9} g/ml produced a half maximal loss of $G_{q/11}\alpha$ immunoreactivity. The effect of 10^{-9} g/ml Buserelin on assayable $G_{q/11}\alpha$ was blocked by 10^{-6} g/ml Antide (a GnRH antagonist; 340 $\pm$ 8 pg/60 μl), as compared to medium treated levels (339 $\pm$ 13 pg/60 μl). Furthermore, 10^{-6} g/ml Antide alone (322 $\pm$ 8 pg/60 μl) did not produce a loss of $G_{q/11}\alpha$ immunoreactivity compared to medium treated levels (339 $\pm$ 13 pg/60 μl).

PMA did not mimic this action of Buserelin treatment (figure 9). GGH_31' cells were treated with 0.33 - 100 ng/ml of PMA for 24 h and total cellular $G_{q/11}\alpha$ was assayed by RIA. Treatment of GGH_31' cells with PMA for 24 h did not result in any significant loss of immunoreactive $G_{q/11}\alpha$ as compared to medium treated levels.

Discussion

The data presented here demonstrate that $G_{q/11}\alpha$ is palmitoylated in a time- and dose-dependent manner when the GnRH receptor is occupied by an agonist. Antagonist occupancy of the receptor does not lead to this effect. Stimulation of hormone release from dispersed pituitary cell cultures with increasing concentrations of GnRH resulted in the concurrent increase in incorporation of [^{3}H]-palmitic acid label into $G_{q/11}\alpha$. This effect was time- and dose-dependent. Similarly, cholera toxin and pertussis toxin did not increase the incorporation of the label into $G_{q/11}\alpha$. $G_{q/11}\alpha$ is cholera- and pertussis-toxin insensitive, and as expected, these agents do not stimulate the incorporation of [^{3}H]-palmitic acid. Phorbol myristic acid, a PKC activator, stimulated palmitate labeling of $G_{q/11}\alpha$, although not to the same extent as GnRH stimulated levels. We also show that the transfection of the $\alpha_{1B}3_i$ loop cDNA resulted in the partial inhibition of GnRH receptor mediated inositol phosphate turnover in dispersed rat pituitary cell cultures. Although the expression of this peptide did not significantly inhibit GnRH stimulated LH release in these cultures. Transfection of the cDNA sequences for $\alpha_{2A}3_i$ loop, M_13_i loop, M_23_i loop, and $D_{1A}3_i$ loop did not significantly inhibit GnRH stimulated IP turnover or LH release. The expression of one of the plasmids containing the cDNA sequence for the $\alpha_{2A}3_i$ loop was confirmed by Western analysis. We were unable to perform Western analysis to confirm the expression of other third intracellular loops due to the unavailability of antisera against these peptides. Since the same expression vector (pRK5; Eaton et al., 1986) was used for all studies, it is reasonable to believe that these would be expressed at similar levels as the $\alpha_{2A}3_i$ loop. The regulation of $G_{q/11}\alpha$ by the GnRH receptor extends towards the GGH_31' cells. Buserelin treatment of these cells resulted in the loss of total cellular $G_{q/11}\alpha$ as assessed by a RIA. This effect of

Buserelin was time- and dose-dependent, and was antagonized by GnRH antagonist, Antide. Neither Antide alone, nor PMA mimicked the actions of Buserelin.

This study was designed to investigate the G-proteins that are regulated by the GnRH receptor. G-protein involvement in GnRH action in the gonadotrope has been suggested by studies which show stimulation of LH release by stable GTP analogs and by IP accumulation in ATP-permeabilized cells (Hawes et al., 1992; Andrews et al., 1986). Although ample evidence is available to suggest G-protein involvement in the gonadotrope, specific G-protein(s) are yet to be identified. In this study, we used G-protein coupled receptor evoked palmitoylation of Gα to identify specific moieties that are regulated by the GnRH receptor. This study demonstrates that GnRH receptor regulates the palmitoylation of $G_{q/11}\alpha$. As palmitoylation of $G_{q/11}\alpha$ is dependent on this moiety being activated by a receptor, it suggests that GnRH receptor is coupled to $G_{q/11}\alpha$. PMA, a PKC activator, stimulated $G_{q/11}\alpha$ incorporation of [^{3}H]-palmitic acid, albeit less than GnRH stimulated levels. The effect of PMA on $G_{q/11}\alpha$ incorporation of [^{3}H]-palmitic acid is puzzling, as PMA did not have any significant effect on the Buserelin evoked loss of $G_{q/11}\alpha$ immunoreactivity in GGH_3 cells. The effect of PMA on $G_{q/11}\alpha$ palmitoylation may be the result of activation of G-protein palmitoyltransferase, the enzyme responsible for addition and removal of palmitate from G-proteins. Alternatively, PMA may activate $G_{q/11}\alpha$ directly or through PKC thereby presenting it as an activated G-protein for palmitoyltransferase to palmitoylate, although there is no prior evidence for this action of PMA. However, GnRH evoked palmitoylation is greater than PMA alone. This may indicate that GnRH-receptor evoked palmitoylation reflects a direct activation of G-proteins, while the action of PMA

(mediated through PKC) may be pharmacologic. The receptor mediated component demonstrates that the GnRH-receptor is coupled to $G_{q/11}\alpha$ in the gonadotrope.

Inhibition of GnRH receptor evoked IP turnover by transfecting the cDNA for the $\alpha_{1B}3_i$ loop, whose cognate receptor mediates IP turnover through a member of the $G_{q/11}\alpha$ family, demonstrated that the GnRH receptor is coupled to $G_{q/11}\alpha$ and further confirmed the palmitoylation studies. Luttrell et al., (1993) showed that $\alpha_{1B}3_i$ loop inhibited heterologous receptor mediated IP turnover, which collaborates with the data presented in this study. Although M_1Ach receptor mediates IP turnover through a pertussis toxin insensitive G-protein, transfection of M_13_i loop cDNA did not inhibit GnRH receptor mediated IP turnover. This may indicate that this loop is less effective at inhibiting GnRH receptor mediated IP turnover, because the inhibition of heterologous receptor mediated activity by third intracellular loop peptides can vary (Hawes et al., 1994). The lack of inhibition may also be due to the fact that this loop is expressed to a lesser extent than $\alpha_{1B}3_i$ loop, although this is unlikely as both the $\alpha_{1B}3_i$ loop and the M_13_i loop is translated by the same regulatory elements, namely, the CMV promoter of the pRK5 vector. Transfection of $\alpha_{2A}3_i$ loop cDNA and M_23_i loop cDNA did not inhibit GnRH receptor mediated signal transduction, although the cognate receptors of these loops couple to $G_i\alpha$ (Hawes et al., 1994), which has been implicated in GnRH receptor mediated pertussis toxin sensitive IP turnover (Hawes et al., 1993). The lack of inhibition may be due to the same reasons that were mentioned earlier, including the fact that GnRH receptor may not couple to these G-proteins. As would be expected, $D_{1A}3_i$ loop, which inhibit $G_s\alpha$ mediated actions, did not inhibit GnRH evoked IP turnover or LH release because these events are not thought to involve $G_s\alpha$. The decrease in maximal effects of GnRH evoked IP turnover seen with $\alpha_{1B}3_i$ loop transfection,

has also been observed by other investigators (Hawes et al., 1994). They have shown that the peptide mediated inhibition of receptor-evoked IP turnover is due to competition between the hormone-receptor complex and the peptide for the common binding site on Gα-subunit. This competition can be overcome by increasing the hormone-receptor coupled by increasing its transfected receptor cDNA.

Previous work done in cell lines have shown that activation of $G_s\alpha$ induces a conformational change that allows a loss of membrane avidity and increase its degradation rate (Levis and Bourne, 1992). Furthermore, in αT3-1 cell lines GnRH agonist treatment results in an increased degradation of $G_{q/11}\alpha$ (Shah et al., 1995). These observations support our findings in the GGH_3 cells; Buserelin treatment resulted in the time- and dose-dependent decrease in cellular $G_{q/11}\alpha$. Using the increased degradation of activated Gα as a marker, our results show that $G_{q/11}\alpha$ is activated by the GnRH receptor. For technical reasons, these studies were done in an immortalized cell line, because gonadotropes are only about 20% of the cells in culture prepared from female weanling rat pituitaries, and changes in G-protein content can be masked by a relatively high background from non-gonadotrope cells. Although this study was done in a cell culture, it corroborates well with our previous findings to show that the GnRH receptor regulates $G_{q/11}\alpha$. Furthermore, the fact that PMA treatment did not mimic the actions of Buserelin on loss of $G_{q/11}\alpha$ may indicate that PKC is not involved, and this action of GnRH agonist is mediated through a direct GnRH-receptor/$G_{q/11}\alpha$ interaction.

This study provides evidence for GnRH receptor regulation of $G_{q/11}\alpha$. We have shown that $G_{q/11}\alpha$ incorporates [^{3}H]-palmitic acid in a dose-and time-dependent manner when treated with GnRH, and the third intracellular loop of the α_{1B}-adrenergic receptor,

which couples to $G_{q/11}\alpha$, act as an intracellular inhibitor of GnRH receptor mediated IP turnover. We have also shown that GnRH agonist treatment results in dose- and time-dependent loss of $G_{q/11}\alpha$ immunoreactivity in GGH_31' cells. The observation made in this study suggest that GnRH receptor regulates the activity of $G_{q/11}\alpha$ in rat gonadotropes and also in GGH_31' cell line. The ability of the GnRH-receptor to regulate $G_{q/11}\alpha$ activity indicates that it is able to couple to this G-protein.

Acknowledgments

We thank Linda Wolf for preparing the manuscript and Dr. Brian Hawes and Dr. Alfredo Ulloa-Aguirre for helpful suggestions.

Figure 4-1. The time course for GnRH stimulated incorporation of [^{3}H]-palmitic acid into $G_{q/11}\alpha$. Dispersed rat pituitary cell cultures were incubated in the presence or absence of 10^{-6} g/ml GnRH for the indicated times. Incorporation of the label into $G_{q/11}\alpha$ was assayed by immunoprecipitation then resolved by 10% SDS-PAGE, fluorography and densitometry. Data show band density in arbitrary optical density units. The data are from one representative experiment. Three separate experiments showed similar results.

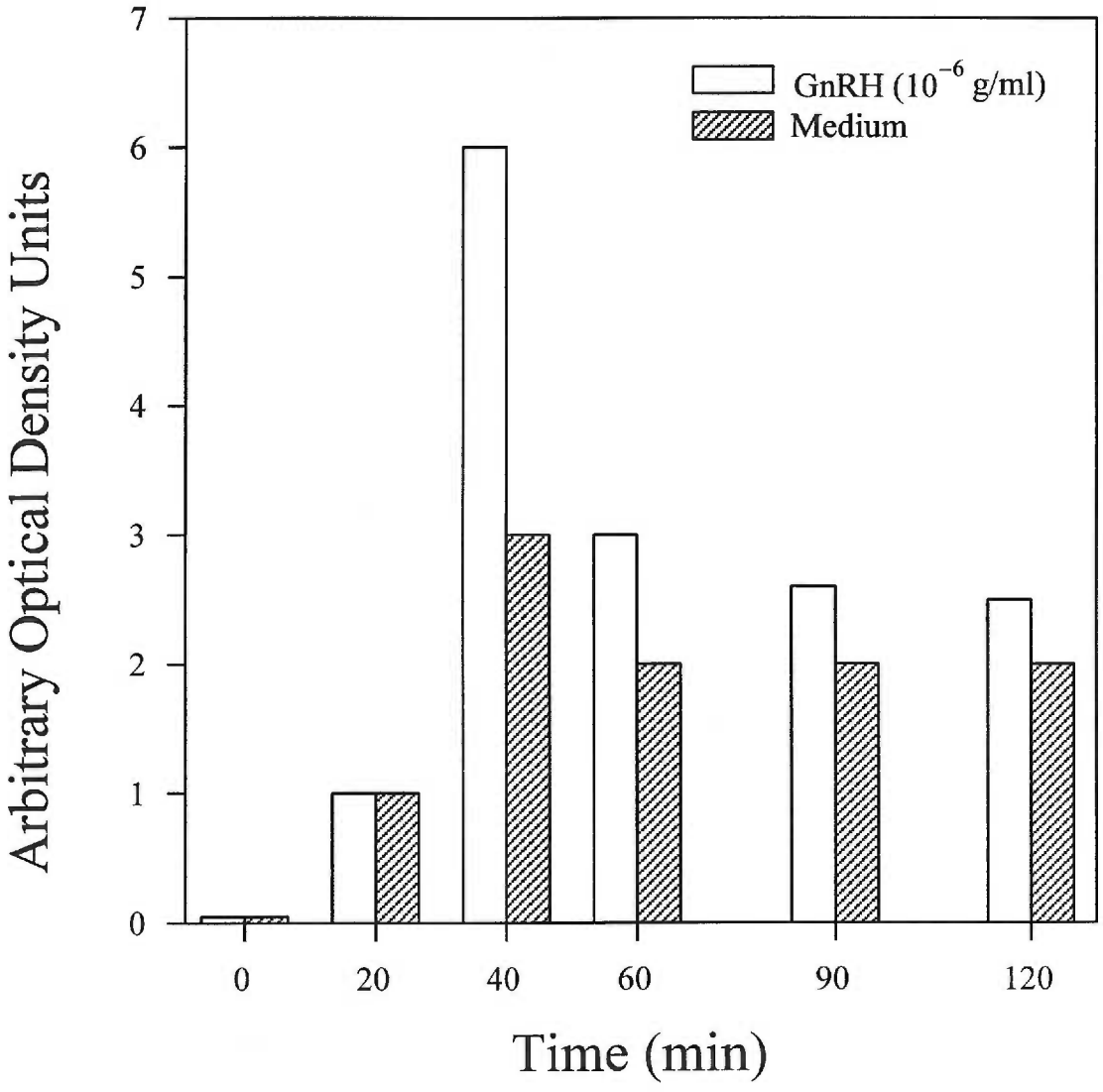

Arbitrary Optical Density Units
7
6
5
4
3
2
1
0
GnRH (10^{-6} g/ml)
Medium
0
20
40
60
90
120
Time (min)

Figure 4-2. The dose-response for GnRH stimulated incorporation of [^{3}H]-palmitic acid into $G_{q/11}\alpha$. Dispersed rat pituitary cell cultures were incubated for 60 min with the indicated doses of GnRH. Incorporation of the label into $G_{q/11}\alpha$ was assayed by immunoprecipitation then resolved by 10% SDS-PAGE, fluorography and densitometry. Data show band density in arbitrary optical density units. The data are from one representative experiment. Three separate experiments showed similar results.

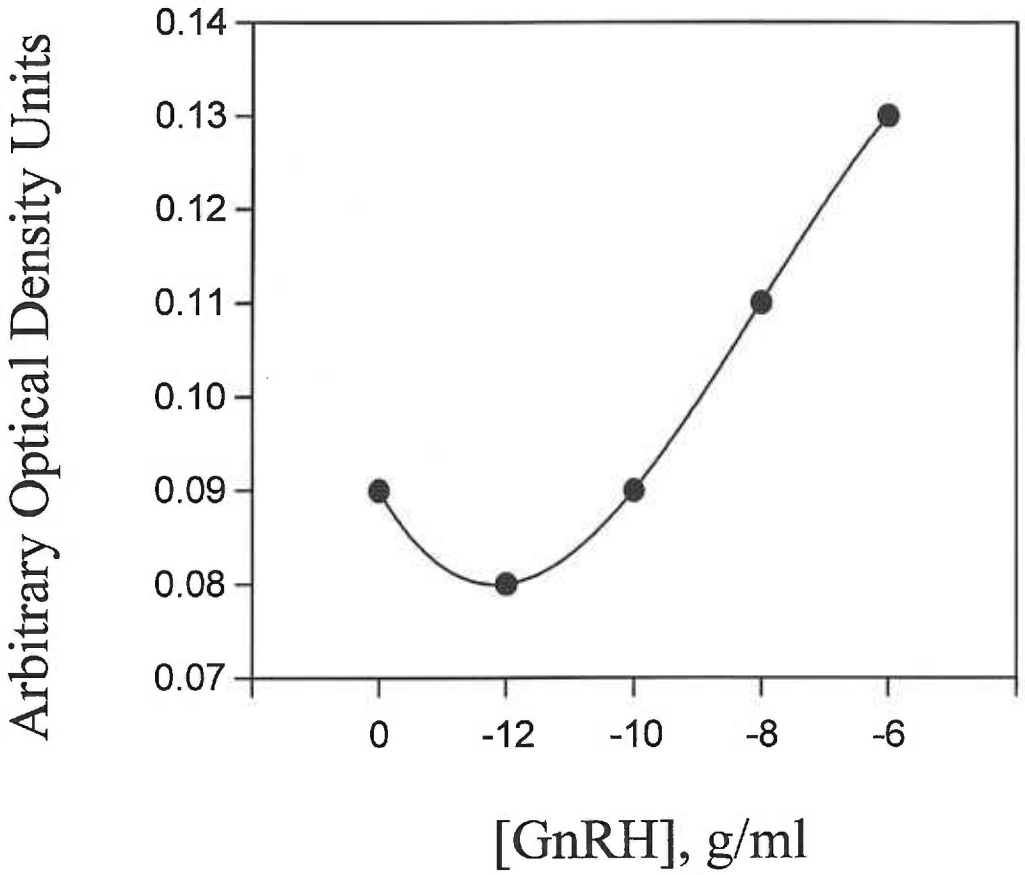

Arbitrary Optical Density Units
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0
-12
-10
-8
-6
[GnRH], g/ml

Figure 4-3. The effect of the indicated agents on $G_{q/11}\alpha$ incorporation of [^{3}H]-palmitic acid. Dispersed rat pituitary cell cultures were incubated in the presence of the indicated agents at the indicated concentrations for 40 min. Incorporation of the label into $G_{q/11}\alpha$ was assayed by immunoprecipitation then resolved by 10% SDS-PAGE, fluorography and densitometry. Data show band density in arbitrary optical density units. The data are from one representative experiment. Three separate experiments showed similar results.

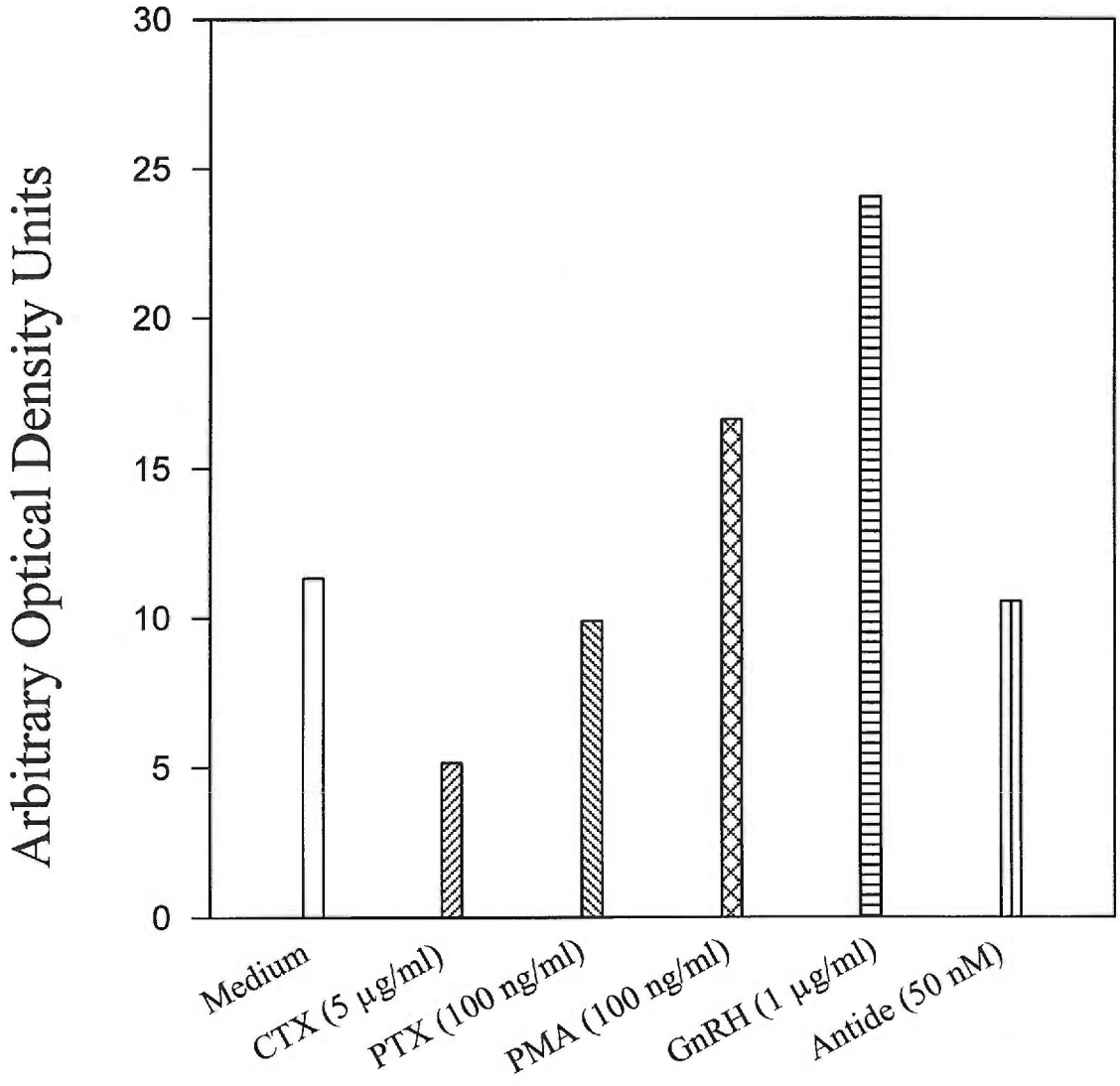

Arbitrary Optical Density Units
30
25
20
15
10
5
0
Medium
CTX (5 µg/ml)
PTX (100 ng/ml)
PMA (100 ng/ml)
GnRH (1 µg/ml)
Antide (50 nM)

Figure 4-4. GnRH stimulated LH release in transiently transfected dispersed rat pituitary cell cultures. Cell cultures were transiently transfected, as described in the methods, with the indicated third intracellular loops of G-protein coupled receptors in pRK5 expression vector. 24 h after transfection, cells were stimulated for 2 h with the indicated concentrations of GnRH, and LH released into the medium was assayed by RIA. The data are the mean of triplicate transfections and error bars show the SEM. Three separate experiments showed similar results.

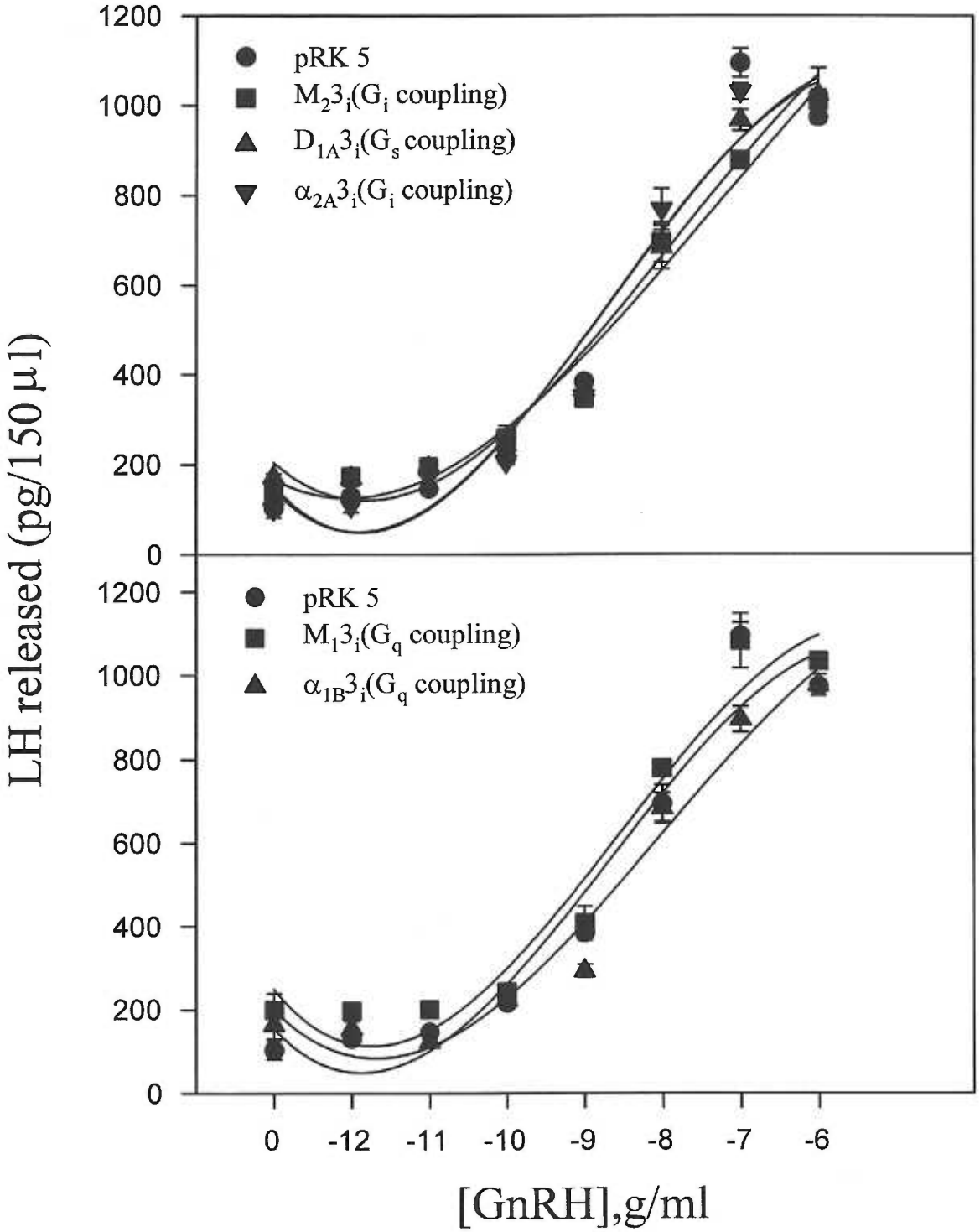

LH released (pg/150 µl)
1200
1000
800
600
400
200
0
pRK 5
M2 3i(Gi coupling)
D1A 3i(Gs coupling)
α2A 3i(Gi coupling)
pRK 5
M1 3i(Gq coupling)
α1B 3i(Gq coupling)
0
-12
-11
-10
-9
-8
-7
-6
[GnRH],g/ml

Figure 4-5. GnRH stimulated IP turnover in transiently transfected dispersed rat pituitary cell cultures. Cell cultures were transiently transfected, as described in the methods, with the indicated third intracellular loops of G-protein coupled receptors in pRK5 expression vector. 24 h after transfection, the cellular inositol was labeled with [^{3}H]-myo-inositol for 18 h. Following which cells were stimulated for 2 h with the indicated concentrations of GnRH, and total [^{3}H]-inositol phosphates were determined by Dowex anion exchange chromatography and liquid scintillation spectroscopy. The data are the mean of triplicate transfections, and error bars show the SEM. Three separate experiments showed similar results.

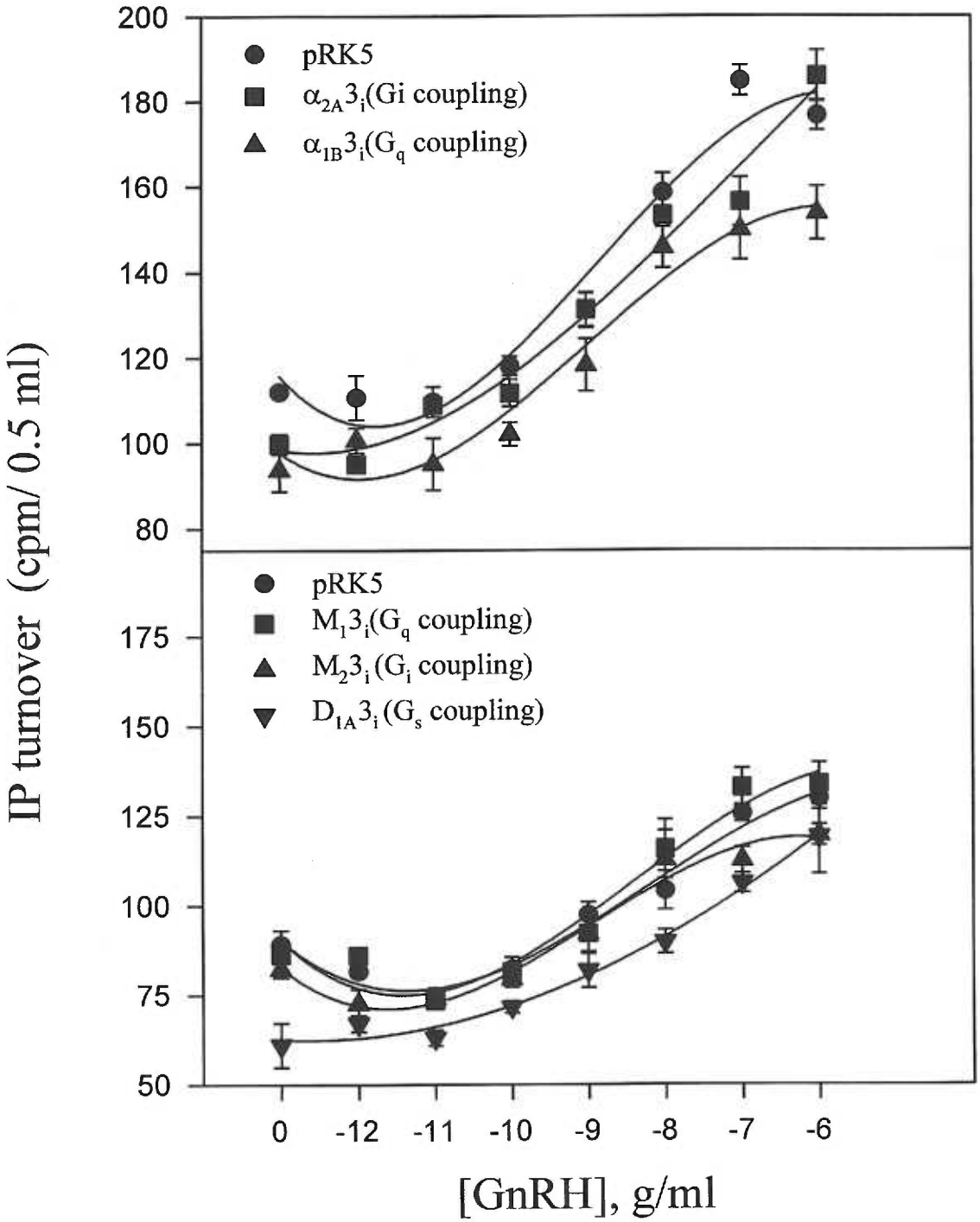

pRK5
$\alpha_{2A}3_i$(Gi coupling)
$\alpha_{1B}3_i$(G_q coupling)
pRK5
$M_1 3_i$(G_q coupling)
$M_2 3_i$ (G_i coupling)
$D_{1A} 3_i$ (G_s coupling)
IP turnover (cpm/ 0.5 ml)
200
180
160
140
120
100
80
175
150
125
100
75
50
0
-12
-11
-10
-9
-8
-7
-6
[GnRH], g/ml

Figure 4-6. Protein immunoblot depicting expression of the third intracellular loop of the α_{2A}-adrenergic receptor. Rat pituitary cell cultures were transiently transfected with the third intracellular loop of the α_{2A}-adrenergic receptor or the empty expression vector pRK5. Twenty-four h after transfection cell cultures were lysed and expression of the peptide was detected with immunoblotting using peptide specific antisera as described under "Methods."

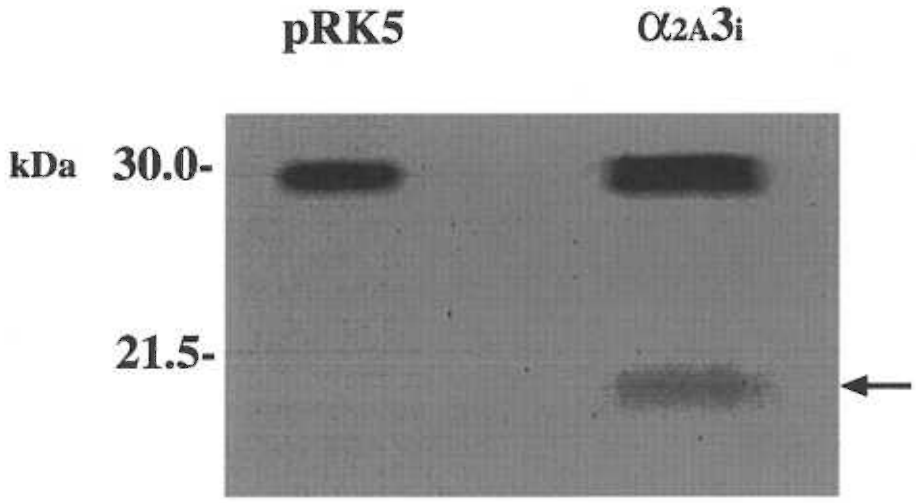

pRK5
α2A3i
kDa
30.0-
21.5-

Figure 4-7. Time course of $G_{q/11}\alpha$ proteins from GGH_31' cells treated with medium alone (●) or 0.1 μg/ml Buserelin (■). The $G_{q/11}\alpha$ proteins were measured by RIA. The data shown are the means ± SEM of quadruplicate determinations. A molar correction factor of 0.03 should be used to adjust the values obtained to account for the ratio of the mol wt of the standard (CTS) to that of $G_{q/11}\alpha$. Three separate experiments showed similar results.

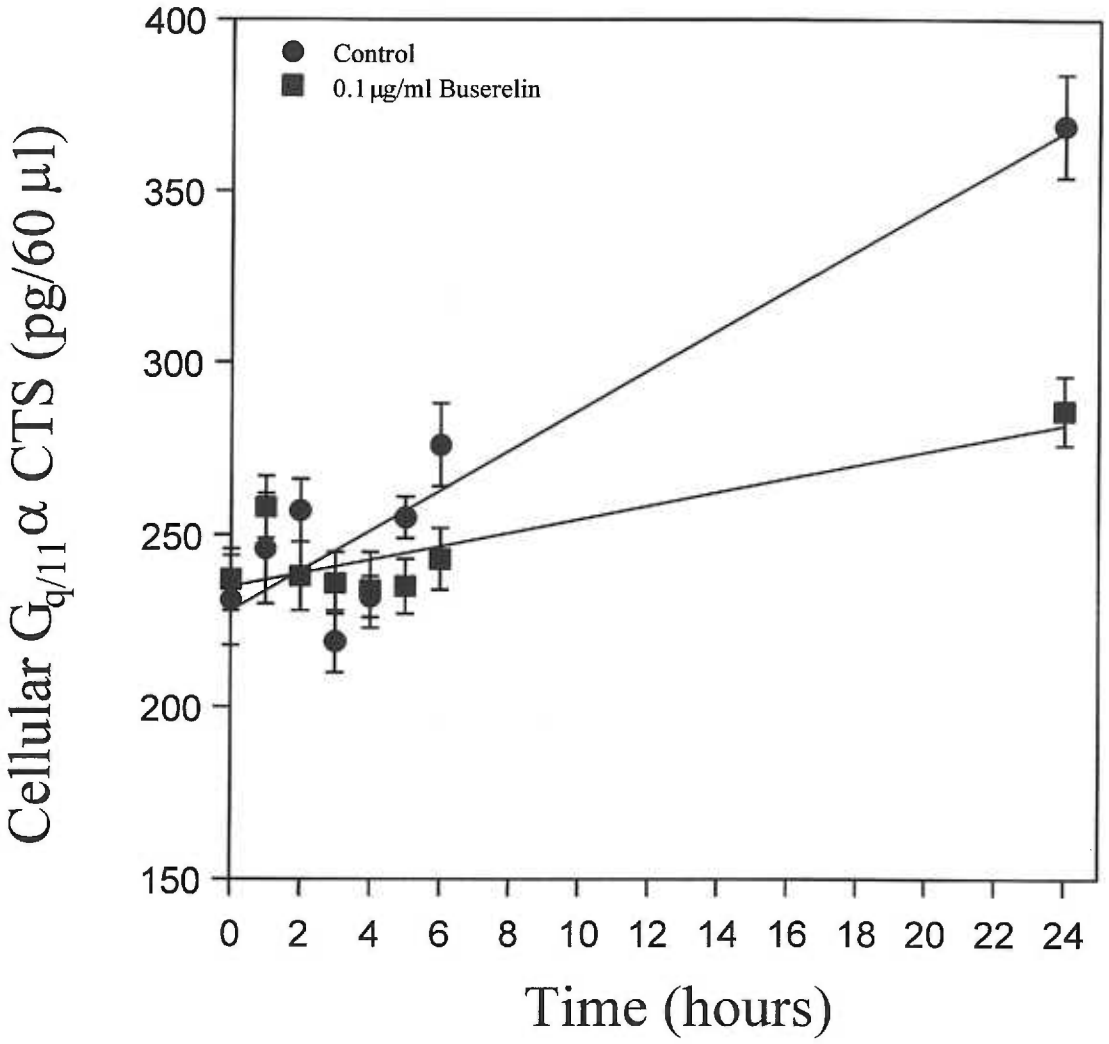

Control
0.1 µg/ml Buserelin
Cellular $G_{q/11}\alpha$ CTS (pg/60 µl)
400
350
300
250
200
150
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (hours)

Figure 4-8. Dose-response curve of $G_{q/11}\alpha$ proteins from GGH_31' cells treated 24 h with Buserelin (●). 10^{-6} g/ml Antide in the presence of 10^{-9} g/ml Buserelin (340 ± 8 pg/60 µl) or in the absence (322 ± 8 pg/µl) did not produce a loss of immunoreactivity as compared to medium treated levels (339 ± 13 pg/60 µl). The $G_{q/11}\alpha$ proteins were measured by RIA. The data shown are the means ± SEM of triplicate determinations. A molar correction factor of 0.03 should be used to adjust the values obtained to account for the ratio of the mol wt of the standard (CTS) to that of $G_{q/11}\alpha$. Three separate experiments showed similar results.

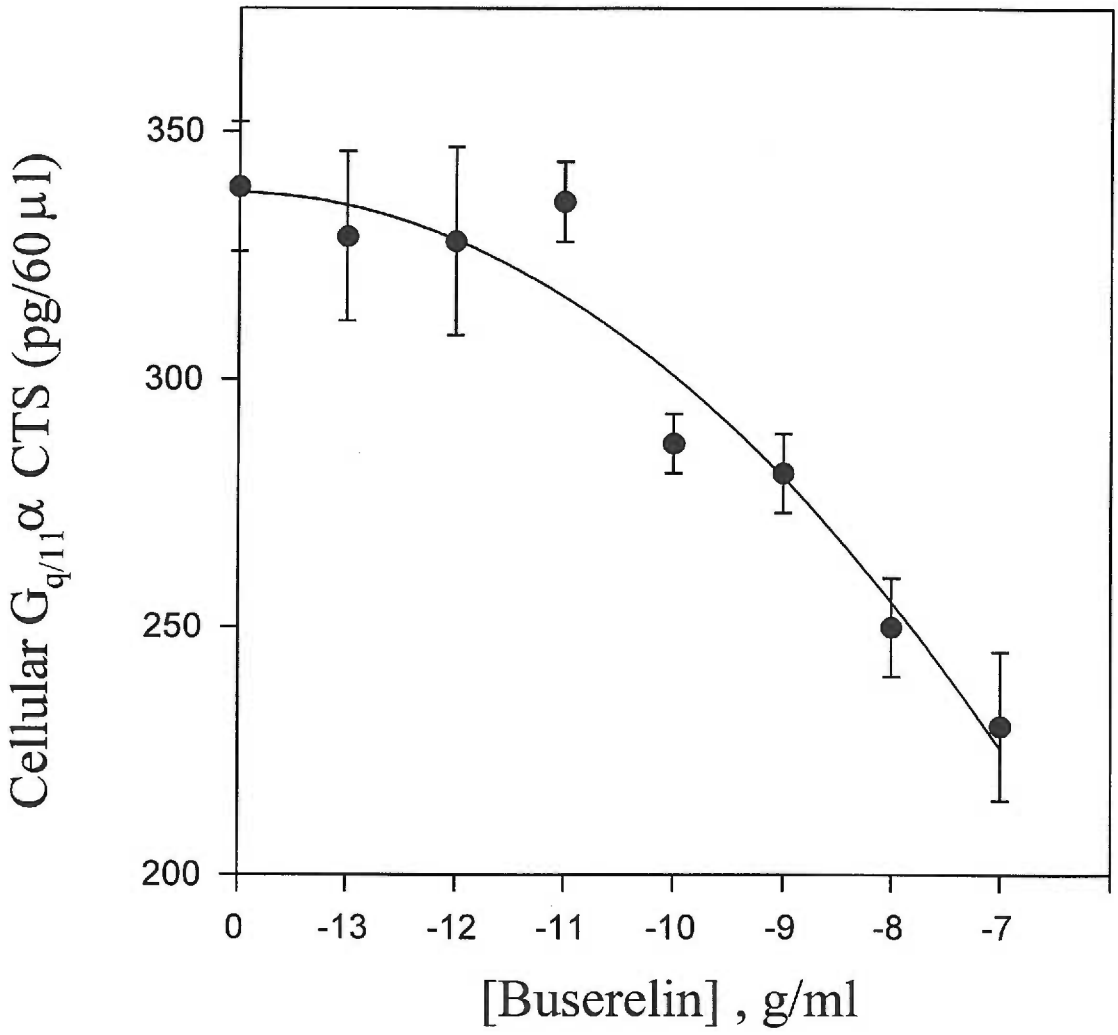
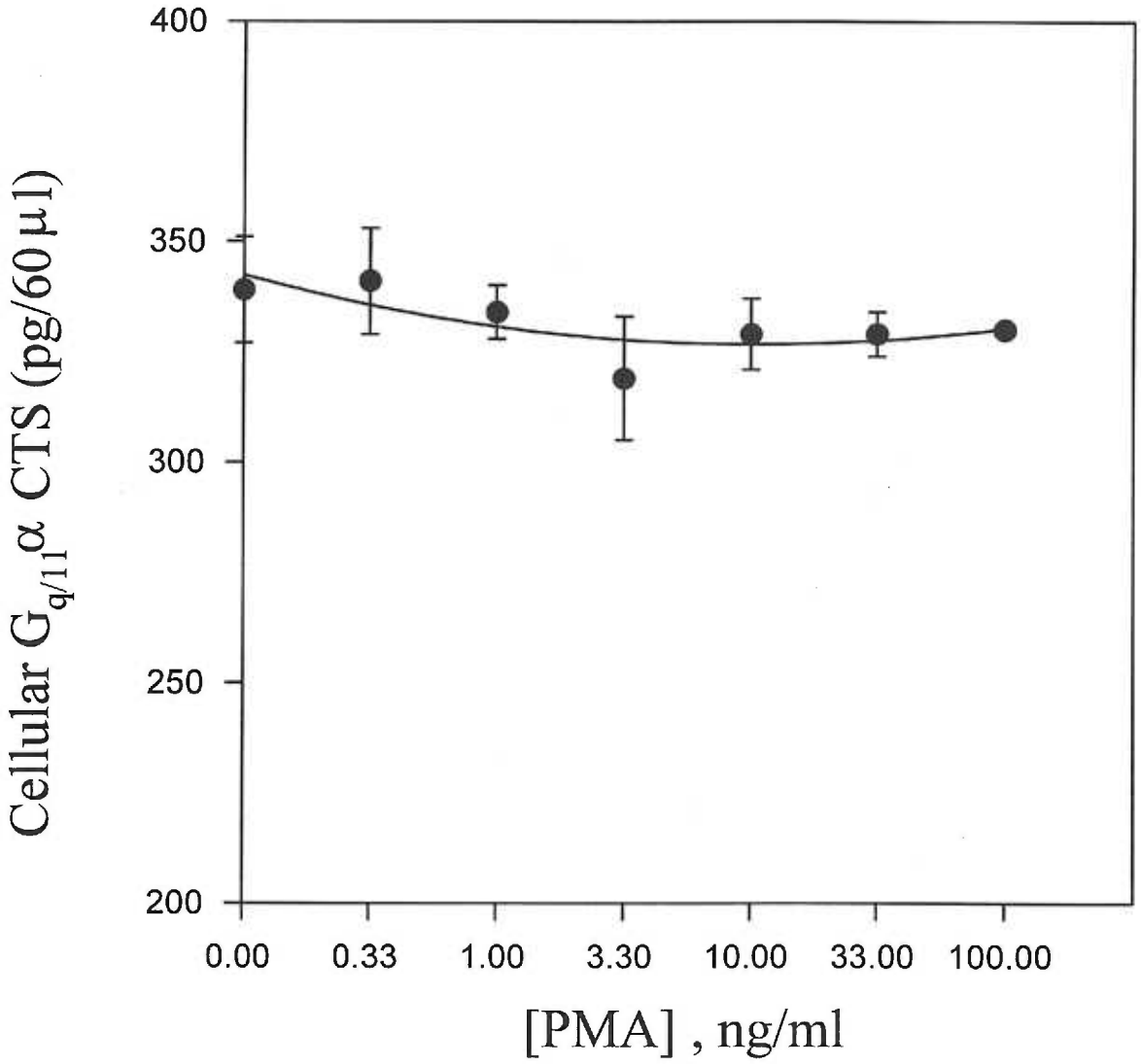

Cellular $G_{q/11}\alpha$ CTS (pg/60 µl)
350
300
250
200
0
-13
-12
-11
-10
-9
-8
-7
[Buserelin] , g/ml

Figure 4-9. Dose-response curve of $G_{q/11}\alpha$ proteins from GGH_31' cells treated for 24 h with PMA. The $G_{q/11}\alpha$ proteins were measured by RIA. The data shown are the means $\pm$ SEM of triplicate determinations. A molar correction factor of 0.03 should be used to adjust the values obtained to account for the ratio of the mol wt of the standard (CTS) to that of $G_{q/11}\alpha$. Three separate experiments showed similar results.


400
350
300
250
200
Cellular $G_{q/11}\alpha$ CTS (pg/60 µl)
0.00
0.33
1.00
3.30
10.00
33.00
100.00
[PMA] , ng/ml

CHAPTER FIVE

GnRH RECEPTOR COUPLES TO MULTIPLE G-PROTEINS IN GONADOTROPES AND IN GGH_3 CELLS: EVIDENCE FROM PALMITOYLATION AND OVEREXPRESSION OF G-PROTEINS

As submitted to

Biology of Reproduction

Abstract

Evidence that suggests GnRH-receptor coupling to multiple G-proteins is present from several cell systems. For example, GnRH receptor couples to $G_i\alpha$ in reproductive tract tumors, $G_s\alpha$ in GGH_3 cell lines and $G_{q/11}\alpha$ in rat pituitary cell cultures. Presently there are no published studies showing GnRH receptor coupling to $G_i\alpha$, $G_s\alpha$ and $G_{q/11}\alpha$ in a single cell type. To examine this possibility we measured palmitoylation of G-proteins in response to GnRH receptor occupancy, since this event is a measure of G-protein activation by cognate receptors. GnRH stimulated time-(0-120 min) and dose-dependent (10^{-12}-10^{-6} g/ml) palmitoylation of both $G_i\alpha$ and $G_s\alpha$. Palmitoylation is G-protein activation dependent; accordingly, pertussis toxin (100 ng/ml; PTX), phorbol myristic acid (100 ng/ml) and Antide (50 nM; a GnRH antagonist) did not stimulate palmitoylation of $G_i\alpha$ or $G_s\alpha$ above basal levels. However cholera toxin (5 μg/ml), an activator of $G_s\alpha$, stimulated palmitoylation of $G_s\alpha$, but not $G_i\alpha$. We used a lactotrope-derived cell line, expressing the GnRH receptor (GGH_3), to examine whether the ability of the receptor to couple multiple G-proteins is gonadotrope specific. GGH_3 cells were transfected with specific cDNA coding for different G-proteins, and agonist stimulated second messenger production was assessed. Buserelin (a GnRH agonist) stimulated increased cAMP release in $G_s\alpha$ cDNA transfected GGH_3 cells, whereas in $G_i\alpha$ cDNA transfected cells, both IP production and cAMP release were decreased in response to Buserelin. Transfection of $G_q\alpha$, $G_{11}\alpha$, $G_{14}\alpha$, and $G_{15}\alpha$ cDNA into GGH_3 cells resulted in an increased IP production in response to Buserelin, indicating that GnRH receptor couples to this PTX insensitive G-protein family. The observations presented in this

study provides evidence for GnRH receptor coupling to multiple G-proteins in a single cell type.

Introduction

Gonadotropin releasing hormone (GnRH) action mediates, luteinizing hormone (LH) and follicle stimulating hormone (FSH) release from the anterior pituitary. GnRH action is mediated through a G-protein coupled, 7-transmembrane segment receptor (7-TMS; Tsutsumi et al., 1992).

Toxin studies in dispersed pituitary cell cultures have shown that G-proteins are present in the gonadotrope and are coupled to effector systems. Pertussis toxin (PTX) pretreatment of rat pituitary cell cultures, which ADP-ribosylates G-proteins of the $G_i\alpha$ family to prevent activation by receptors, inhibits GnRH stimulated inositol phosphate production (Hawes et al., 1992; Andrews et al., 1986; Hawes et al., 1993). In addition, in human reproductive tract tumors, GnRH receptor couples to $G_i\alpha$ proteins indicating an intrinsic ability of the receptor to couple to this family of proteins (Imai et al., 1996). However in gonadotrope-derived cell lines (αT3-1 cells) and in lactotrope-derived cell lines stably expressing the GnRH receptor (GGH_3 cells), $G_{q/11}\alpha$ couples to the GnRH receptor (Hsieh and Martin, 1992; Janovick and Conn, 1994). More recent evidence suggests that $G_{q/11}\alpha$ is palmitoylated in a dose- and time-dependent manner in rat pituitary cell cultures (Stanislaus et al., 1997), and is redistributed in response to treatment with GnRH, providing more direct evidence for the activation of $G_{q/11}\alpha$ by the GnRH receptor (Cornea, et al., 1998). In addition to $G_i\alpha$ and $G_{q/11}\alpha$ coupling to the GnRH receptor, evidence from cholera toxin (CTX) pretreatment studies indicate that $G_s\alpha$ may also be coupled to the GnRH receptor. Pretreatment of rat pituitary cell cultures with CTX (an activator of $G_s\alpha$) results in the movement of LH to a releasable pool, potentiating GnRH stimulated LH release (Janovick and Conn, 1993).

The coupling of 7-TMS receptors to different signal transduction systems could depend on several factors, such as receptor/G-protein affinity, receptor density, and G-protein concentration. In Chinese hamster ovary cells stably expressing M_2 muscarinic receptor, agonist stimulated phosphoinositide hydrolysis is less efficient than adenylyl cyclase inhibition, suggesting differential affinities of the receptor to the G-proteins involved in this mechanism (Askenazi et al., 1987). Moreover, receptor density has a profound influence on the signal transduction characteristics of a cell. For example, strong adenylyl cyclase stimulators, such as β-adrenergic receptors and type 2 vasopressin receptors, activate phospholipase C at very high receptor densities (Zhu et al., 1994).

These studies suggest that multiple G-protein coupling observed with GnRH receptor may be a result of experimental conditions that alter receptor/G-protein affinity, receptor density and G-protein concentration. Depending on the type of cell, the concentration of G-proteins, the receptor density, and the compartmentalization of signal transduction machinery the accessibility of G-proteins to receptors may change, leading to unexpected interactions. Indeed, transfection of alpha 1D adrenergic receptor cDNA or NPY receptor cDNA in different cell lines resulted in differential coupling to second messenger systems (Perez et al., 1993; Herzog, et al., 1992). Therefore investigations of GnRH receptor/G-protein coupling in primary pituitary cell cultures maybe more indicative of physiological interactions than those measured in cell lines.

Agonist stimulated second messenger production can be used to indicate which G-proteins are involved in signal transduction. However, different G-proteins can give rise to the same second messenger. For example, both G_i and $G_{q/11}$ proteins can stimulate

inositol phosphate production (IP). Another complication in using second messengers to identify G-proteins is that the second messenger production may be too low to be detectable. For example, in the rat pituitary, CTX ($G_s\alpha$ activator) potentiates GnRH stimulated LH release, although even the most sensitive radioimmunoassay (RIA) has difficulty in detecting GnRH-stimulated cAMP (Janovick and Conn, 1993), resulting in an ambiguity about the involvement of $G_s\alpha$ in GnRH receptor action. Therefore, it is important that a more direct method be used to investigate $G_i\alpha$ and $G_s\alpha$ coupling to GnRH receptor.

Previous studies performed to identify the G-proteins coupled to receptors have utilized photoreactive GTP analogs, [α^{32}P]GTP azidoanilide (Offermanns et al., 1991), or ADP-ribosylation (Eason et al., 1992) of membrane G-proteins. By necessity these studies were done in membrane preparations and not in intact cells. The protocols used to isolate membranes can disrupt membrane organization, and produce an environment for receptor-G-protein interactions that would not normally occur in an intact cell. Furthermore, the enzymatic steps used in membrane preparations may lead to partial receptor or G-protein degradation which may result in a loss of specificity between receptor-G-protein interactions. For example, in HEK 293 cells a severely truncated PTH receptor had differential signaling characteristics when compared with the full length receptor (Schneider et al., 1994), suggesting that cleavage of receptors can alter G-protein coupling specificity. In the present study, G-proteins coupled to the GnRH receptor were identified by GnRH receptor evoked palmitoylation of these proteins; this technique had minimal impact on the intracellular environment, as it did not rely on membrane preparations or transfected cell lines.

Palmitoylation of G-protein α-subunits, near the N-terminal Cys, through a thioester linkage is a dynamic process. G-protein activation results in palmitic acid turnover (Degtyarev et al., 1993; Wedegaertner et al., 1993). Except for G-proteins of the transducin family, all other known G-proteins have a N-terminus consensus sequence for palmitoylation (Degtyarev et al., 1993). Receptor-evoked palmitoylation of G-proteins is a well-characterized phenomenon that occurs in a time- and ligand dose-dependent manner (Wedegaertner et al., 1993; Mumby et al., 1994; Iri et al., 1996). G-protein palmitoylation may have physiological significance. For example, palmitoylation of G-proteins appears to be required for membrane association (Wedegaertner et al., 1993), and furthermore has been shown to correlate with $G_{q/11}\alpha$ relocalization in response to GnRH in rat gonadotropes (Cornea et al., 1998). These studies suggest that palmitoylation is integral to G-protein activation, and can serve as an excellent marker for receptor mediated G-protein activation.

This study was undertaken to identify the G-proteins that are activated by GnRH receptor in the rat gonadotrope. As mentioned earlier, different cell types expressing the GnRH receptor has been shown to couple different kinds of G-proteins. However, this is the first study of this kind to show multiple G-protein coupling to the GnRH receptor in the same cell type. In this study we used a method, selected because it minimally disrupted the intracellular environment of the gonadotrope, to label G-proteins that are activated by the GnRH receptor. Taken together with previous work (Stanislaus et al., 1997), the present data show that the GnRH receptor couples to $G_{q/11}\alpha$, $G_i\alpha$ and $G_s\alpha$ proteins in the rat gonadotrope.

Materials and Methods

Horse and fetal calf sera (Hyclone Laboratories, Logan, UT), bovine serum albumin (BSA fraction V; Irvine Scientific, Santa Ana, CA), HEPES buffer (United States Biochemical, Cleveland, OH), collagenase (Worthington Biochemical, Freehold, NJ), formic acid (Mallinkrodt, McGraw Park, IL), ammonium formate, sodium deoxycholate and EDTA (Fisher Scientific, Fairlawn, NJ), Nonidet P-40 (Particle Data Laboratory, Elm Hurst, IL), gentamicin sulfate (Gemini, Bio-products, Calabasas, CA), hyaluronidase, and phorbol myristic acid (PMA, Sigma, St. Louis, MO), Antide (Ares-Serono, Geneva, Switzerland) were obtained as indicated. Other reagents were obtained at the highest grade available from commercial vendors.

Preparation of pituitary cell cultures

Pituitary cell cultures were prepared as previously described (Conn et al., 1979). Briefly, pituitary glands were removed from 28 day old female Sprague-Dawley rats (B&K Universal Inc, Kent, WA) and placed in medium 199 (Irvine Scientific, Santa Ana, CA) containing 0.3% (w/v) BSA and 10 mM HEPES, pH 7.4 (M199/BSA). The pituitaries were minced and incubated in sterile M199/BSA containing 0.125% (w/v) collagenase and 0.1% (w/v) hyaluronidase in a 37° C shaking water bath for 15 min. The dissociated cells were filtered through organza cloth, and the remaining tissue was incubated a second time with a similar enzyme solution for an additional 15 min. The combined cells were collected by centrifugation (10 min at 200 x g) and resuspended in M199/BSA containing 10% (v/v) horse serum, 2.5% (v/v) fetal calf serum, and 20 μg/ml gentamicin sulfate then filtered through organza cloth. For palmitoylation studies, cell

suspensions were plated at a cell density of 2.5 x 10^6 cells/well in six-well culture plates (Costar, Cambridge, MA). Cells were maintained for approximately 48 h at 37° C in a water-saturated atmosphere prior to beginning experiments.

Metabolic labeling of G-proteins with [9,10-^{3}H]-palmitic acid and immunoprecipitation

Pituitary cell cultures were washed twice with M199/BSA 2 h before labeling with [9,10-^{3}H]-palmitic acid (specific activity 30-60 Ci/mmol, 0.5 mCi/ml of M199/BSA; DuPont NEN, Boston, MA) containing the indicated compounds for the indicated times. Labeling was stopped by aspirating the labeling medium and washing once with cold Dulbecco's-PBS; the cells were lysed for 1 h on ice with 750 µl of cold RIPA buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 1 mM EDTA and 2.5 mM $MgCl_2$). The insoluble material and nuclei were removed by centrifugation at 12,000 x g (Eppendorf microcentrifuge) for 3 min. Non-specific binding was removed by rocking the cell extract in 1.5 ml Eppendorf tubes containing 75 µl of Protein A-Sepharose 6MB (Pharmacia Biotech), previously coupled to IgG from normal rabbit serum, for 30 min at 4° C. After this step, the cell extract was transferred to 1.5 ml Eppendorf tubes containing 75 µl of Protein A-Sepharose coupled to rabbit polyclonal antibody specific for $G_i\alpha$ and $G_s\alpha$ (prepared in our laboratory, see below), and immunoprecipitated overnight at 4° C. The cell extract was centrifuged briefly to pellet the beads, and the supernate was discarded. The pelleted beads were washed three times with 750 µl of cold RIPA buffer. Finally the beads were resuspended in SDS-polyacrylamide gel electrophoresis sample buffer (reducing agents were omitted

to prevent the hydrolysis of thioester linked fatty acids) and heated at 100° C for 2 min. The immunoprecipitates were resolved by 10% SDS-PAGE, fixed and prepared for fluorography with Fluoro-Hance (RPI, Mt. Prospect, IL). The gels were exposed to Kodak X-OMAT autoradiography film for approximately 30 days at -70° C. In parallel experiments, gels were treated with 1 M hydroxylamine (pH 7.0) after a 15 min fixative period, before fluorography and exposure to autoradiography film (Mumby and Buss, 1990). Treatment with hydroxylamine cleaves the thioester bonds of palmitic acids to G-proteins, but not the amide bonds of myristic acids, indicating palmitate labeling of G-proteins as opposed to myristate labeling (Mumby and Buss, 1990). Densitometric analyses was performed with NIH Image 1.47 software to obtain band density.

Cell culture and transfection

GGH_31' cells were derived from GH_3 cells stably transfected with the rat GnRH receptor cDNA as previously reported (Kaiser et al., 1994). The GGH_31' cells were maintained in an atmosphere of 5% CO_2 at 37°C in DMEM (GIBCO, Grand Island, NY) containing 10% fetal calf serum and 20 μg/ml gentamicin. Cells were grown to confluence in 162-cm^2 or 75-cm^2 T-flasks (Costar), then scraped and plated at a density of 50,000 - 100,000 cells/well in a 24-well culture plate for 24 h at 37°C in 5% CO_2. Cells were washed once in OPTI-MEM (GIBCO), before transfecting with the cDNA for specific G-proteins or control plasmid. Approximately 24 h after seeding, cells were washed with OPTI-MEM (pH 7.4), and 0.4 μg of DNA mixed with 2 μl of lipofectamine (GIBCO) in 0.25 ml of OPTI-MEM was added to wells in triplicate. After 5 h at 37° C, 0.25 ml of DMEM containing 20% fetal calf serum was added to each well. After 24 h

from start of transfection, transfection medium was replaced for another 24 h with DMEM/ 10% fetal calf serum/ and 20 μg/ml gentamicin. Approximately 48 h after start of transfection, plates were washed twice with DMEM/ 0.1% BSA/ 20 μg/ml gentamicin before examining Buserelin-stimulated (GnRH agonist) inositol phosphate, cAMP or prolactin production.

Measurement of inositol phosphate accumulation

After plates were washed twice with DMEM/ 0.1% BSA/ 20 μg/ml gentamicin to remove serum and unattached cells, cellular inositol lipids were labeled in DMEM (inositol free) supplemented with [^{3}H]-myo-inositol (specific activity 30-60 Ci/mmol, 4 μCi/ml; Dupont NEN) for 18 h. After inositol labeling, cells were washed twice with DMEM (inositol free) containing 5 mM LiCl (DMEM/LiCl), and stimulated for 2 h with the indicated Buserelin concentrations prepared in DMEM/LiCl. A 2 h Buserelin treatment produced a maximal response in GGH_3 cells (Janovick and Conn, 1994). The treatment solutions were removed, and 1 ml of 0.1 M formic acid was added to each well. The cells were freeze-thawed once to disrupt the cell membranes, and the total [^{3}H]-inositol phosphates were determined by Dowex anion exchange chromatography and liquid scintillation spectroscopy (Huckle and Conn, 1987).

Measurement of prolactin and cAMP accumulation

After plates were washed twice with DMEM/ 0.1% BSA/ 20 μg/ml gentamicin to remove serum and unattached cells, prolactin and cAMP accumulation was measured following a 24 h incubation in a 1 ml volume of DMEM/ 0.1% BSA/ 20

μg/ml gentamicin containing the indicated concentrations of Buserelin. In GGH_3 cells prolactin and cAMP release in response to Buserelin is clearly detectable at 24 h from the start of treatment (Kuphal et al., 1994). At this time period the prolactin and cAMP response is not desensitized (Kuphal et al., 1994). To prevent degradation of cAMP by phosphodiesterases, 0.2 mM methyl isobutyl xanthine (MIX) was included in the medium. Aliquants from the fluid bathing the cells were used for prolactin and cAMP assays. After stimulating GGH_3 cells, the samples were collected in tubes containing a final theophylline concentration of 1 mM. The samples were heated (95 C) for 5 min to destroy phosphodiesterase activity. There was no detectable effect on the prolactin RIA when the samples were heated in this fashion. Prolactin release was measured by RIA using materials obtained from the Hormone Distribution Program of the National Pituitary Agency, NIDDK. Prolactin was radioiodinated by standard procedures (Hunter and Greenwood, 1962). RIA of cyclic AMP was performed by a modification of the method of Steiner et al. (1972), with the addition of the acetylation step described by Harper and Brooker (1975). Cyclic AMP antiserum C-1B (prepared in our laboratory, Andrews et al., 1986) was used at a titer of 1:5,100. This antiserum showed less than 0.1% cross reactivity with cyclic GMP, 2':3'-cyclic AMP, 5'-cyclic AMP, 3'-cyclic AMP, ADP, GDP, ATP, CTP, MIX, or theophylline.

Production of polyclonal $G_i\alpha$ and $G_s\alpha$ antisera

Antisera was raised in rabbits using the C-terminal amino acid sequence for the alpha subunit of G_{i2} and G_{i3} ("KENLKDCGLF") and the C-terminal amino acid sequence for the alpha subunit of G_s ("RMHLRQYELL"), and coupled to keyhole limpet

hemocyanin. The antisera was characterized by probing the immunoblots of cell extracts with previously characterized antisera (kindly provided to us by Dr. Allen Spiegel; NIH, Bethesda, MD), along with our own, and looking for comigrating stained bands. The $G_i\alpha$ antisera recognized one major band at an apparent molecular weight of 40 kDa. In immunoblots probed with the $G_s\alpha$ anitsera two major bands at 45 and 52 kDa, respectively, were detected. These bands corresponded to the short and the long form of $G_s\alpha$ (Sternweis et al., 1981).

Expression vectors

Complimentary DNAs corresponding to G-protein α-subunits $G_q\alpha$, $G_{11}\alpha$, $G_{14}\alpha$, $G_{15}\alpha$, and $G_i\alpha$ were carried by the cytomegalovirus vector pCIS (Offermanns et al., 1996). The $G_s\alpha$ was in pCMV. A β-galactosidase construct inserted into pCIS was used as a transfection control (lac z;).

Statistical analyses

The results are presented as the mean ± standard error (SEM) of the indicated number of samples. Data was analyzed by one-way ANOVA, followed by students t-test with Bonferoni correction for multiple comparisons.

Results

Rat pituitary cell cultures were treated with 10^{-6} g/ml GnRH or cell culture medium alone in the presence of [^{3}H]-palmitate for 0, 20, 40, 60, 90 or 120 min. The time course of GnRH-stimulated palmitoylation of $G_i\alpha$ and $G_s\alpha$ in pituitary cell cultures is shown in figures 1 and 2, respectively. Immunoprecipitation of $G_i\alpha$ and $G_s\alpha$ with antisera directed against the individual G-proteins showed an increase in [^{3}H]-palmitate incorporation with GnRH treatment (figures 1A and 2A). The earliest detectable incorporation of the [^{3}H]-palmitic acid was measurable 20 min after the addition of GnRH. Shorter time intervals (5 and 10 min) did not show any difference from medium treated values (data not shown). GnRH stimulated incorporation of the label is detectable up to 120 min, the longest time examined. $G_i\alpha$ and $G_s\alpha$ proteins in figures 1A and 2A, respectively, were detected in immunoblots with our antisera, and these bands comigrated with a standard antisera obtained from Dr. A. Spiegel. Our own $G_i\alpha$ antisera, and standard antisera for $G_i\alpha$ each recognized a comigrating band at an apparent molecular weight of 40 kDa. Two major bands at 45 and 52 kDa, respectively, were detected in immunoblots probed with our own $G_s\alpha$ anitsera, and standard antisera for $G_s\alpha$. Figures 1B and 2B show the arbitrary optical density of the bands in the autoradiographs.

Palmitate incorporation into $G_i\alpha$ and $G_s\alpha$ was dependent on GnRH (figures 3 and 4, respectively). Pituitary cell cultures were treated with medium, 10^{-12}, 10^{-10}, 10^{-8} or 10^{-6} g/ml of GnRH in the presence of [^{3}H]-palmitate for 60 min, and [^{3}H]-palmitic acid incorporation was assayed by autoradiography as described in methods (figures 3A and 4A). A concentration of 10^{-9} g/ml GnRH produced a half maximal incorporation of

palmitate label to either $G_i\alpha$ or $G_s\alpha$. Figures 3B and 4B show the arbitrary optical density of the bands in the autoradiographs.

Rat pituitary cell cultures showed increased incorporation of the palmitate label above medium treated levels in $G_i\alpha$ and $G_s\alpha$ when stimulated with GnRH (10^{-6} g/ml, figure 5 and 6, respectively). Figures 5A and 6A show the autoradiographs of $G_i\alpha$ and $G_s\alpha$ proteins, respectively. Only $G_s\alpha$ showed increased incorporation of palmitate label when treated with cholera toxin (5 μg/ml, figure 6). Incubation with pertussis toxin (100 ng/ml), phorbol myristic acid (PMA, a protein kinase C activator; 100 ng/ml), or Antide (GnRH antagonist; 50 nM) did not result in an increased palmitoylation of $G_i\alpha$ or $G_s\alpha$ compared to medium treated levels (figures 5 and 6, respectively). Rat pituitary cell cultures were incubated in the presence of the above agents for 60 min, and $G_i\alpha$ and $G_s\alpha$ was immunoprecipitated as described in methods. Figures 5B and 6B show the arbitrary optical density of the bands in the autoradiographs.

Treatment with 1 M hydroxylamine solubilized all measurable radioactivity from gels indicating alkali sensitive characteristic of a thioester linked palmitate moiety (not shown).

We used lactotrope derived GGH_3 cells to examine whether the ability of the receptor to couple multiple G-proteins is gonadotrope specific. GGH_3 cells were transfected with specific cDNA coding for different G-proteins, and agonist stimulated second messenger production was assessed. Figure 7 shows Buserelin dose-dependent stimulation of inositol phosphate production in GGH_3 cells transfected with the cDNA of $G_{q/11}\alpha$ family ($G_q\alpha$, $G_{11}\alpha$, $G_{14}\alpha$, and $G_{15}\alpha$), $G_{i2}\alpha$ and $G_s\alpha$. Stimulation of cells transfected with the cDNA for $G_{q/11}\alpha$ family with the indicated doses of Buserelin

resulted in enhanced IP production compared to lac z transfected cells (control plasmid; Offermanns et al., 1996). However, in cells transfected with the $G_{i2}\alpha$ cDNA, Buserelin-stimulated IP production was decreased from control levels. As expected, cells transfected with cDNA for $G_s\alpha$ resulted in no significant change in Buserelin-stimulated IP production over lac z transfected cells (figure 7). GGH_3 cells were transiently transfected as described in Methods, and stimulated for 2 h with the indicated concentrations of Buserelin.

Figures 8 and 9 show the effect of transfection of indicated G-protein cDNA on Buserelin-stimulated prolactin and cAMP release, respectively. Transfections were performed as described in Methods and the cells were stimulated with the indicated concentrations of Buserelin for 24 h before assaying for the released prolactin and cAMP by RIA. Transient transfection of the cDNA for $G_{q/11}\alpha$ family, $G_{i2}\alpha$ and $G_s\alpha$ did not show a significant change in Buserelin-stimulated prolactin release in these cells (figure 8) compared to lac z transfected cells. Only $G_s\alpha$ cDNA transfected cells showed an increased level of cAMP production compared to lac z transfected cells when stimulated with the indicated doses of Buserelin (figure 9). $G_{i2}\alpha$ cDNA transfected cells showed a decrease in Buserelin-stimulated cAMP production compared to the control lac z transfected cells, however, transfection of cDNA from the $G_{q/11}\alpha$ family resulted in no significant change in the Buserelin stimulated cAMP production when compared to control cells (figure 9).

Discussion

This study provides evidence for GnRH receptor regulation of $G_i\alpha$ and $G_s\alpha$ in rat pituitary cell cultures. The data presented here demonstrate that $G_i\alpha$ and $G_s\alpha$ are palmitoylated in a time- and dose-dependent manner by GnRH receptor occupancy by the releasing hormone. Antide, a GnRH receptor antagonist which binds but does not activate the receptor, did not measurably stimulate palmitoylation above background levels. Increasing the GnRH concentration resulted in a dose-dependent increase in palmitoylation of $G_i\alpha$ and $G_s\alpha$. Cholera toxin, an activator of $G_s\alpha$, stimulated palmitoylation of $G_s\alpha$ in the absence of GnRH. These observations taken together with previous work (Stanislaus et al., 1997) suggest that the GnRH receptor couples to $G_i\alpha$, $G_s\alpha$ and $G_{q/11}\alpha$ in the rat gonadotrope.

We have used a measure of G-protein activation, i.e. palmitoylation, to identify regulation of $G_i\alpha$ and $G_s\alpha$ by the GnRH receptor in the rat pituitary. Palmitic acid turnover on G-proteins occurs when the protein is activated by a receptor or by a pharmacological agent. Therefore, regulation of palmitic acid turnover on $G_i\alpha$ and $G_s\alpha$, when primary pituitary cell cultures are stimulated by GnRH, suggests that the GnRH-receptor couples to these proteins. The observation that CTX-evoked palmitoylation was seen in $G_s\alpha$ and not in $G_i\alpha$, suggests that palmitoylation of G-proteins is activation dependent and not a non-specific event.

The ability of the GnRH receptor to couple multiple G-proteins is not specific to the gonadotrope. In this study we show that overexpression of G-proteins in GGH_3 cells results in changes in the cognate signal transduction cascades. This suggests the ability of the GnRH receptor to couple to the respective G-proteins. Previous studies have

shown that in GGH_3 cells, GnRH receptor activation results in dose-dependent production of prolactin, cAMP and IP (Janovick and Conn, 1994;Kuphal et al., 1994). Transfection with $G_s\alpha$ cDNA resulted in an increased Buserelin (GnRH agonist) evoked cAMP production. An increase in the free $G_s\alpha$ proteins would enable more of these proteins to be activated by the GnRH receptor, thereby increasing the production of cAMP. Conversely, transfection of $G_i\alpha$ inhibited Buserelin-stimulated cAMP. This indicates that GnRH receptor occupancy results in the activation of $G_i\alpha$ to inhibit cAMP production by the adenylate cyclase enzyme. Transfection of $G_i\alpha$ cDNA resulted in a decrease in GnRH-evoked IP turnover. G-proteins of the $G_i\alpha$ family works through its associated $\beta\gamma$ subunits to activate PLCβ, and to produce inositol phosphates and diacylglycerols (Katz et al., 1992). Transfecting with $G_i\alpha$ cDNA presumably increased levels of this protein, which in turn would compete with PLCβ for the $\beta\gamma$ subunits resulting in a decrease in IP production. These observations suggest that GnRH receptor is able to couple $G_s\alpha$ to produce an increase in GnRH evoked cAMP production in GGH_3 cells, but also couple $G_i\alpha$ to inhibit adenylate cyclase to produce a decrease in cAMP levels in GGH_3 cells.

In this study we were able to lend support to the view that the GnRH receptor couples to $G_{q/11}\alpha$ by showing that the GnRH receptor was able to activate PLCβ through all the known members of the $G_{q/11}\alpha$ family. The α-subunit of these G-proteins activates PLCβ directly, when the trimeric G-protein is activated by a receptor (Blank et al., 1991). Buserelin stimulation of cells transfected with the cDNA of $G_q\alpha$, $G_{11}\alpha$, $G_{14}\alpha$ and $G_{15}\alpha$ resulted in an increased IP production over control cells.

In GGH_3 cells GnRH-evoked prolactin release is mediated through a cAMP dependent signal transduction pathway (Kuphal et al., 1994). However we did not observe an increase in Buserelin-evoked prolactin release in cells transfected with $G_s\alpha$ cDNA, even though Buserelin-evoked cAMP levels were higher than in control cells. Moreover, in $G_i\alpha$ transfected cells, although the Buserelin-stimulated cAMP levels were below control levels, there was no significant difference in the prolactin level. The lack of change seen with Buserelin-stimulated prolactin release may be due to the fact that the amount of cAMP needed to stimulate prolactin release is low. Therefore an increase or decrease in cAMP seen in transiently transfected cells may still be above the levels of the second messenger needed to stimulate prolactin release.

Overexpression of G-proteins in the transfected cells were not clearly detectable in immunoblots. This is due to the fact that only a maximum 30% transfection rate is unable to produce G-proteins in excess of the non-transfected control cells. However the expression of these plasmids were verified in a previous publication (Offermanns et al., 1996).

Receptor stimulated palmitoylation of G-proteins is a convenient in vivo method to identify G-protein activation. However, due to low specific activity of ^3H, the exposure time for autoradiographs are prolonged. The long exposure times result in high, and varied background exposure levels. Due to these reasons, and experimental artifacts arising from cell culture preparations, differences in labeling medium and in other experimental procedures, combing data from different experiments were not appropriate.

The studies in the GGH_3 cells were undertaken to verify the observations in the gonadotrope, and to examine whether the ability of the GnRH receptor to couple multiple

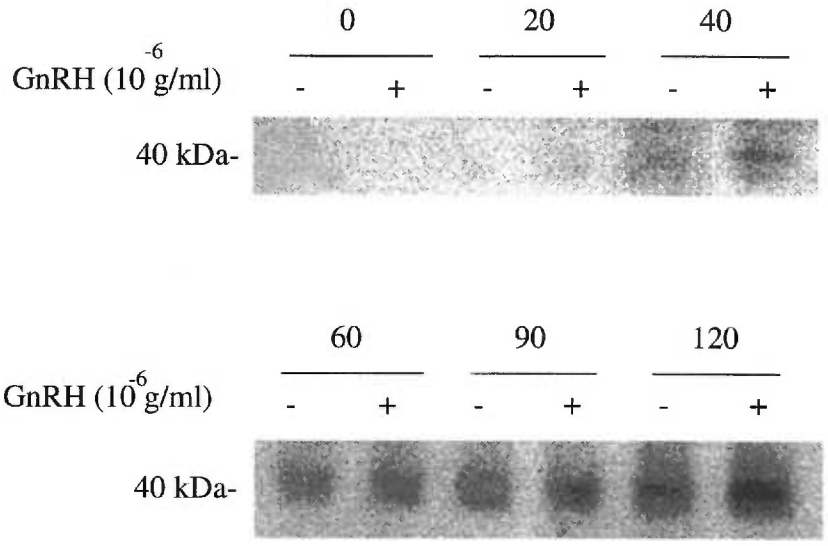
G-proteins is restricted to the gonadotrope. GGH_3 cells were an ideal model because the progenitor cell line, GH_3, does not express GnRH receptor endogenously, and these cells have been characterized extensively (Janovick and Conn, 1994; Kuphal et al., 1994; Kaiser et al., 1994). This enabled us to examine whether the ability to couple multiple G-proteins is receptor specific or gonadotrope specific. The answer to this question would explain the different observations investigators have seen with different cell lines with respect to GnRH receptor/G-protein coupling. Our paradigm used one cell type to show GnRH receptor coupling to $G_i\alpha$, $G_s\alpha$, and $G_{q/11}\alpha$. Moreover we provide evidence to show that this ability is not gonadotrope specific, but more a receptor specific ability. The gonadotrope may control the availability of G-proteins to the receptor, but as far as the GnRH receptor is concerned it has the ability to couple to multiple G-proteins. The ability to couple multiple G-proteins enables the GnRH receptor to activate multiple signal transduction pathways. The activation of multiple signal transduction pathways with different end-points maybe useful for GnRH to stimulate multiple responses from the gondotrope, such as LH and FSH release.

The ability of 7-TMS receptors to couple multiple G-proteins is documented for other members of this family. The human thyrotropin receptor is known to couple to all four classes of G-proteins: $G_i\alpha$, $G_s\alpha$ $G_{q/11}\alpha$ and $G_{12}\alpha$ (Laugwitz et al., 1996), and the α_{2A}-adrenergic receptor couples to $G_i\alpha$ and to $G_s\alpha$ (Eason et al., 1992). This is the first study of this nature to identify the G-proteins that are activated by the GnRH receptor in its native environment, and to show multiple G-protein coupling to the GnRH receptor in a single cell type.

The mechanism by which multiple G-proteins interact with the GnRH receptor is unknown. However the second and the third intracellular loops appear to be involved in signal transduction (Stanislaus et al., 1997; Arora et al., 1995), suggesting that multiple sites on the receptor may interact with G-proteins. Another possibility is that receptors in different cells may interact with different G-proteins, but cell by cell heterogeneity, in the same cell population, with respect to GnRH receptor G-protein coupling has not been identified. These studies do not indicate whether the GnRH receptor is able to couple to multiple G-proteins simultaneously or couple each protein one at a time. The propensity of the GnRH receptor to couple multiple G-proteins may explain how the activation of the GnRH receptor by the releasing hormone can regulate multiple cellular events in a coordinate fashion.

Figure 5-1. The time course of GnRH stimulated palmitoylation of $G_i\alpha$ in pituitary cell cultures. The rat pituitary cell cultures were treated with either GnRH (10^{-6} g/ml) or medium alone for the indicated times in the presence of [^{3}H]-palmitic acid. The labeled G-proteins were immunoprecipitated and visualized by autoradiography (figure 1A) as described in Methods. Figure 1B show the band intensity in arbitrary optical density units. The data are from one representative experiment. Three or more experiments were performed with similar results.

A


0
20
40
GnRH (10 -6 g/ml)
- + - + - +
40 kDa-
60
90
120
GnRH (10-6g/ml)
- + - + - +
40 kDa-

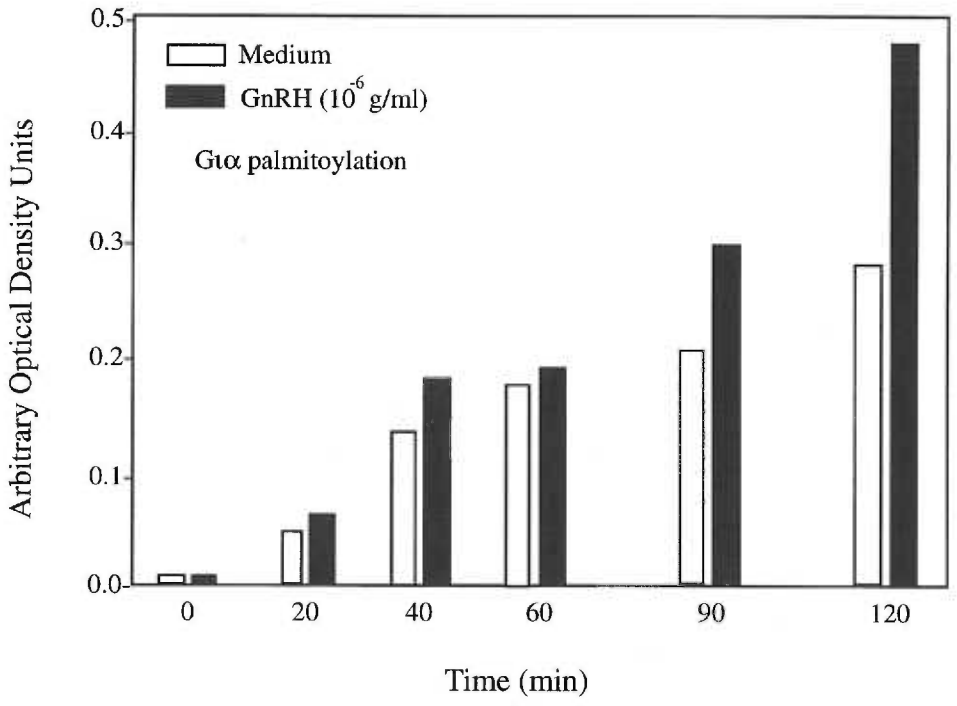
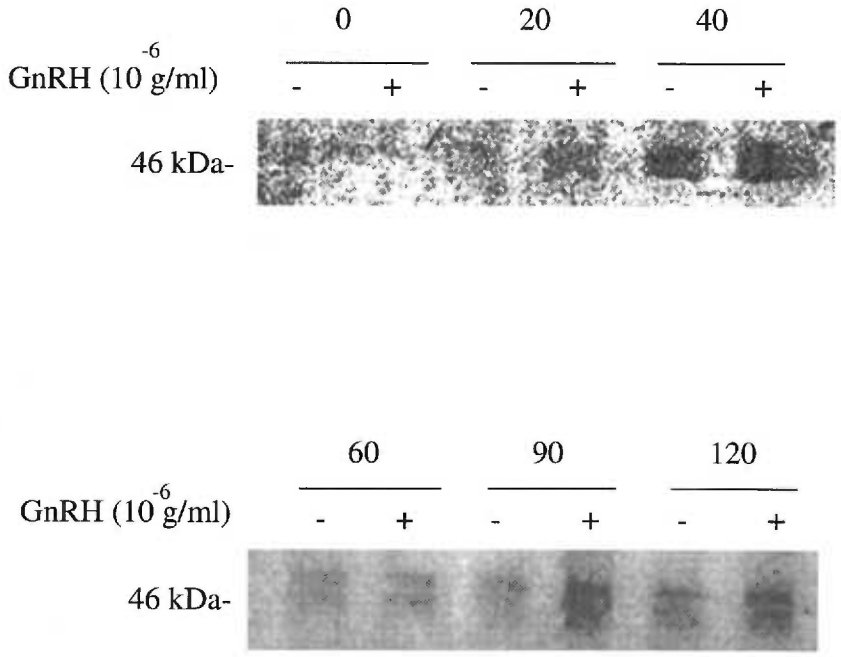

Medium
GnRH (10-6 g/ml)
Gtα palmitoylation
Arbitrary Optical Density Units
0.5
0.4
0.3
0.2
0.1
0.0
0
20
40
60
90
120
Time (min)

Figure 5-2. The time course of GnRH stimulated palmitoylation of $G_s\alpha$ in pituitary cell cultures. The rat pituitary cell cultures were treated with either GnRH (10^{-6} g/ml) or medium alone for the indicated times in the presence of [^{3}H]-palmitic acid. The labeled G-proteins were immunoprecipitated and visualized by autoradiography (figure 2A) as described in Methods. Figure 2B show the band intensity in arbitrary optical density units. The data are from one representative experiment. Three or more experiments were performed with similar results.


0
20
40
GnRH (10 -6 g/ml)
- + - + - +
46 kDa-
60
90
120
GnRH (10 -6 g/ml)
- + - + - +
46 kDa-

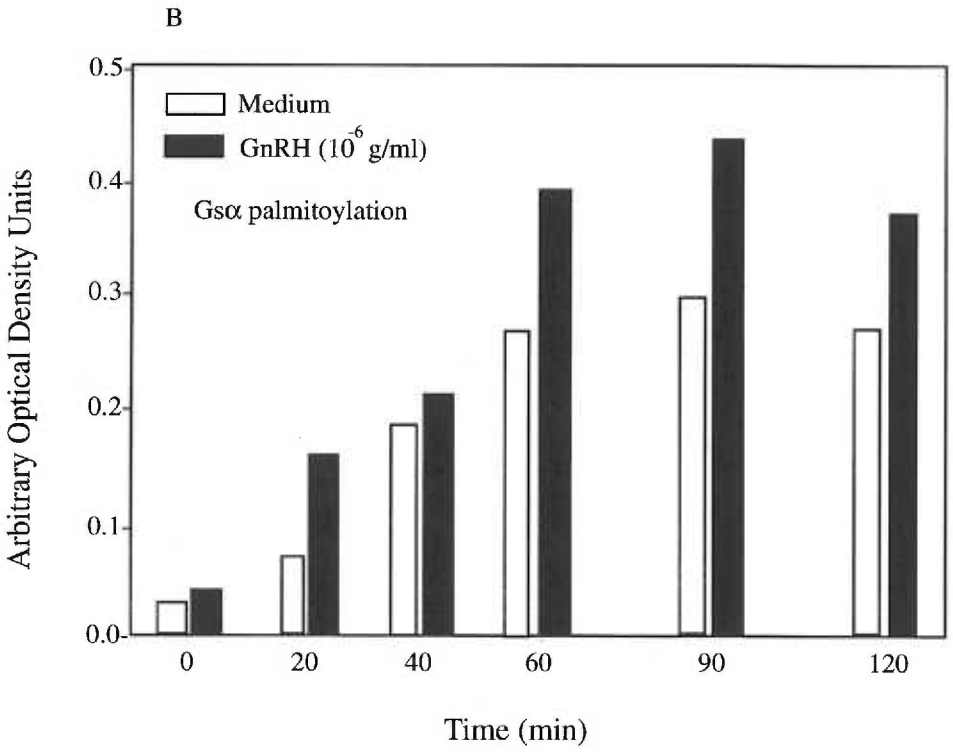
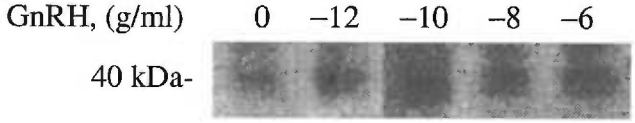

B
Medium
GnRH (10-6 g/ml)
Gsα palmitoylation
Arbitrary Optical Density Units
0.5
0.4
0.3
0.2
0.1
0.0
0
20
40
60
90
120
Time (min)

Figure 5-3. The dose-dependent palmitoylation of $G_i\alpha$ in response to the indicated doses of GnRH in pituitary cell cultures. The rat pituitary cell cultures were treated with the indicated doses of GnRH for 1 h in the presence of [^{3}H]-palmitic acid. The labeled G-proteins were immunoprecipitated and visualized by autoradiography (figure 3A) as described in Methods. Figure 3B show the band intensity in arbitrary optical density units. The data are from one representative experiment. Three or more experiments were performed with similar results.

A Giα palmitoylation



B

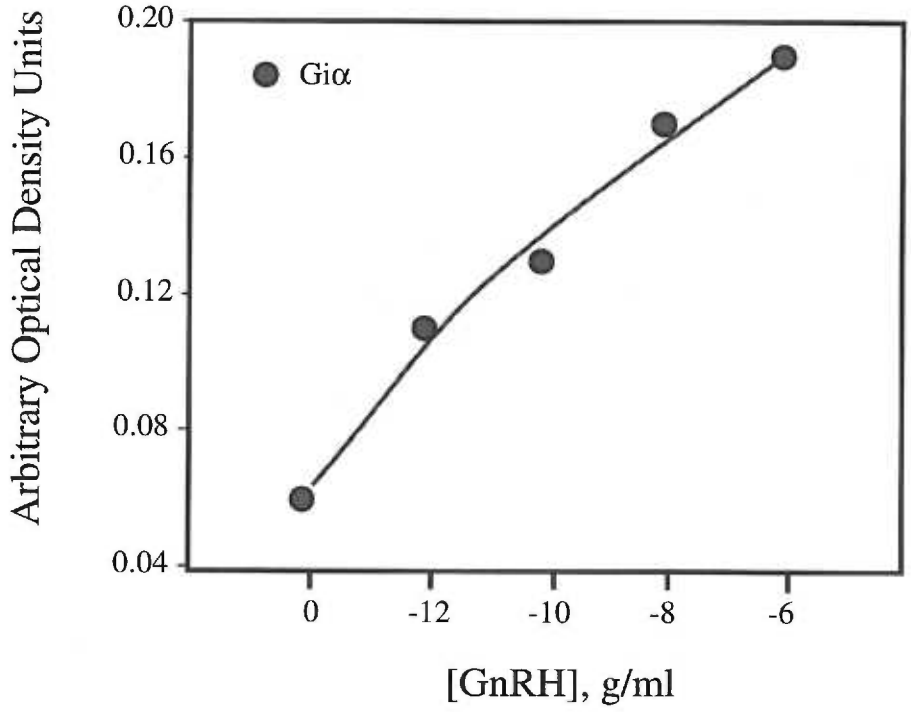
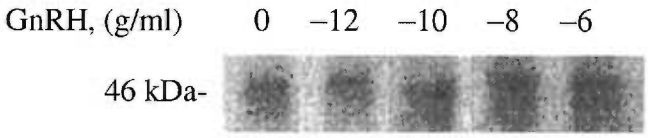


Figure 5-4. The dose-dependent palmitoylation of $G_s\alpha$ in response to the indicated doses of GnRH in pituitary cell cultures. The rat pituitary cell cultures were treated with the indicated doses of GnRH for 1 h in the presence of [^{3}H]-palmitic acid. The labeled G-proteins were immunoprecipitated and visualized by autoradiography (figure 4A) as described in Methods. Figure 4B show the band intensity in arbitrary optical density units. The data are from one representative experiment. Three or more experiments were performed with similar results.

A Gsα palmitoylation



B

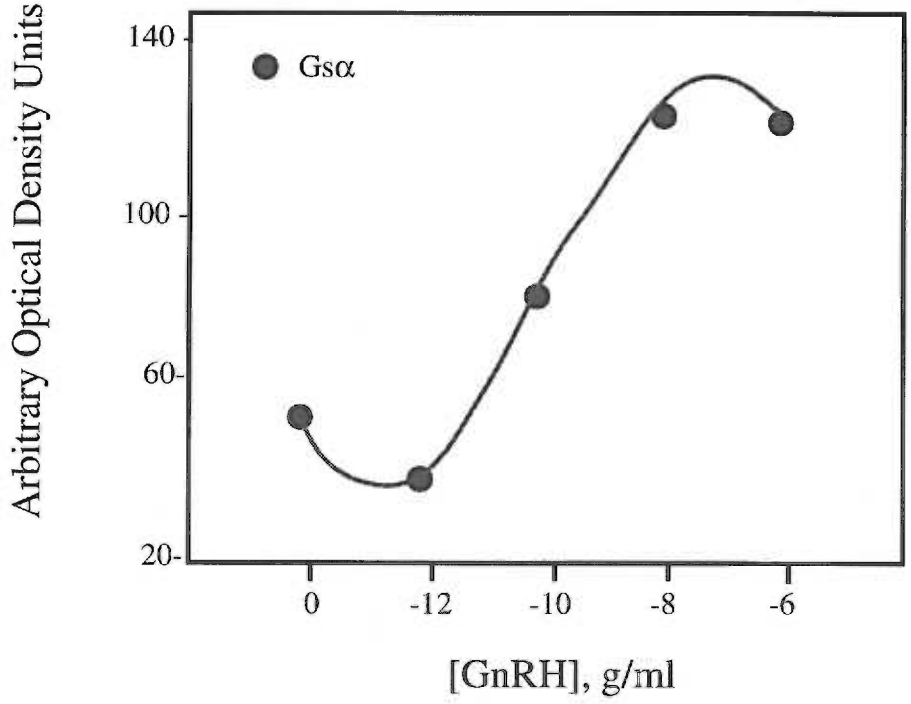
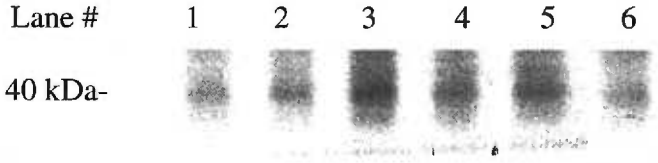


Figure 5-5. Palmitoylation of $G_i\alpha$ in response to the indicated pharmacological agents in pituitary cell cultures. The rat pituitary cell cultures were treated with the indicated agents for 1 h in the presence of [^{3}H]-palmitic acid. The labeled G-proteins were immunoprecipitated and visualized by autoradiography (figure 5A) as described in Methods. Data show the band intensity in arbitrary optical density units (figure 5B). The lane numbers correspond to the numbers on figure 5B. The data are from one representative experiment. Three experiments were performed with similar results.

A Giα palmitoylation



B

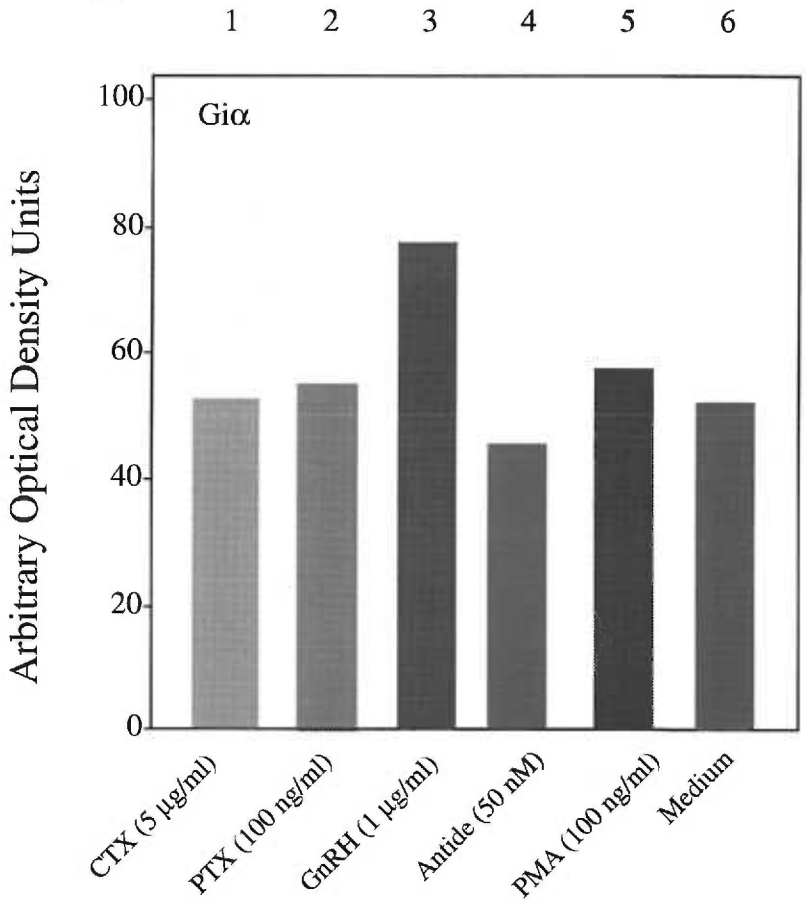
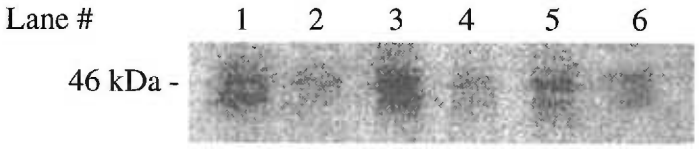


Figure 5-6. Palmitoylation of $G_s\alpha$ in response to the indicated pharmacological agents in pituitary cell cultures. The rat pituitary cell cultures were treated with the indicated agents for 1 h in the presence of [^{3}H]-palmitic acid. The labeled G-proteins were immunoprecipitated and visualized by autoradiography (figure 6A) as described in Methods. Data show the band density in arbitrary optical density units (figure 6B). The lane numbers correspond to the numbers on figure 6B. The data are from one representative experiment. Three experiments were performed with similar results.

A Gsα palmitoylation



B

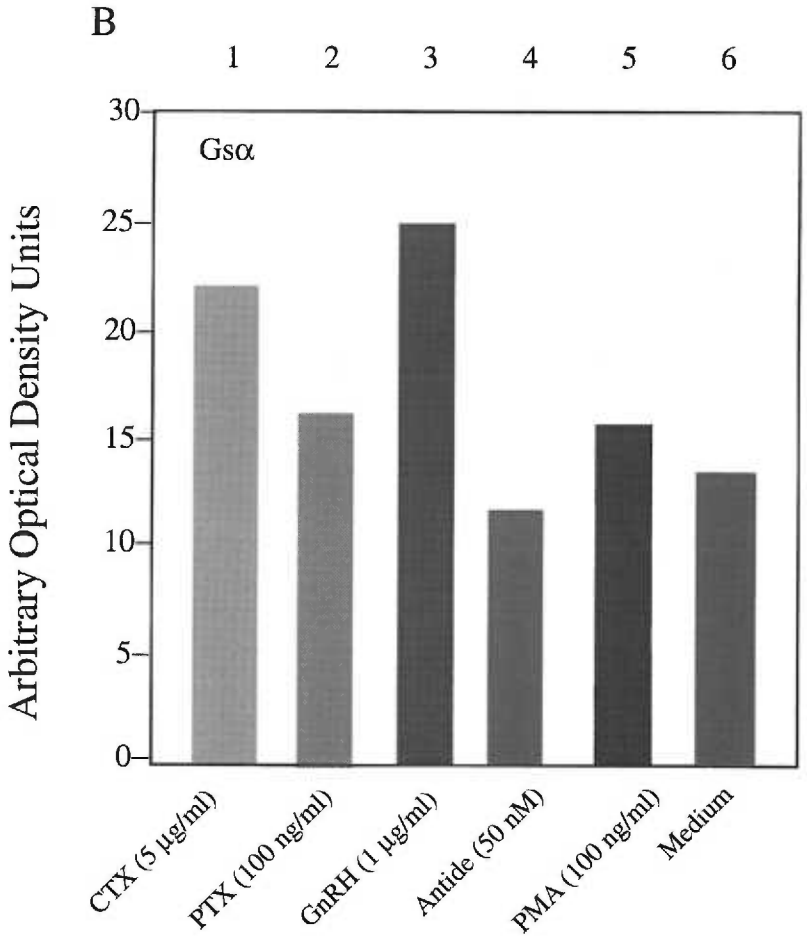


Figure 5-7. Dose-dependent stimulation of inositol phosphate production in response to Buserelin in GGH_3 cells transiently transfected with the cDNA for $G_{q/11}\alpha$ family, $G_{i2}\alpha$ and $G_s\alpha$. Cells were transfected as described, and stimulated with the indicated doses of Buserelin for 2 h. Inositol production was assessed as described in Methods. Data represent the average of triplicate wells, and the error bars represent the SEM. The experiments were performed three or more times with similar results. *, $P<0.05$ compared to the corresponding lac z transfected values.

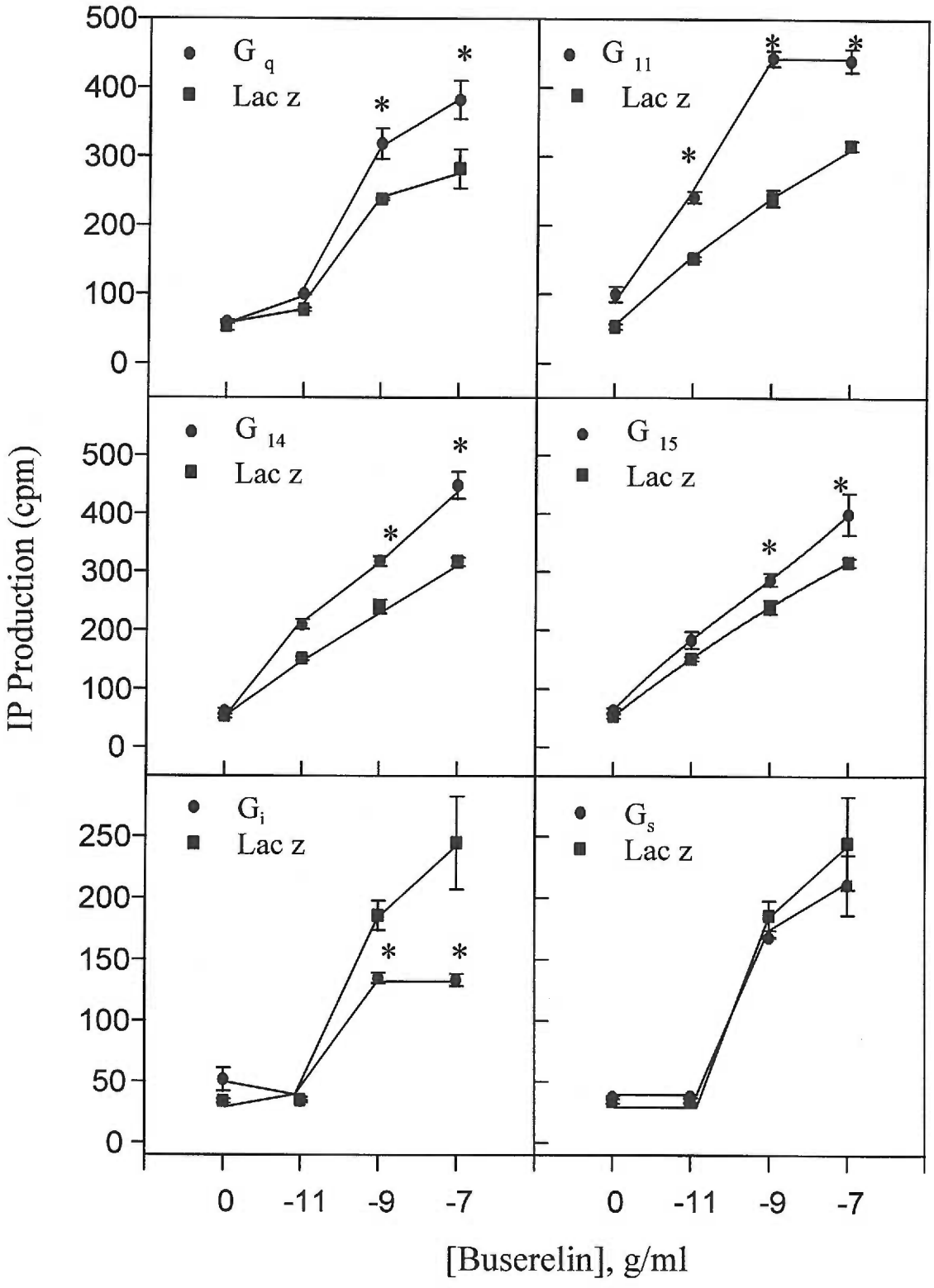

IP Production (cpm)
G q
Lac z
G 11
Lac z
G 14
Lac z
G 15
Lac z
Gi
Lac z
Gs
Lac z
500
400
300
200
100
0
250
150
50
0
0
-11
-9
-7
[Buserelin], g/ml

Figure 5-8. Dose-dependent stimulation of prolactin release in response to Buserelin in GGH_3 cells transiently transfected with the cDNA for $G_{q/11}\alpha$ family, $G_{i2}\alpha$ and $G_s\alpha$. Cells were transfected as described in Methods, and stimulated with the indicated doses of Buserelin for 24 h. Prolactin release was assessed by RIA as described in Methods. Data represent the average of triplicate wells, and the error bars represent the SEM. The experiments were performed at least three or more times with similar results. The experiments were performed three or more times with similar results.

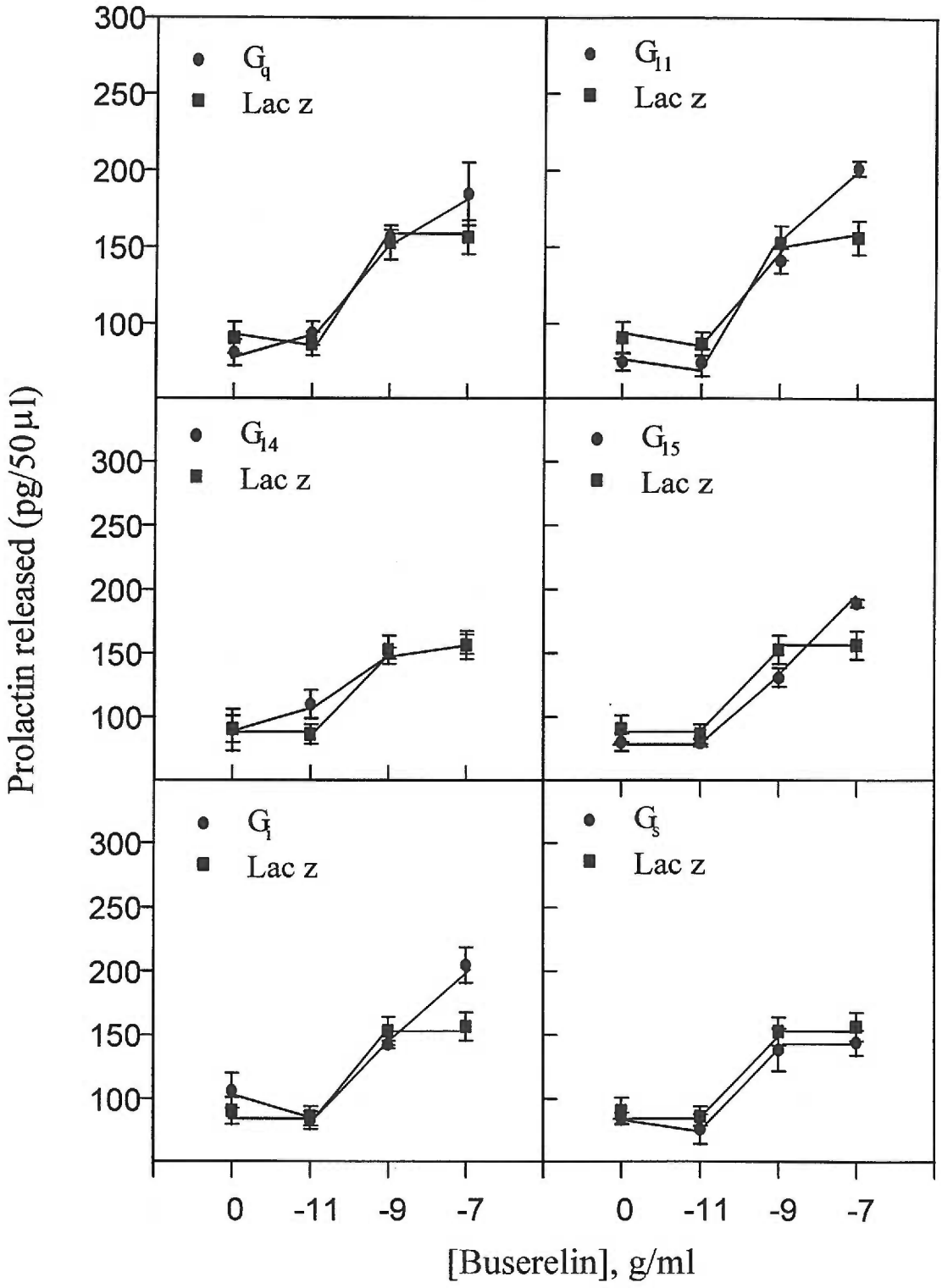
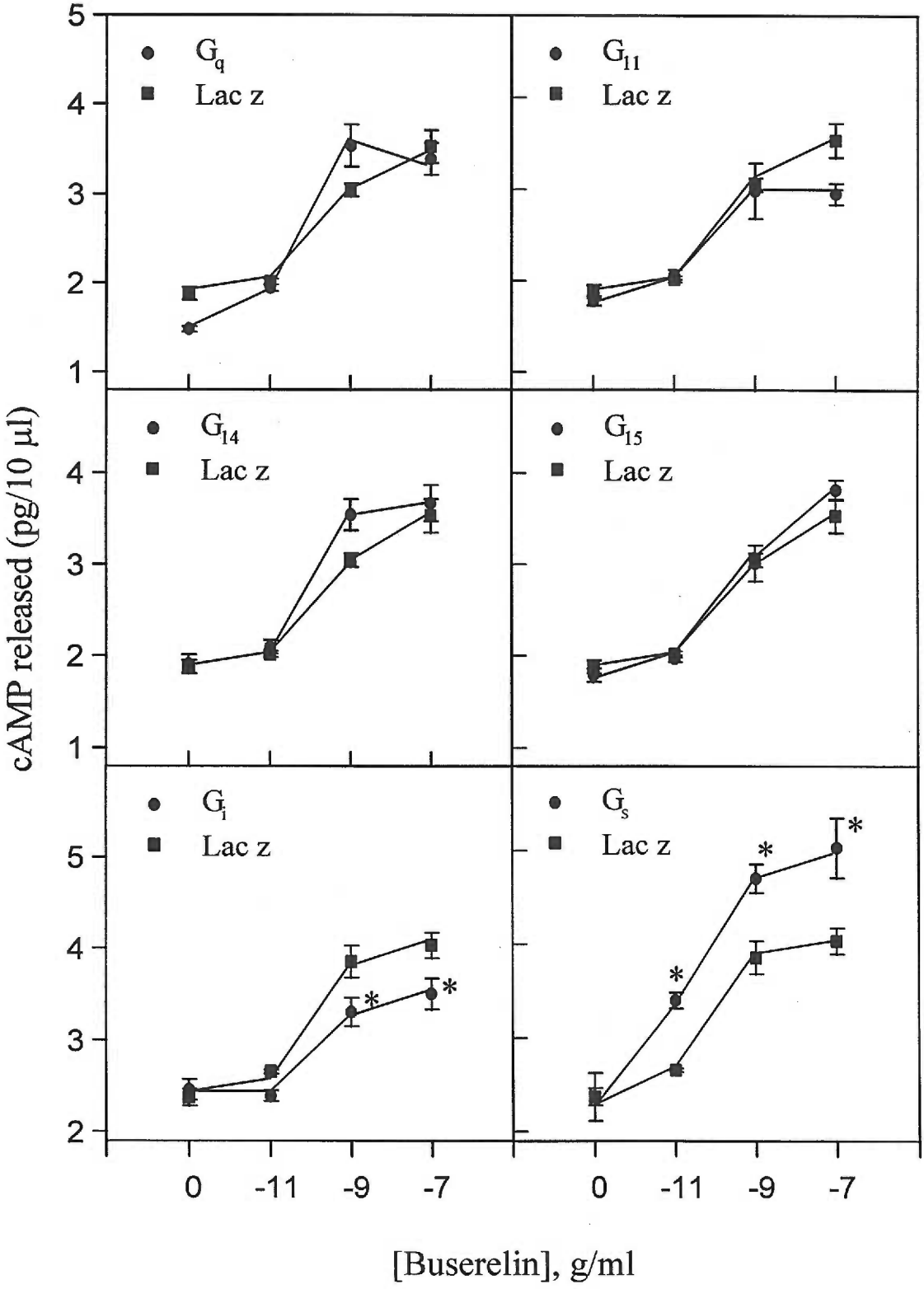

Prolactin released (pg/50 µl)
300
250
200
150
100
G_q
Lac z
G_{11}
Lac z
G_{14}
Lac z
G_{15}
Lac z
G_i
Lac z
G_s
Lac z
0
-11
-9
-7
[Buserelin], g/ml

Figure 5-9. Dose-dependent stimulation of cAMP release in response to Buserelin in GGH_3 cells transiently transfected with the cDNA for $G_{q/11}\alpha$ family, $G_{i2}\alpha$ and $G_s\alpha$. Cells were transfected as described, and stimulated with the indicated doses of Buserelin for 24 h. Cyclic AMP release was assessed by RIA as described in Methods. Data represent the average of triplicate wells, and the error bars represent the SEM. The experiments were performed three or more times with similar results. *, $P<0.05$ compared to the corresponding lac z transfected values.


cAMP released (pg/10 µl)
G_q
Lac z
G_{11}
Lac z
G_{14}
Lac z
G_{15}
Lac z
G_i
Lac z
G_s
Lac z
*
0
-11
-9
-7
[Buserelin], g/ml

CHAPTER SIX

GONADOTROPIN AND GONADAL STEROID RELEASE IN RESPONSE TO A GnRH AGONIST IN $G_q\alpha$ AND $G_{11}\alpha$ KNOCKOUT MICE

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Abstract

In this study we used mice lacking the $G_{11}\alpha$ (G_{11} KO) or $G_q\alpha$ gene (G_q KO) to examine LH release in response to a metabolically stable GnRH agonist (Buserelin). Mice homozygous for the absence of $G_{11}\alpha$ and $G_q\alpha$ appear to breed normally. Treatment of (5 wk old) female KO mice with the GnRH agonist Buserelin (2 μg/100 μl, sc) resulted in a rapid increase of serum LH levels (reaching 328 ± 58 pg/25 μl for G_{11} KO; 739 ± 95 pg/25 μl for G_q KO) at 75 min. Similar treatment of the control strain, 129SvEvTacfBr for G_{11} KO or the heterozygous mice for G_q KO, resulted in an increase in serum LH levels (428 ± 57 pg/25 μl for G_{11} KO; 884 ± 31 pg/25 μl for G_q KO) at 75 min. Both G_{11} KO and G_q KO male mice released LH in response to Buserelin (2μg/100 μl of vehicle; 363 ± 53 pg/25 μl and 749 ± 50 pg/25 μl 1 h after treatment, respectively). These values were not significantly different from the control strain. In a long-term experiment, Buserelin was administered every 12 h, and LH release was assayed 1 h later. In female G_{11} KO mice and control strain, serum LH levels reached ~500 pg/25 μl within the first hour, then subsided to a steady level (~100 pg/25 μl) for 109 h. In male G_{11} KO mice and in control strain, elevated LH release lasted for 13 h, however, LH levels in the G_{11} KO male mice did not reach control levels for ~49 h. In a similar experimental protocol, the G_q KO male mice released less LH (531 ± 95 pg/25 μl) after 13 h from the start of treatment than the heterozygous male mice (865 ± 57 pg/25 μl), but the female knockout mice released more LH (634 ± 56 pg/25 μl) after 1 h from the start of treatment than the heterozygous female mice (346 ± 63 pg/25 μl). However, after the initial LH flare, the LH levels in the heterozygous mice never reached the basal levels

achieved by the knockout mice. G_{11} KO mice were less sensitive to low doses (5 ng/per animal) of Buserelin than the respective control mice. Male G_{11} KO mice produced more testosterone than the control mice after 1 h of stimulation by 2 μg of Buserelin, whereas there was no significant difference in Buserelin stimulated testosterone levels between G_q KO and heterozygous control mice. There was no significant difference in Buserelin stimulated estradiol production in the female G_q KO mice compared to control groups of mice. However, female G_{11} KO mice produced less estradiol in response to Buserelin (2 μg) compared to control strain. Although there were differences in the dynamics of LH release and steroid production in response to Buserelin treatment compared to control groups of mice, the lack of complete abolition of these processes, such as stimulated LH release, and steroid production, suggests that these G-proteins are either not absolutely required or are able to functionally compensate for each other.

Introduction

Gonadotropin releasing hormone (GnRH) stimulates luteinizing hormone (LH) and follicle stimulating hormone (FSH) release from the anterior pituitary. Several pieces of evidence implicated G-protein involvement in GnRH receptor mediated actions. First, GTP and its analogs stimulate LH release and inositol phosphate production in permeabilized pituitary cell cultures (Andrews et al., 1986) and provoke the characteristic decrease in affinity of the GnRH receptor for its ligand (Perrin et al., 1989). Second, the GnRH receptor has seven transmembrane segments characteristic of G-protein coupled receptors (Tsutsumi et al., 1992).

Multiple G-proteins appear to be involved in mediating the response of the gonadotrope to GnRH (Hawes et al., 1992; Hawes et al., 1993). GnRH is capable of stimulating IP release through pertussis toxin sensitive (PTX) G-proteins, while a cholera toxin sensitive (CTX) G-protein appears to provoke gonadotrope sensitization to GnRH and other secretagogues. Immunedepletion studies from αT3-1 cell membranes suggest that the GnRH receptor is coupled to $G_{q/11}\alpha$ protein (Hsieh and Martin, 1992), and more recently we have shown that the GnRH receptor regulates $G_{q/11}\alpha$ in rat pituitary cell cultures and in GGH_3 cells which stably express the GnRH receptor (Stanislaus et al., 1997). Stimulation with GnRH also provokes palmitoylation and redistribution of $G_{q/11}\alpha$ in primary pituitary cultures (Stanislaus et al., 1997; Cornea et al., 1998).

The $G_q\alpha$ subfamily includes $G_q\alpha$, $G_{11}\alpha$, $G_{14}\alpha$, $G_{15}\alpha$ and $G_{16}\alpha$ (Wilkie et al., 1992). These G-proteins are capable of activating phospholipase C-β (PLCβ) and are unmodified by pertussis toxin (Strathmann and Simon, 1990; Smrcka et al., 1991). $G_q\alpha$ and $G_{11}\alpha$ have 88% amino acid sequence identity (Strathmann and Simon, 1990). In

SDS-polyacrylamide gel electrophoresis, $G_q\alpha$ and $G_{11}\alpha$ migrate at 41-42 kDa (Hepler et al., 1993). Due to the structural similarities between $G_q\alpha$ and $G_{11}\alpha$, it is technically difficult to discriminate between the two G-proteins by biochemical approaches.

Very few studies are available to attribute a specific activity to either $G_q\alpha$ or $G_{11}\alpha$, and the likelihood exists that these two proteins functionally compensate for each other. Studies done on Swiss 3T3 cells, for example, indicate that bombesin and vasopressin receptors concurrently activate both $G_q\alpha$ and $G_{11}\alpha$ (Offermanns et al., 1994), suggesting that with respect to PLCβ activation, these two proteins may function interchangeably. Whereas, when $\alpha_{1A/D}$, α_{1B} and α_{1C} adrenergic receptors are activated by agonists, $G_q\alpha$ and $G_{11}\alpha$ proteins, which are activated by these receptors, are degraded at a similar rate, an observation suggesting that the G-proteins may couple to these receptors without any preference (Rodbell et al., 1971). Furthermore, in Xenopus oocytes, $G_q\alpha$ and $G_{11}\alpha$ show similar modulation of response to thyrotropin releasing hormone (Quick et al., 1994), suggesting that these two proteins activate similar effectors, although an earlier report in the same system suggests that the response to thyrotropin-releasing hormone is differentially coupled to downstream effectors by $G_q\alpha$ and $G_{11}\alpha$ proteins (Lipinsky et al., 1992).

All of these studies were performed either in transfected cell lines or in Xenopus oocytes. Therefore, little is known about the G-protein coupling to the GnRH receptor in intact animals, particularly under endocrine systems with complex feedback mechanisms. To examine this question with respect to G-protein coupling to the GnRH receptor, we examined the role of $G_q\alpha$ and $G_{11}\alpha$ in the mouse gonadotrope by utilizing knockout

mice, lacking either the $G_q\alpha$ or the $G_{11}\alpha$ protein. This study provides evidence to suggest that $G_q\alpha$ and $G_{11}\alpha$ proteins can functionally compensate for each other in GnRH analog stimulated LH release and steroidogenesis.

Materials and Methods

Preparation of mice lacking the $G_{11}\alpha$ gene

$G_{11}\alpha$ knockout mice were prepared from 129/SvEv strain of mice as previously described (Wilkie, manuscript in preparation). For control experiments 129/SvEvTacfBR strain of mice (Taconic Farms, Germantown, NY) were used. Both control mice and knockout mice were 5 weeks old, and weighed ~16-18 g. The breeding pair of $G_{11}\alpha$ knockout mice were homozygous for the knockout gene. The weight to age curves, litter size and other external characteristics were indistinguishable between 129/SvEvTacfBR mice and knockout mice. Therefore we used aged matched 129/SvEvTacfBR strain as controls.

Preparation of mice lacking the $G_q\alpha$ gene

$G_q\alpha$ knockout mice were prepared as previously described (Offermanns et al., 1997). Male mice homozygous for the $G_q\alpha$ knockout were mated to female mice heterozygous for the $G_q\alpha$ knockout. Offspring which were heterozygous for the $G_q\alpha$ knockout were used as control mice. The experiments were performed at 5 weeks of age, and at this time, both homozygous and heterozygous mice for the knockout gene weighed ~ 15-18 g each. The mice were genotyped at 3 weeks to identify the homozygous knockout mice in the litter. To keep the variations between the animals to a minimum, heterozygous mice from the same litter was used as controls. Heterozygous mice, who have only one functioning $G_q\alpha$ gene, do not show any adverse effects. These mice have weight to age curves similar to the wild-type mice, and were indistinguishable from the

wild-type mice. Thus, heterozygous mice from the same litter were an appropriate control.

Genotyping of $G_q\alpha$ knockout mice

Approximately 0.5 cm size section of the mouse tails were digested overnight under constant agitation at 50 C in 300 µl of digestion buffer (100 mM EDTA, 0.5 % SDS, 0.5 mg/ml proteinase K and 50 mM Tris-HCl; pH 8). The digested samples were spun at 12,000 x g for 2 min, and 200 µl of the supernatant was mixed with 100 µl of 7.5 M ammonium acetate to obtain a final concentration of 2.5 M. Genomic DNA was precipitated by adding 600 µl of ice cold ethanol and collected at 12,000 x g for 10 min. The DNA pellet was washed once with 70% ethanol and dried. Finally the DNA pellet was dissolved in 300 µl of Tris-EDTA buffer (pH 8).

A 150 bp sequence of the disrupted $G_q\alpha$ gene containing the neomycin gene was amplified by PCR with flanking primers NEO4 (5' GATTCGCAGCGCATCGCCTTC TAT 3') and QNEO (5' TTCAAAGTATCACACTCACATCACAG 3'). A 150 bp sequence of the wild type $G_q\alpha$ gene was amplified with the flanking primers 5EXQ(5' GAACCGCATGGAGGAGAGCAAAGC 3') and 3EXQ (5' CTGGGAAGTAG TCGACTAGGTGGG 3'). The PCR protocol used is as follows: 5 min at 94 C, 35 cycles of 1 min at 94 C, 1 min at 63 C and 3 min at 72 C, and finally 10 min at 72 C.

Serum collection and LH RIA

Each mouse received 2 μg of Buserelin (GnRH agonist, Hoechst) in 100 μl of vehicle (PBS/0.3% BSA) or vehicle alone. This is a saturating dose of Buserelin with respect to LH release (Conn et al., 1981). For the dose-response studies, indicated doses of Buserelin in 100 μl of vehicle was given. Buserelin was injected subcutaneously (sc) into the skin behind the neck. Mice were anesthetized with methoxyflurane, and serum collected at the indicated times by intra-orbital puncture. In long time course experiments, serum was collected 1 h after injection of Buserelin. Serum was aliquoted and stored at -20 C before assay.

The RIA used a highly purified rat LH for iodination (NIDDK; Hunter and Greenwood, 1962) and a mouse reference preparation obtained as a kind gift from Dr. Al Parlow (Harbor-UCLA Hospital, Torrance, CA). LH antisera (C102) was prepared and characterized as previously described (Smith et al., 1982). Bound and free proteins were separated using the second antibody technique (Gupta and Morton, 1979). The minimum detectable dose for the RIA was 6 $\pm$ 1 pg (n=6) and the inter- and intra-assay variance was less than 10%. The rat and mouse LH standards were approximately identical; the rat ED_{20} never varied more than 9% from the mouse ED_{20}.

Steroid assays for testosterone and estradiol

Serum estradiol (Resko et al., 1975; Goodman, 1978) and testosterone (Resko et al., 1980) levels were measured by RIA in the ORPRC Radioimmunoassay Laboratory using previously described methods. Antisera for estradiol (Resko et al., 1975; Goodman, 1978) and testosterone (Resko et al., 1980) were obtained as previously

described. For the estradiol assay, the minimum detectable dose was ~0.5 pg. The intra- and inter-assay variance was ~7% and ~13%, respectively. For the testosterone assay, the minimum detectable dose was 5 pg. The intra- and inter-assay variance was ~5% and ~8%, respectively.

Statistical analyses

The results are presented as the mean $\pm$ standard error (SEM) of the indicated number of animals. Data was analyzed by one-way ANOVA, followed by students t-test with Bonferoni correction for multiple comparisons. To examine the overall LH release in mice over time, a two-way ANOVA for repeated measures was performed.

Results

Figures 1A and 1B show, respectively, serum LH levels in male and female $G_{11}\alpha$ knockout mice, after a sc injection of 2 μg of Buserelin or vehicle. Samples were collected at 0, 15, 30, 45 and 75 min after the injection of drug or the vehicle. In male $G_{11}\alpha$ knockout mice, serum LH levels increased from 7 ± 2 pg/ 25 μl (n=6) to 363 ± 53 pg/ 25 μl (n=6), when administered with 2 μg of Buserelin (figure 1A). In the control strain, the same treatment of Buserelin resulted in an increase of serum LH from 10 ± 1 pg/ 25 μl (n=5) to 458 ± 59 pg/ 25 μl (n=5). When treated with the vehicle (PBS/0.1 % BSA), the serum LH levels in male $G_{11}\alpha$ knockout mice and in the male control strain remained between 7-10 pg/ 25 μl. In female $G_{11}\alpha$ knockout mice, serum LH levels increased from 6 ± 1 pg/ 25 μl (n=7) to 328 ± 58 pg/ 25 μl (n=7), when administered with 2 μg of Buserelin (figure 1B). In the control strain, the same treatment of Buserelin resulted in an increase of serum LH from 10 ± 1 pg/ 25 μl (n=6) to 428 ± 57 pg/ 25 μl (n=6). LH released in response to Buserelin was not significantly different ($p<0.05$) in both male and female $G_{11}\alpha$ knockout mice compared to control mice. When treated with the vehicle (PBS/0.1 % BSA), the serum LH levels in female $G_{11}\alpha$ knockout mice and in female control strain remained between 6-10 pg/25 μl.

Figures 2A and 2B show serum LH levels in mice 1 h after a sc injection of 2 μg of Buserelin given every 12 h for 108 h. In male $G_{11}\alpha$ knockout mice, the serum LH levels reached a maximum of 753 ± 29 pg/ 25 μl (n=7) after 13 h from the start of treatment (figure 2A). In the male control strain, a similar treatment resulted in a maximum LH level of 507 ± 27 pg/ 25 μl (n=6) after 13 h from the start of treatment.

After 61 h from the start of treatment, the serum LH levels in the male $G_{11}\alpha$ knockout mice reached the serum LH level of the control mice. Male $G_{11}\alpha$ knockout mice released significantly ($p<0.05$) more LH over time than male control mice. In female $G_{11}\alpha$ knockout mice, the serum LH levels reached a maximum of 579 ± 88 pg/ 25 µl (n=5) after 1 h from the start of treatment (figure 2B). In the female control strain a similar treatment resulted in a maximum LH level of 461 ± 45 pg/ 25 µl (n=7) after 1 h from the start of tratment. The serum LH levels in female $G_{11}\alpha$ knockout mice and control strain were mantained at similar levels after 13 h from start of treatment. There was no significant ($p<0.05$) differences in LH release over time between the female $G_{11}\alpha$ knockout mice and female control mice.

Figures 3A and 3B show, respectively, the serum LH level in male and female $G_q\alpha$ knockout mice after a sc injection of 2 µg of Buserelin or vehicle. Samples were collected at 0, 15, 30, 45 and 75 min after the injection of drug or the vehicle. In male $G_q\alpha$ knockout mice, serum LH levels increased from 8 ± 0 pg/ 25 µl to 749 ± 50 pg/ 25 µl , when administered with 2 µg of Buserelin (figure 3A). In the heterozygous strain same treatment of Buserelin resulted in an increase of serum LH from 10 ± 2 pg/ to 765 ± 85 pg/ 25 µl (n=5). When treated with vehicle (PBS/0.1 % BSA), the serum levels in male $G_q\alpha$ knockout mice and in male control strain remained between 9-10 µl (data not shown). In female $G_q\alpha$ knockout mice, serum LH levels increased from 7 ± 0 pg/ 25 µl to 740 ± 95 pg/ 25 µl (n=9), when administered with 2 µg of Buserelin (figure 3B). In the female heterozygous strain, the same treatment of Buserelin resulted in an increase of serum LH from 9 ± 1 pg/ 25 µl to 884 ± 31 pg/ 25 µl

(n=6). LH released in response to Buserelin was not significantly different ($p<0.05$) in both male and female $G_q\alpha$ knockout mice compared to heterozygous mice. When treated with the vehicle (PBS/0.1 % BSA), the serum LH levels in female $G_q\alpha$ knockout mice and in female heterozygous mice remained between 9-10 pg/ 25 µl (data not shown).

Figures 4A and 4B show serum LH levels in $G_q\alpha$ knockout mice 1 h after a sc injection of 2 µg of Buserelin given every 12 h for 108 h. In male $G_q\alpha$ knockout mice, the serum LH level reached a maximum of 556 ± 43 pg/ 25 µl (n=7) after 1 h from the start of treatment (figure 4A). In the heterozygous male, a similar treatment resulted in LH levels of 570 ± 60 pg/ 25 µl (n=4) after 1 h from the start of treatment, a maximum serum LH level of 865 ± 57 pg/ 25 µl was achieved after 13 h from the start of treatment. The serum LH levels of the heterozygous male mice never returned to the serum LH levels of the $G_q\alpha$ knockout mice during the treatment period. In female $G_q\alpha$ knockout mice, the serum LH level reached a maximum of 634 ± 56 pg/ 25 µl (n=4) after 1 h from the start of treatment (figure 4B). In the heterozygous strain a similar treatment resulted in a maximum LH level of 346 ± 63 pg/ 25 µl (n=7) after 1 h from the start of treatment. The serum LH level in female $G_q\alpha$ knockout mice was maintained after the initial LH flare below the heterozygous serum LH levels. There was a significant ($p<0.05$) difference in LH release over time (both males and females) between $G_q\alpha$ knockout and heterozygous mice.

To examine whether the sensitivity of the gonadotrope is different between the knockout and control mice, we investigated the amount of LH released in response to different doses of Buserelin. Figures 5A and 5B show the LH released after 1 h in

response to the indicated doses of Buserelin in $G_{11}\alpha$ knockout male and female mice, respectively. There is no significant difference between the knockout mice and control mice at doses above 0.05 μg of Buserelin. The control mice responded more robustly to a 5 ng/per animal dose of Buserelin than the knockout mice; 448 ± 18 pg/25 μl (n=5) vs 198 ± 25 pg/ 25 μl (n=6) in male knockout mice, 417 ± 23 pg/25 μl (n=5) vs. 175 ± 24 pg/ 25 μl (n=7) in female knockout mice.

Figures 6A and 6B show the LH released after 1 h in response to the indicated doses of Buserelin in $G_q\alpha$ knockout male and female mice, respectively. There was a significant difference in LH release in female $G_q\alpha$ knockout mice when 2 μg of Buserelin was administered; 634 ± 56 pg/25 μl (n=4) for $G_q\alpha$ knockout vs 346 ± 63 pg/25 μl (n=4) for control. However, unlike the $G_{11}\alpha$ knockout mice, there was no significant ($p<0.05$) difference between $G_q\alpha$ knockout mice and heterozygous mice with respect to LH release in response to 0.05 μg of Buserelin.

In order to examine whether there is a differential response to GnRH with respect to steroid production between the knockout mice and the control strain, we examined the production of testosterone and estradiol in mice when injected with 2 μg of Buserelin. Figure 7A and 7B show the testosterone and estradiol levels in the serum, respectively, of male and female $G_{11}\alpha$ knockout mice, before and 1 h after a sc injection of 2 μg of Buserelin. In male $G_{11}\alpha$ knockout mice, serum testosterone levels increased from 2 ± 1 ng/ml to 23 ± 4 ng/ml (n=10) in response to a sc injection of 2 μg of Buserelin. In the male control strain a similar treatment of Buserelin increased serum testosterone levels from 1 ± 1 ng/ml to 14 ± 3 ng/ml (n=5). In female $G_{11}\alpha$ knockout mice, we were unable

to detect a change in serum estradiol levels in response to 2 μg of Buserelin; 8 ± 1 pg/ml before Buserelin treatment, and 6 ± 1 pg/ml (n=12) 1 h after Buserelin treatment. In the female control strain, serum estradiol levels increased in response to 2 μg of Buserelin from 12 ± 1 pg/ml (n=4) to 17 ± 2 pg/ml (n=5).

Figures 8A and 8B show the serum testosterone and estradiol levels, respectively, of male and female mice, before and 1 h after a sc injection of 2 μg of Buserelin in $G_q\alpha$ knockout mice. In male $G_q\alpha$ knockout mice, serum testosterone levels increased from 1 ± 0 ng/ml to 14 ± 2 ng/ml (n=7) in response to a sc injection of 2 μg of Buserelin. In the male heterozygous mice, a similar treatment of Buserelin increased serum testosterone levels from 1 ± 0 ng/ml to 17 ± 2 ng/ml (n=5). In female $G_q\alpha$ knockout mice there was no significant change in serum estradiol levels in response to 2 μg of Buserelin; 5 ± 2 pg/ml before Buserelin treatment, and 4 ± 2 pg/ml (n=3) 1 h after Buserelin treatment. The same was true for female heterozygous mice; 4 ± 1 pg/ml before Buserelin treatment and 7 ± 1 pg/ml (n=3) after Buserelin treatment. There was no significant difference between $G_q\alpha$ knockout mice and heterozygous mice, with respect to Buserelin stimulated steroid (testosterone and estradiol) release.

Discussion

In this study we investigated the role of $G_q\alpha$ and $G_{11}\alpha$ in mouse gonadotrope function using knockout mice lacking the $G_q\alpha$ or the $G_{11}\alpha$ protein. The fact that these knockout mice breed relatively normally, immediately suggested that either protein is not essential in the regulation of the gonadotrope; potentially $G_q\alpha$ and $G_{11}\alpha$ can substitute for each other. In order to examine the role of these two proteins in GnRH-stimulated LH release, we examined the dynamics of GnRH-stimulated LH release in mice. We used two different protocols to address this question. First, we gave each mouse (control and knockout) a single dose of Buserelin and subsequently measured the serum LH levels at the given times. This protocol would show any differences in the release of LH between the knockout mice and control mice in response to acute GnRH analog treatment. Alternatively, we administered Buserelin every 12 h for 108 h, and 1 h after each administration, measured the serum LH levels. The second protocol would address any differences in the LH release between the control mice and knockout mice in response to chronic GnRH analog treatment, and allow assessment of the development of desensitization. To determine the sensitivity of the gonadotrope, we performed an *in vivo* dose-response of Buserelin-stimulated LH release in the knockout and control mice. Furthermore, to determine whether any differences in Buserelin-stimulated LH release between knockout mice and control mice are due to different levels of gonadal steroids, producing varying levels of negative feedback, we assayed the serum testosterone and estradiol levels in male and female mice respectively.

In the short time course study, in both sexes, we did not observe any significant differences between the control strain and the $G_{11}\alpha$ knockout mice. In the long time

course study, the male $G_{11}\alpha$ knockout mice had a rapid increase in serum LH levels after Buserelin treatment compared to the control strain. Furthermore, in the male $G_{11}\alpha$ knockout mice, the serum LH levels were elevated above those of the control mice up to 61 h after the start of treatment, indicating that the knockout mice did not become refractory to the GnRH analog as rapidly as did the control strain. Another possible interpretation would be that LH is elevated to such an extent, that it takes a longer time to return to control plasma levels.

The short time course studies, in both sexes of the $G_q\alpha$ knockout mice, showed no significant differences in LH release in response to a GnRH analog when compared with the control heterozygous strain. In the long time course studies, both sexes showed a significant difference in the LH release compared to heterozygous mice. The $G_q\alpha$ knockout male mice released less LH than the heterozygous mice, and after the initial LH flare, the LH levels in the heterozygous mice never reached the basal levels achieved by the knockout mice. Paradoxically, in the female $G_q\alpha$ knockout mice, Buserelin stimulated a higher level of LH release compared to the control heterozygous strain.

Only $G_{11}\alpha$ knockout mice and not $G_q\alpha$ knockout mice were less sensitive to low doses of Buserelin with respect to LH release. Stimulation of knockout mice with 5 ng of Buserelin resulted in substantially less LH release than in the control mice in $G_{11}\alpha$ knockout mice. This may indicate different roles for $G_{11}\alpha$ and $G_q\alpha$ in the gonadotrope.

To determine whether the differential responses to Buserelin, with respect to LH release, was due to different levels of gonadal steroids being released resulting in differing levels of negative feedback on the gonadotrope, we examined the serum testosterone and estradiol levels in male and female mice, respectively. Buserelin-

stimulated serum testosterone levels in male $G_{11}\alpha$ knockout mice was significantly higher than in control mice, although Buserelin stimulated estradiol levels in female $G_{11}\alpha$ knockout mice were lower than in control mice. In male and female $G_q\alpha$ knockout mice, there were no significant differences in Buserelin stimulated gonadal steroid levels compared to control heterozygous strain.

The fact that there was a difference in Buserelin-stimulated LH release between the short and long time course studies, suggests that there is a differential response to Buserelin under different treatment protocols. It is possible that mice in the short time course protocol maybe stressed, resulting in a retarded response to Buserelin.

In the gonadotrope LH release is under the negative feedback control of estrogen and testosterone. Therefore if gonadal steroid production was affected in the knockout mice, this could produce different intensities of negative feedback on the gonadotrope resulting in differential levels of LH release in response to Buserelin. The steroid data suggest that negative feedback cannot account for the high LH release in male $G_{11}\alpha$ knockout mice in the long time course study, because in the $G_{11}\alpha$ knockout mice, the testosterone levels are higher than in control mice. In $G_q\alpha$ knockout male mice, Buserelin-stimulated LH release is lower than in the control mice in the long time course study, although there is no significant difference in testosterone production compared to the control strain, at 1 h or 13 h after the start of Buserelin treatment. Therefore the differences in the Buserelin-stimulated LH release in the knockout mice compared to the control mice cannot be clearly explained with the differing steroid levels. In the female $G_{11}\alpha$ knockout mice we could not detect an increase in the serum estradiol production after Buserelin stimulation, although in the control wild type mice we measured a

Buserelin-stimulated increase in serum estradiol production. The absence of a Buserelin-stimulated estradiol increase in female $G_{11}\alpha$ knockout mice maybe a physiological manifestation of $G_{11}\alpha$ absence, or maybe a technical artifact due to serum estradiol levels too low to detect with our assay. In the female $G_q\alpha$ knockout mice we could not detect an increase in serum estradiol in response to Buserelin. However, there were no significant differences in the serum estradiol production between the knockout mice and the control heterozygous mice. This may indicate that $G_q\alpha$ does not play a role in Buserelin-stimulated estradiol production in these mice, although, as mentioned earlier, this observation may be due to low serum estradiol levels compared to the sensitivity of our assay.

The fact that in the long time course study, Buserelin-stimulated LH release is higher in male $G_{11}\alpha$ knockout mice than in control (figure 2), whereas in male $G_q\alpha$ knockout mice, Buserelin-stimulated LH release is lower than in the control strain (figure 4), suggests that these proteins may play a different role in LH synthesis. A disruption in LH synthesis, manifested as a difference in Buserelin-stimulated LH release in the long time course experiments cannot be discounted in these knockout mice.

The differences between male control mice and male $G_{11}\alpha$ knockout mice, with regard to Buserelin-stimulated LH release and testosterone production, may be due to the fact that the control mice and the knockout mice were from two different breeding groups. However, this is unlikely, as the strain of the two groups of mice were the same (129/SvEvTacfBR). Similarly, the differences between the heterozygous mice and $G_q\alpha$ knockout mice, with respect to LH release, are not likely due to differences in strain, age or pubertal status, as we tried to use mice from the same litter group.

The reason for a sex based difference in Buserelin-stimulated LH release in $G_q\alpha$ and $G_{11}\alpha$ knockout mice is unclear. One possibility is that there may be differences in the sexual maturation time between males and females, although how this may affect Buserelin-stimulated LH release is not known. It would be interesting to see whether there are other sex based differences in these G-protein knockout mice.

The changes in serum LH levels observed between the knockout mice and the control mice is not due to differences in degradation, because serum LH clearance is dependent on sialation of the protein. Changes in sialation is observed in different aged animals, and we used mice that were of similar age (Conn et al., 1980).

This study shows that there are differences in the dynamics of LH release in response to chronic GnRH analog treatment. The lack of complete abolition of processes, such as stimulated LH release and steroid production, suggest that these G-proteins are either not required or are able to functionally compensate for each other. Experiments similar to this study will have to be performed with conditional double knockouts of $G_{11}\alpha$ and $G_q\alpha$ to determine whether these proteins are required for GnRH activity in the pituitary.

Figure 6-1 (A & B) show, respectively, serum LH levels in male and female $G_{11}\alpha$ knockout mice, after a sc injection of 2 μg of Buserelin or vehicle. Samples were collected at the indicated times and LH released was assayed by RIA as mentioned in the methods. Data represent the mean ± SEM.

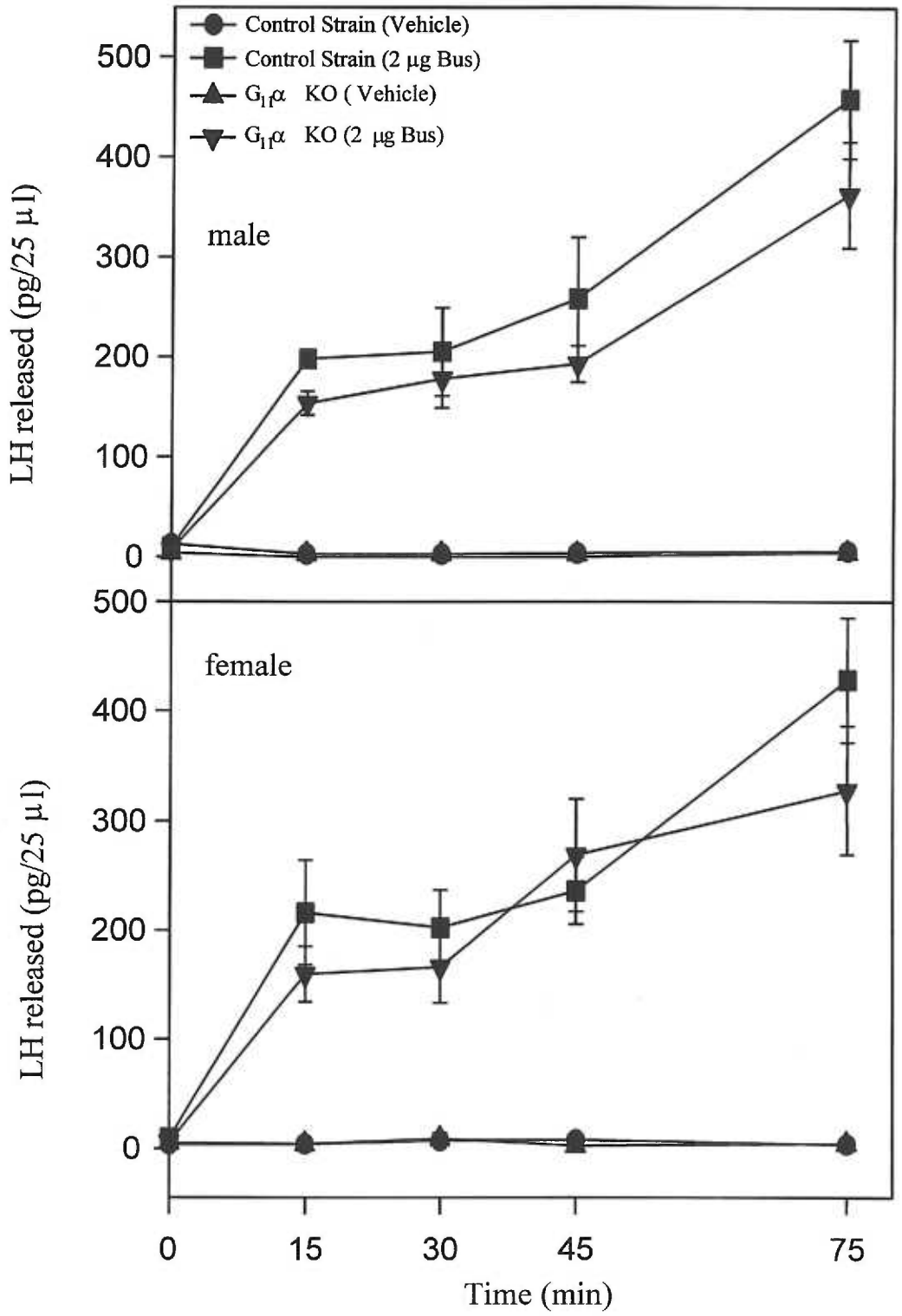

Control Strain (Vehicle)
Control Strain (2 µg Bus)
$G_{i1}\alpha$ KO (Vehicle)
$G_{i1}\alpha$ KO (2 µg Bus)
male
female
LH released (pg/25 µl)
LH released (pg/25 µl)
0
100
200
300
400
500
0
15
30
45
75
Time (min)

Figure 6-2 (A & B) show serum LH levels in $G_{11}\alpha$ knockout male and female mice, respectively, 1 h after a sc injection of 2 μg of Buserelin given every 12 h for 108 h. Samples were collected and LH released was assayed by RIA as mentioned in the methods. The data represent the mean ± SEM. Data points with asterisks (*) are significantly different ($P<0.05$) from the corresponding data points for the control strain.

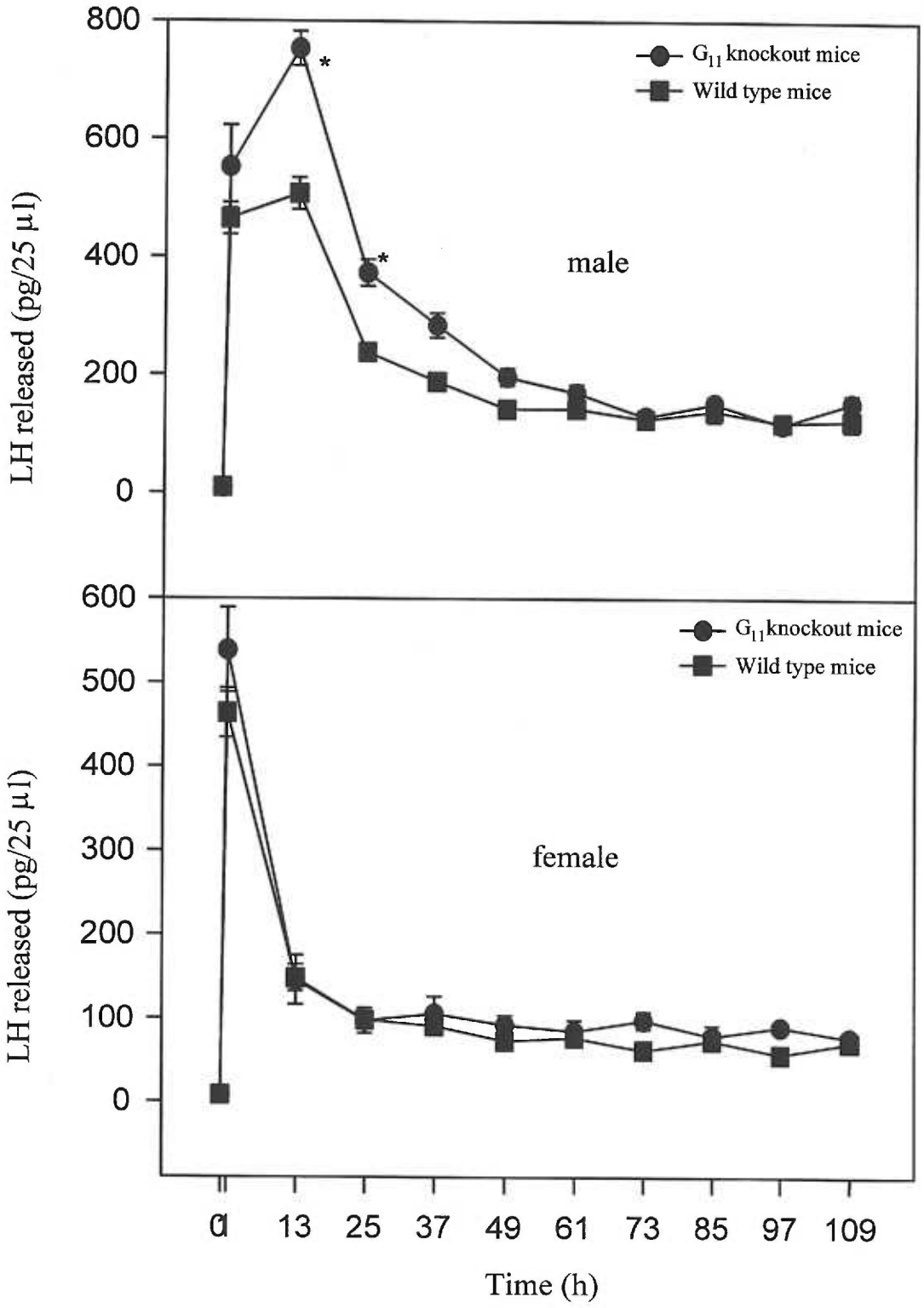

800
600
400
200
0
LH released (pg/25 µl)
G_{11} knockout mice
Wild type mice
male
*
*
600
500
400
300
200
100
0
LH released (pg/25 µl)
G_{11} knockout mice
Wild type mice
female
0
13
25
37
49
61
73
85
97
109
Time (h)

Figure 6-3 (A & B) show, respectively, serum LH levels in male and female $G_q\alpha$ knockout mice, after a sc injection of 2 μg of Buserelin or vehicle. Samples were collected at the indicated times and LH released was assayed by RIA as mentioned in the methods. Data represent the mean ± SEM.

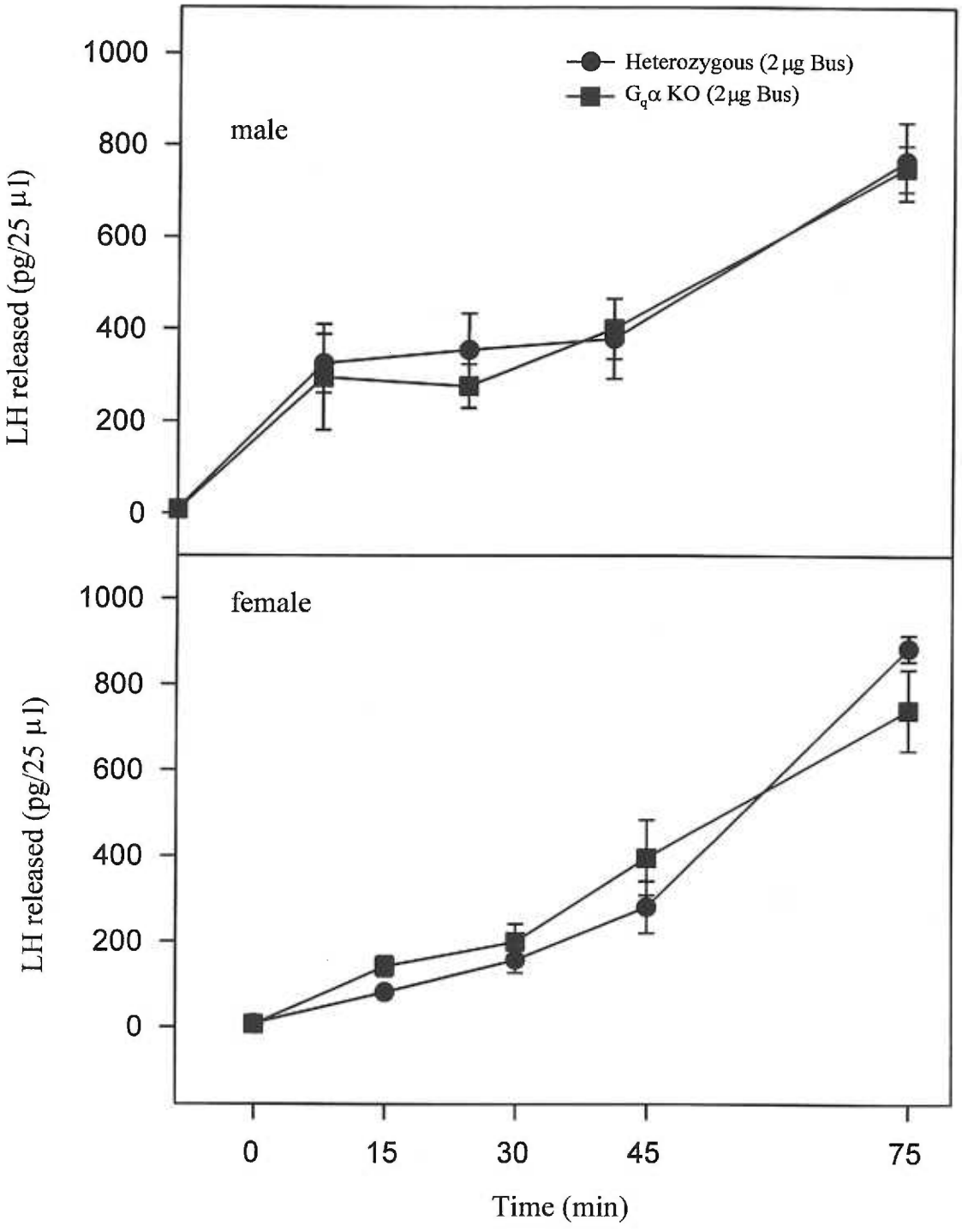

Heterozygous (2 µg Bus)
Gqα KO (2µg Bus)
male
female
LH released (pg/25 µl)
LH released (pg/25 µl)
1000
800
600
400
200
0
0
15
30
45
75
Time (min)

Figure 6-4 (A & B) show serum LH levels in $G_q\alpha$ knockout male and female mice, respectively, 1 h after a sc injection of 2 μg of Buserelin given every 12 h for 108 h. Samples were collected and LH released was assayed by RIA as mentioned in the methods. The data represent the mean ± SEM. Data points with asterisks (*) are significantly different ($P<0.05$) from the corresponding data point for the heterozygous mice.

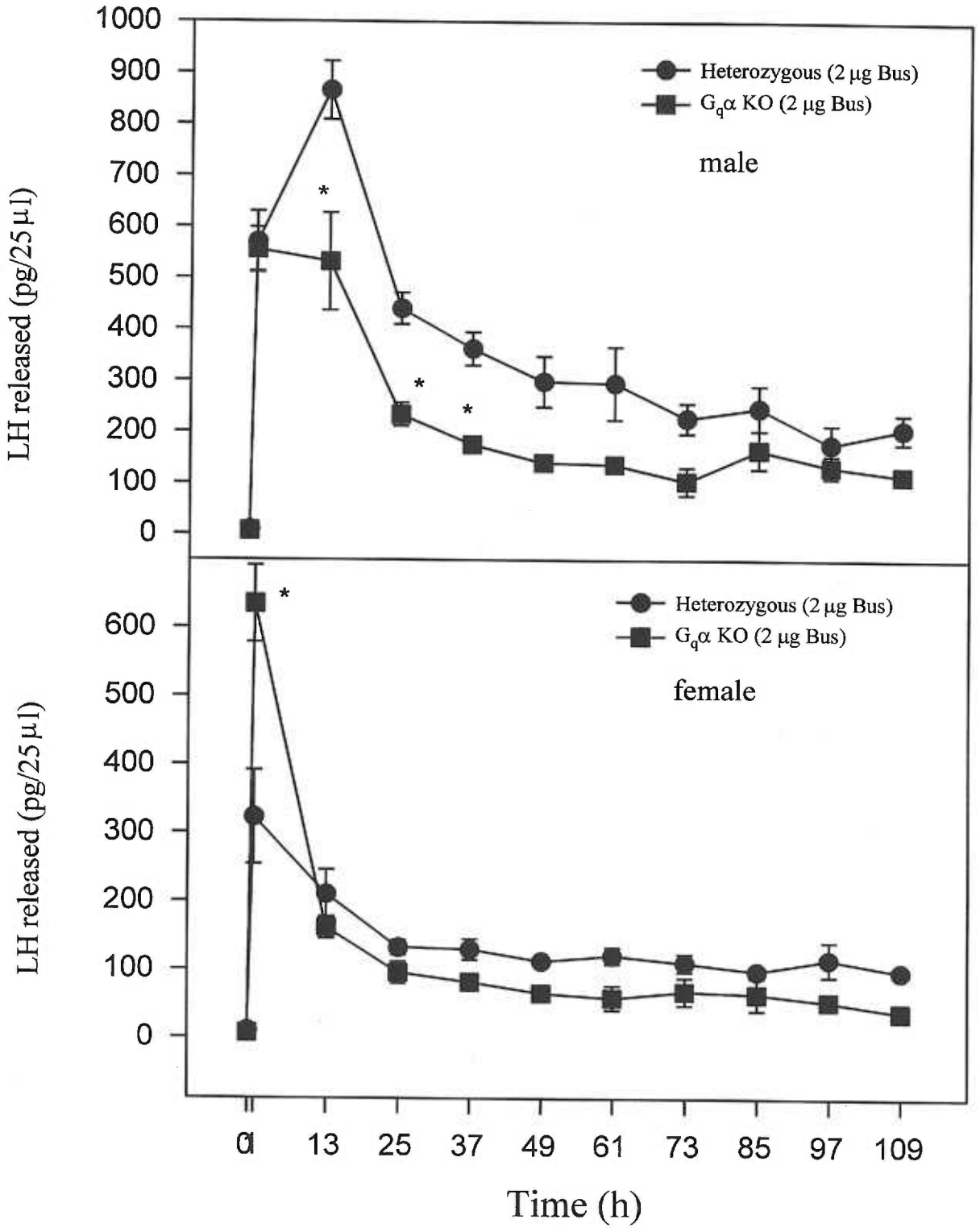
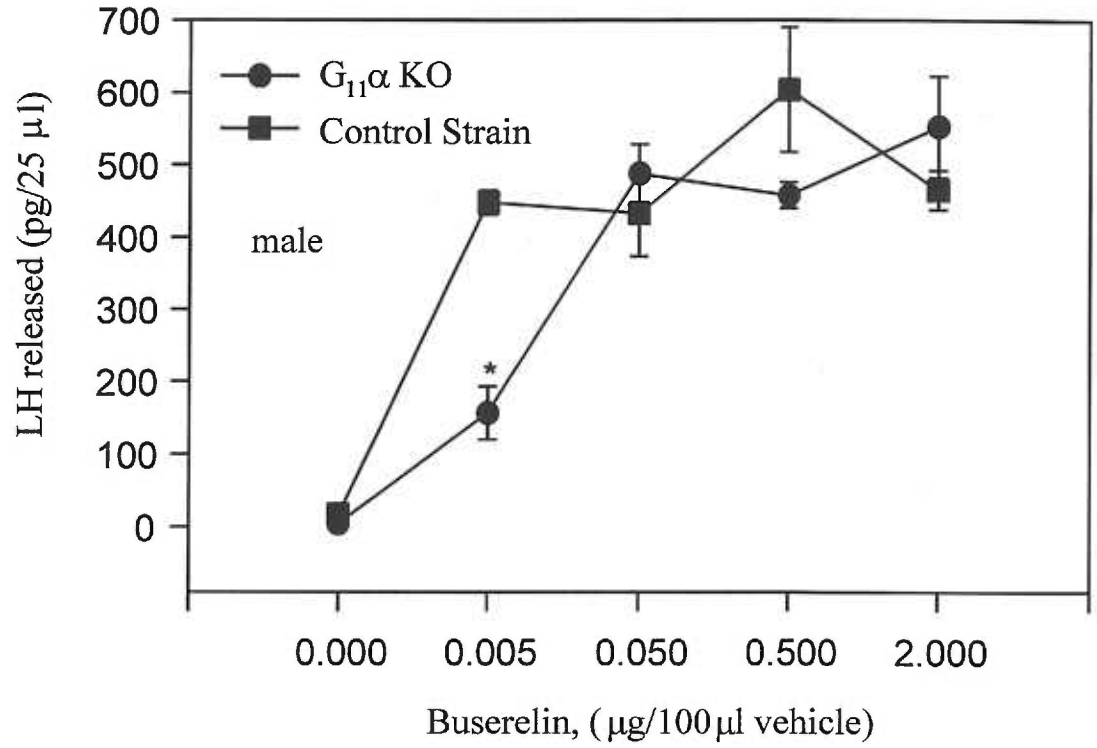

LH released (pg/25 µl)
1000
900
800
700
600
500
400
300
200
100
0
Heterozygous (2 µg Bus)
Gqα KO (2 µg Bus)
male
*
*
*
LH released (pg/25 µl)
600
500
400
300
200
100
0
Heterozygous (2 µg Bus)
Gqα KO (2 µg Bus)
female
*
0
13
25
37
49
61
73
85
97
109
Time (h)

Figure 6-5 (A & B) shows the LH released, after 1 h, in response to the indicated doses of Buserelin in $G_{11}\alpha$ knockout male and female mice respectively. Samples were collected and LH released was assayed by RIA as mentioned in the methods. The data represent the mean $\pm$ SEM. Data points with asterisks (*) are significantly different ($P<0.05$) from the corresponding data points for the control strain.


700
600
500
400
300
200
100
0
LH released (pg/25 µl)
$G_{11}\alpha$ KO
Control Strain
male
*
0.000
0.005
0.050
0.500
2.000
Buserelin, (µg/100 µl vehicle)

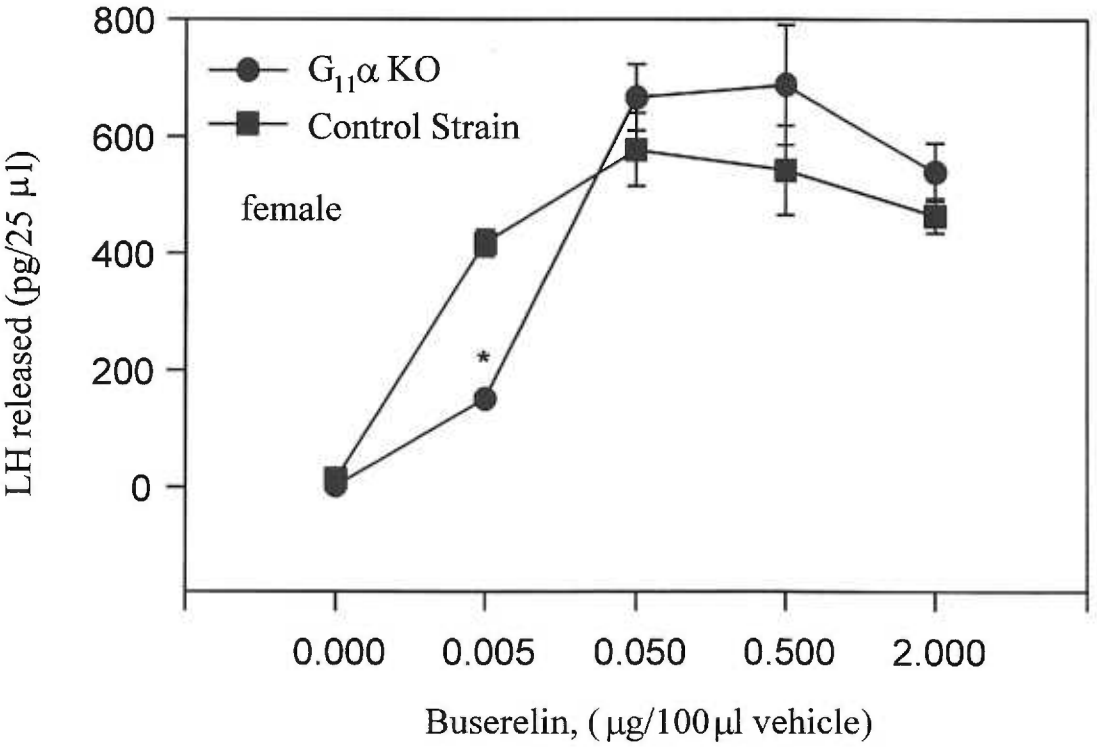
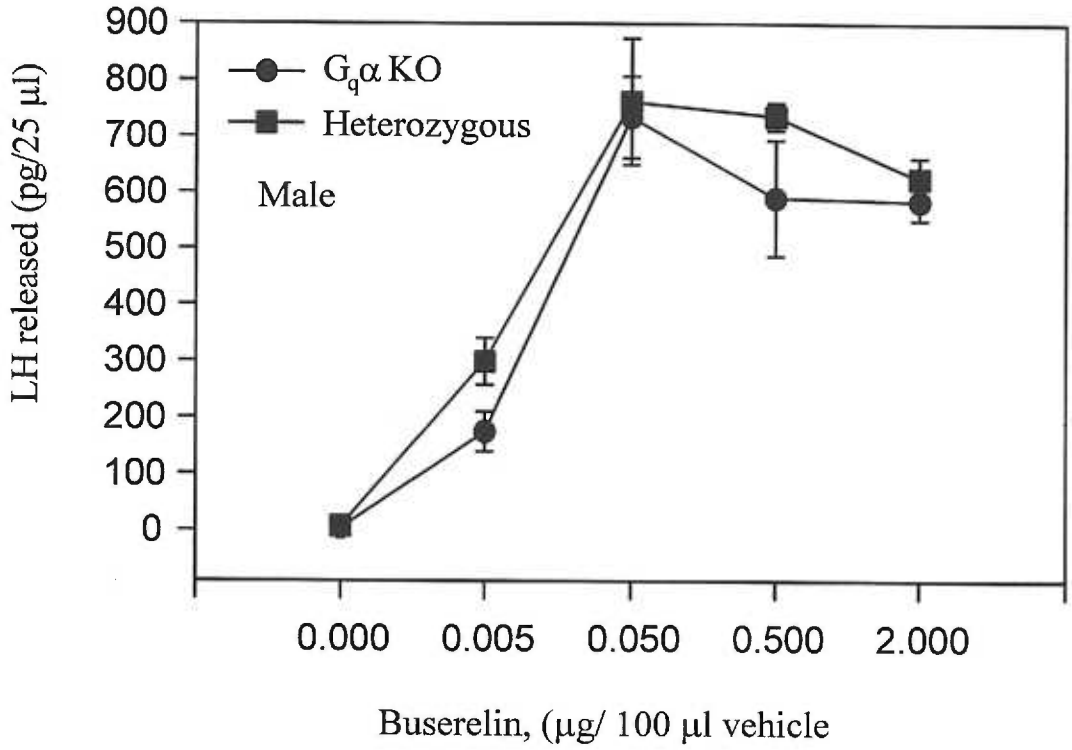

800
600
400
200
0
LH released (pg/25 µl)
$G_{11}\alpha$ KO
Control Strain
female
*
0.000
0.005
0.050
0.500
2.000
Buserelin, (µg/100 µl vehicle)

Figure 6-6 (A & B) shows the LH released, after 1 h, in response to the indicated doses of Buserelin in $G_q\alpha$ knockout male and female mice respectively. Samples were collected and LH released was assayed by RIA as mentioned in the methods. The data represent the mean $\pm$ SEM. Data points with asterisks (*) are significantly different ($P<0.05$) from the corresponding data points for the heterozygous mice.


900
800
700
600
500
400
300
200
100
0
LH released (pg/25 µl)
$G_q\alpha$ KO
Heterozygous
Male
0.000
0.005
0.050
0.500
2.000
Buserelin, (µg/ 100 µl vehicle

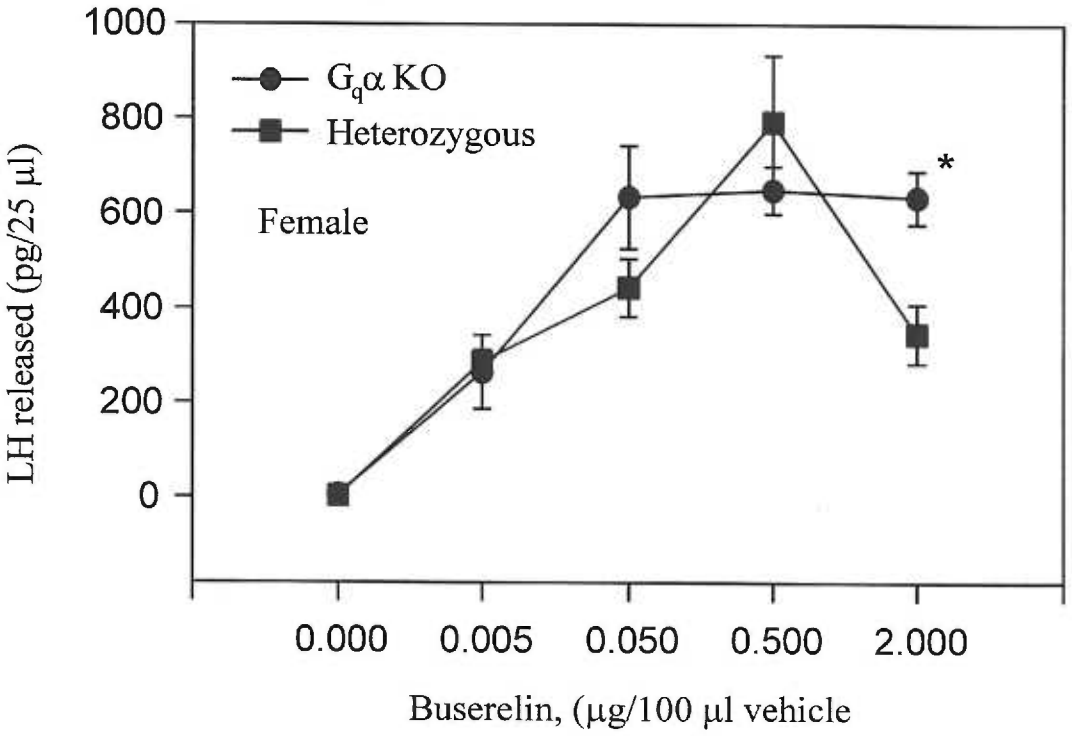
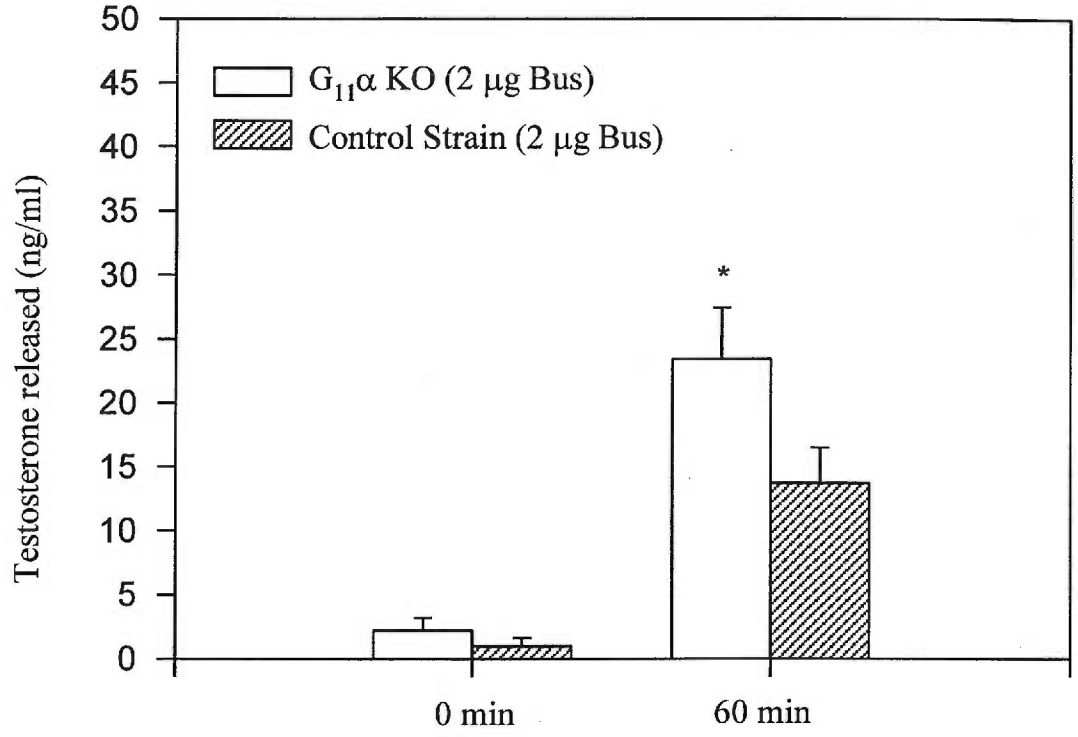

1000
800
600
400
200
0
LH released (pg/25 µl)
$G_q\alpha$ KO
Heterozygous
Female
*
0.000
0.005
0.050
0.500
2.000
Buserelin, (µg/100 µl vehicle

Figure 6-7 (A & B) show the testosterone and estradiol levels in the serum, respectively, of male and female mice, before and 1 h after a sc injection of 2 μg of Buserelin in $G_{11}\alpha$ knockout mice. Samples were collected and serum testosterone and estradiol levels were assayed as mentioned in the methods. The data represent the mean ± SEM. Data points with asterisks (*) are significantly different ($P<0.05$) from the corresponding data point for the control strain.


Testosterone released (ng/ml)
50
45
40
35
30
25
20
15
10
5
0
$G_{11}\alpha$ KO (2 μg Bus)
Control Strain (2 μg Bus)
*
0 min
60 min

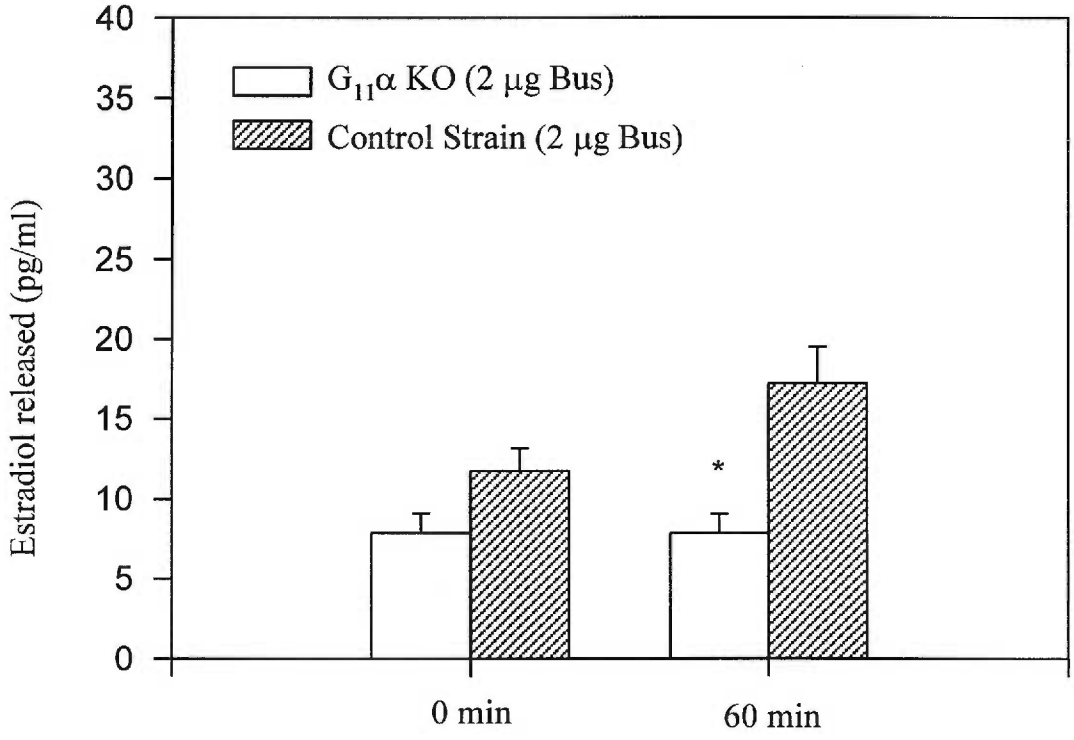
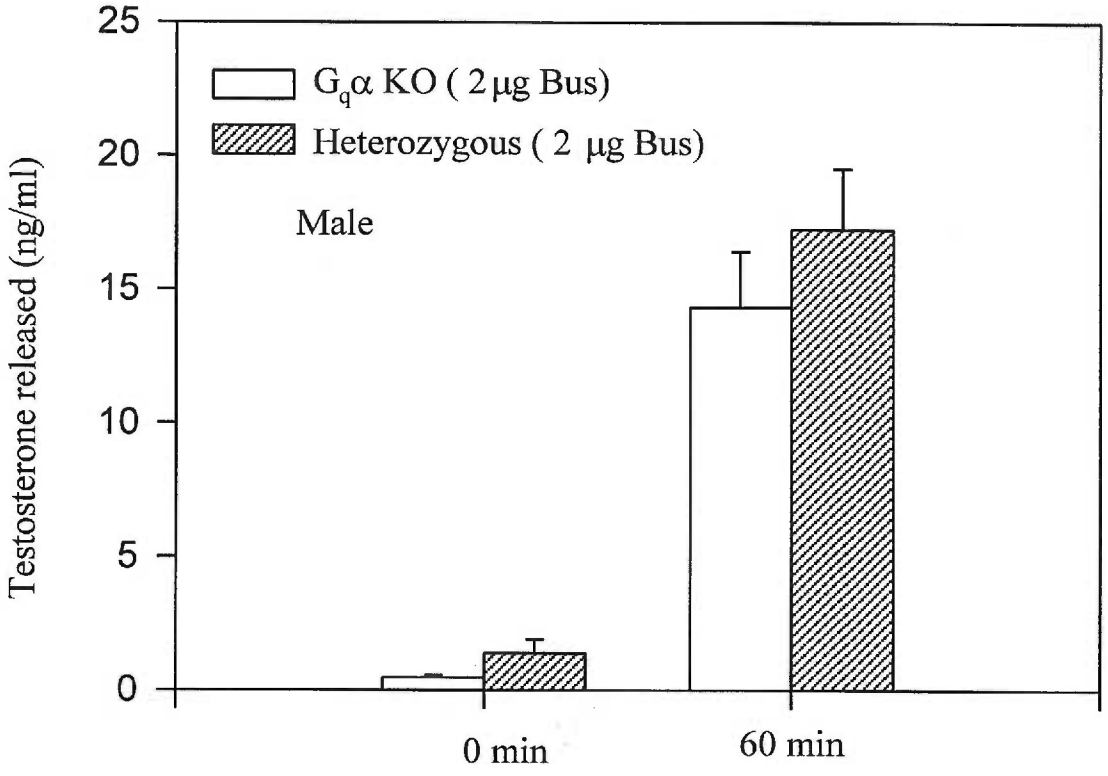
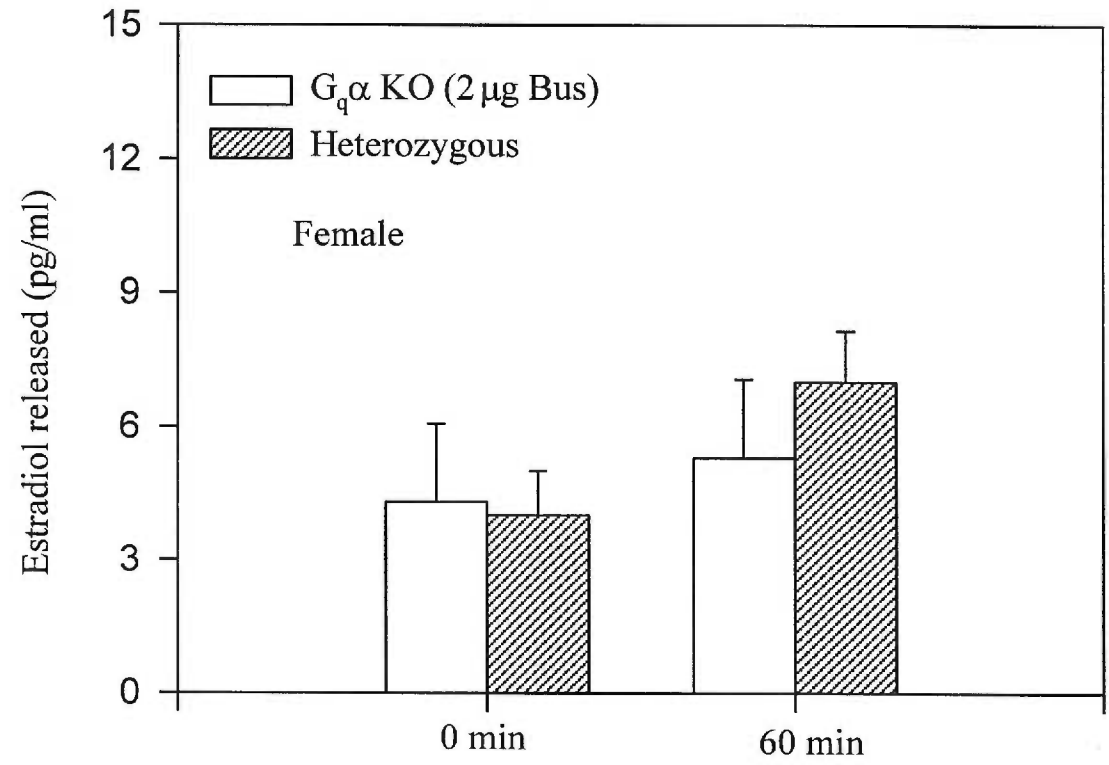

Estradiol released (pg/ml)
40
35
30
25
20
15
10
5
0
$G_{11}\alpha$ KO (2 μg Bus)
Control Strain (2 μg Bus)
*
0 min
60 min

Figure 6-8 (A & B) show the serum testosterone and estradiol levels, respectively, of male and female mice, before and 1 h after a sc injection of 2 μg of Buserelin in $G_q\alpha$ knockout mice. Samples were collected and serum testosterone and estradiol levels were assayed as mentioned in the methods. The data represent the mean ± SEM.


Testosterone released (ng/ml)
25
20
15
10
5
0
$G_q\alpha$ KO (2 μg Bus)
Heterozygous (2 μg Bus)
Male
0 min
60 min


Estradiol released (pg/ml)
15
12
9
6
3
0
$G_q\alpha$ KO (2 μg Bus)
Heterozygous
Female
0 min
60 min

CHAPTER SEVEN

Mechanisms Mediating Multiple Physiological Responses to Gonadotropin-Releasing Hormone

as prepared for submission in

Molecular and Cellular Endocirnology

"Cutting Edge Review"

Introduction

Gonadotropin releasing hormone (GnRH) is a decapeptide synthesized by the neuroendocrine cells in the pre-optic area of the brain. GnRH is released from nerve terminals in a pulsatile manner into the hypophysial-portal circulation and stimulates gonadotrope target cells in the anterior pituitary. The gonadotrope responds to GnRH stimulation by releasing the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Both LH and FSH are glycoproteins comprised of a common α-subunit and a distinct β-subunit. The gonadotropins are themselves released in a pulsatile manner into the systemic circulation, and act on the gonads to regulate gametogenesis and hormogenesis (synthesis of progesterone, testosterone, estrogen, inhibin, activin and follistatin). These gonadal hormones have local effects on gametogenesis, and feedback on the hypothalamus and pituitary. In addition some of the sex steroids feedback on the GnRH "pulse-generator" to control the GnRH pulse frequency.

In the gonadotrope GnRH activates a single class of G-protein coupled receptors to produce several distinct physiological responses: LH, FSH, and secretogranin (SII) release, regulated synthesis of the α and β subunits of the gonadotropins, GnRH receptor up- and down-regulation, and target cell sensitivity. The underlying mechanism by which one hormone stimulates a variety of physiological responses through a single class of receptors is a provocative question in endocrinology.

For several distinct physiological events to occur in response to GnRH, multiple signal transduction pathways are likely to be utilized by the GnRH receptor. It is reasonable to imagine that changes in receptor number, G-protein activity and availability

occur in order for these signal transduction pathways to be activated via a single receptor type. In the gonadotrope, GnRH receptor number, G-protein availability and G-protein activity are dynamically regulated. Therefore it is attractive to consider that coordinated regulation of various components of the GnRH receptor mediated signal transduction pathways may lead to several distinct physiological responses.

Regulation of GnRH receptor number as a mechanism for initiating multiple signal transduction pathways

Pulsatile release of GnRH is critical for maintaining gonadotropin secretion and gene activity in the gonadotrope. The frequency and amplitude of these pulses change throughout the reproductive cycle in response to sex steroids and gonadal peptides (Shupnik et al., 1996). GnRH pulse frequency has differential effects on GnRH receptor number, and the rate of LHβ and FSHβ synthesis in the pituitary (Katt et al., 1985, Shupnik et al., 1996). For example, GnRH receptor synthesis and LHβ synthesis is favored at high GnRH pulse frequencies (pulse/30 min), whereas FSHβ synthesis is favored at low GnRH pulse frequencies (pulse/120 min; Kaiser et al., 1997). These studies suggest that modulation of the GnRH pulse frequency enables GnRH to regulate multiple physiological effects. It is reasonable to postulate that GnRH receptor number may play a role in the multiple effects of the releasing hormone, as GnRH occupancy of the receptor is needed for its physiological effects. Indeed, this is supported by the observation that the pre-ovulatory LH surge is associated with an increase in GnRH receptor number (Marian et al., 1981). However this increase in receptor number does not correlate with an increase FSH release, suggesting that changes in receptor number

may play a role in differential signaling and mediate different cellular effects in response to GnRH. Thus, modulation of GnRH receptor concentration, via changing GnRH pulse frequency, could result in differential activation of signal transduction pathways that lead to different cellular processes.

Calcium is both necessary and sufficient for homologous up-regulation of the GnRH receptor (Conn et al., 1983). Drugs which increase intracellular calcium levels also stimulate receptor up-regulation and LH release, even in the absence of added GnRH. Such drugs are effective at receptor up-regulation below the concentrations needed for LH release (Conn et al., 1983). This suggests a mechanism by which small numbers of GnRH receptor could respond to releasing hormone so as to increase its receptor number, resulting in a gonadotrope that is more responsive to the releasing hormone. Such receptor up-regulation may lead to differential activation of varied signal transduction pathways at different receptor numbers.

The differential effects of GnRH receptor concentration on signal transduction are further illustrated by its effects on LHβ or FSHβ promoter activity (Kaiser et al., 1995). In these experiments, GnRH-stimulated LHβ promoter activity was optimally stimulated at relatively high GnRH receptor numbers, while FSHβ promoter activity was optimally stimulated at relatively low GnRH receptor numbers. This study suggests that receptor number affects the signal transduction pathways in the cell. More recent work by Haisenleder and co-workers (1997) showed that modulation of the frequency of intracellular calcium pulses may play a role in differential regulation of LHβ and FSHβ mRNA synthesis. It is possible that the modulation of the frequency and amplitude of intracellular calcium pulses are the result of changes in GnRH receptor number, and that

modulation of GnRH receptor number could regulate different physiological processes. As discussed below, GnRH receptors are coupled to G-proteins, and the changes in receptor number may result in differential coupling to G-proteins. This event itself could enable GnRH, acting through one class of receptor, to modulate different signal transduction pathways, and ultimately different physiological endpoints.

Studies on how receptor number affects the activation of different signal transduction pathways have shown that the ability and the extent to which a particular signal transduction pathway is activated depends on the receptor number in the cell (Zhu et al., 1994, Tsuda et al., 1997, Ashkenazi et al., 1987). For example, G_s coupled receptors can also couple to G_q at high receptor numbers. This suggests that a change in receptor number results in the activation of different signal transduction pathways. Studies have shown that at least two distinct signal transduction pathways may be involved in GnRH stimulated α-, LHβ- and FSHβ-subunit synthesis (Saunders et al., 1998; Weck et al., 1998). Furthermore, GnRH-stimulated inositol phosphate production, but not cAMP production, is sensitive to GnRH receptor number in GH_3 cells expressing the GnRH receptor cDNA. In these studies it was seen that GnRH agonist-stimulated cAMP production did not increase with the increase in GnRH receptor number. However, GnRH agonist-stimulated IP production increased with GnRH receptor number (Pinter et al., manuscript submitted).

Certain receptors have an intrinsic capacity to couple to a certain class of G-proteins and thereby appear to activate these proteins almost exclusively. The α_{2A}-adrenergic receptor coupling to G_i proteins is a good example (Federman et al., 1992). However, the GnRH receptor can couple to multiple G-proteins (Hawes et al., 1992;

Stanislaus et al., 1998), and the receptor may have different affinities for these G-proteins. Increasing the receptor number may provide a mechanism by which G-proteins with less affinity to the receptor is utilized, thus differentially activating signal transduction pathways as a function of receptor number.

In the gonadotrope maximal LH release occurs when only 20% of the GnRH receptors are occupied by the agonist (Naor et al., 1980). This indicates that there are "spare" receptors with respect to LH release; however, whether there are "spare" receptors for other functions of the gonadotrope is unclear, since this is a difficult question to approach experimentally (Conn et al., 1995). The presence of spare receptors may suggest that modulation of receptor number may not be important for initiation of differential signal transduction pathways. However this may also suggest that only 20% of the receptors, for example, may be available for signal transduction and any increase in receptor number would in fact change the number of receptors available for signal transduction, and thereby modulate differential signal transduction pathways.

Multiple G-protein coupling to the GnRH receptor; evidence for activation of differential signal transduction pathways

A variety of experiments indicate that GnRH receptor action is mediated through G-proteins (Snyder et al., 1982; Andrews et al., 1986). In these studies addition of GTP or a metabolically stable analog (guanylimidodiphosphate) to permeabilized pituitary cells resulted in time and dose-dependent increases in inositol phosphate accumulation and LH release. Perrin and co-workers (1989) have shown that binding affinity of a GnRH agonist to the GnRH receptor is decreased in the presence of guanine nucleotides,

a characteristic common to other G-protein coupled receptors (Gilman 1987). More recent evidence from hydropathy analyses of amino acid sequences of the GnRH receptor, from both mammalian and non-mammalian sources, identified the typical 7-transmembrane spanning (7-TMS) segments of G-protein coupled receptor family. (Eidne et al., 1992; Perrin et al., 1993; Tsutsumi et al., 1992).

It is now generally accepted that the first step in GnRH action is binding of the hormone to a G-protein coupled 7-TMS receptor. Evidence from toxin and second messenger studies indicate that multiple G-proteins mediate the actions of the GnRH receptor (Hawes et al., 1992; Andrews et al., 1986). In dispersed pituitary cell cultures, pertussis toxin (PTX) pre-treatment decreases inositol phosphate (IP) production in response to GnRH (Hawes et al., 1993), suggesting that a PTX sensitive G-protein (such as G_i or G_o) couples the receptor to IP turnover. Immuno-depletion studies in membranes derived from αT3-1 cells indicate that PTX-insensitive $G_{q/11}\alpha$ is coupled to GnRH receptors (Hsieh et al., 1992). Moreover, in GH_3 cells stably transfected with the GnRH receptor, GnRH agonist-evoked IP production is mediated through a PTX-insensitive G-protein (Janovick et al., 1994). Studies with cholera toxin (CTX; G_s activator) gave the first indications of G_s mediated functions in the gonadotrope. CTX pretreatment of rat pituitary cell cultures increases LH release in response to GnRH (Weiss et al., 1987; Janovick et al., 1993), which is mimicked by dibutryl cAMP, indicating that the effect of CTX is mediated by cAMP. The involvement of cAMP in GnRH receptor action in gonadotropes suggested that this receptor may couple to G_s, although GnRH receptor stimulated cAMP release has not been detected in rat pituitary cell cultures to date (Conn et al., 1979).

Recently we utilized GnRH-stimulated palmitoylation of G-proteins to label endogenously G-proteins coupled to the GnRH receptor in rat pituitary cell cultures (Stanislaus et al., 1997; Stanislaus et al., 1998). G-protein activation is necessary for palmitic acid turnover (Degtyarev et al., 1993; Wedegaertner et al., 1993). Except for G-proteins of the transducin family, all other known G-proteins have a N-terminus consensus sequence for palmitoylation (Wedegaertner et al., 1993). GnRH stimulation of rat pituitary cell cultures resulted in the dose- and time-dependent palmitoylation of $G_{q/11}\alpha$, $G_i\alpha$ and $G_s\alpha$. Furthermore, this effect was dependent upon receptor activation, as the GnRH receptor antagonist, Antide, did not stimulate palmitoylation of these G-proteins (Stanislaus et al., 1997; Stanislaus et al., 1998). These studies indicate that the GnRH receptor is able to couple to and regulate multiple G-proteins in the rat gonadotrope.

The multiple signal transduction pathways mediated by $G_{q/11}\alpha$, $G_i\alpha$ and $G_s\alpha$ in the gonadotrope

Binding of GnRH to its receptor results in the activation of the signal transduction pathways that ultimately result in multiple physiological endpoints. These physiological endpoints are thought to involve multiple G-protein mediated signal transduction pathways (figure 1).

Activation of $G_{q/11}\alpha$ by cognate receptors results in the subsequent activation of phospholipase Cβ (PLCβ; Berstein et al., 1992). PLCβ is a GTPase activating protein (Berstein et al., 1992) that mediates the hydrolysis of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3; Downes

et al., 1983) and diacylglycerol (DAG; Kishimoto et al., 1980). Intracellular increases in IP_3 leads to the elevation of intracellular calcium derived from non-mitochondrial (Streb et al., 1983) and extracellular sources (Kuno et al., 1987).

GnRH receptor-stimulated gonadotropin release is dependent on intra- and extracellular derived calcium (Conn et al., 1980, Conn et al., 1980b and Hansen et al., 1987). Studies on rat gonadotropes indicate that the GnRH receptor is coupled to $G_{q/11}\alpha$ (Hawes et al., 1992; Stanislaus et al., 1997), and is able to increase phospholipid metabolism (Huckle et al., 1985) and intracellular calcium concentration (Clapper et al., 1985) in a hormone-dependent manner. A logical conclusion of these studies is that a $G_{q/11}\alpha$-mediated pathway produces a rise in intracellular calcium resulting in gonadotropin release. However, inhibition of IP_3 turnover does not inhibit hormone-stimulated gonadotropin release (Hawes et al., 1992b), suggesting that this pathway may not be critical for the secretory process. Therefore, $G_{q/11}\alpha$-mediated signal transduction may be involved in physiological functions of the gonadotrope other than GnRH-stimulated LH release.

PLCβ activation results in the production of DAG, a well-characterized second messenger. In the presence of DAG, protein kinase C (PKC) becomes more sensitive to activation by intracellular calcium (Kishimoto et al., 1980). Thus, the activation of PLCβ by $G_{q/11}\alpha$ may give rise to molecules that elevate intracellular calcium and activate PKC in a coordinate manner. GnRH stimulation of rat pituitary cell cultures results in the activation of PKC (Hirota et al., 1985; McArdle et al., 1986). Although direct PKC activation by phorbol esters releases LH, GnRH can stimulate LH release in rat pituitary cell cultures depleted of PKC (McArdle et al., 1988). This indicates that GnRH-

stimulated LH release can be uncoupled from PKC (Johnson et al., 1988; Andrews et al., 1990). While PKC may not be involved in GnRH-mediated LH release, the fact that it is activated by the GnRH receptor suggests that PKC may have other roles in the pituitary, such as to modulate gonadotrope responsiveness to GnRH (Andrews, et al., 1988).

Studies in our laboratory indicate that PKC activation may have a modulatory effect on the GnRH receptor (Huckle et al., 1989). Pretreatment of rat pituitary cell cultures with PKC activators increases GnRH receptor number and affinity (Huckle et al., 1988). In the gonadotrope, PKC is also thought to regulate the efficiency of coupling of the GnRH receptor to LH release (McArdle et al., 1988). In addition, PKC could play a role in the enhanced LH release in gonadotropes exposed to elevated GnRH concentrations (Waters et al., 1992). PKC is also implicated in gonadotropin biosynthesis. Long-term pulsatile exposure of pituitaries to GnRH results in a two fold increase in LHα and LHβ mRNA levels (Papavasiliou et al., 1986). Furthermore, depletion of PKC in rat pituitary cell cultures inhibits the GnRH-stimulated increase in LHβ mRNA (Andrews et al., 1988). These studies indicate that the PKC-dependent effects seen in the gonadotrope after GnRH treatment are potentially mediated through a $G_{q/11}\alpha$ dependent pathway.

GnRH receptor is able to couple to G_s proteins in the gonadotrope, although we have not been able to measure GnRH-stimulated cAMP release in pituitary cell cultures prepared from female weanlings (figure 1). However, it is not unusual to observe G-protein coupling, without detecting the production of second messengers. For example, in preparations of dog thyroid membranes, thyroid stimulating hormone (TSH) increased the photolabeling of $G_{q/11}\alpha$, suggesting TSH receptor coupling to this G-protein.

However, in thyroid slices or in cells, TSH-stimulated IP production was not detected (Graff et al., 1987; Raspe et al., 1992). In the gonadotrope, cAMP mediates the movement of LH from a nonreleasable pool to a releasable pool. Therefore, GnRH receptor mediated activation of $G_s\alpha$ may help to maintain a constant releasable pool of LH when stimulated by pulsatile GnRH under conditions that do not lead to homologous desensitization. This suggests that uncoupling of $G_s\alpha$ could participate in homologous desensitization.

The G_i family of G-proteins are PTX sensitive, meaning that PTX catalyzed ADP-ribosylation prevents their activation by the receptor, and pre-treatment with this toxin disrupts G_i-mediated signal transduction. GnRH stimulates PTX-sensitive IP production, implicating the proteins of the G_i family (figure 1). Furthermore, GnRH receptor activation results in a dose- and time-dependent palmitoylation of $G_i\alpha$, suggesting that in the rat pituitary these proteins are able to couple to the GnRH receptor. The G_i proteins mediate IP production through their associated $\beta\gamma$ subunits by stimulating the phospholipase Cβ2 isozyme to hydrolyze PIP_2 to IP_3 (Camps et al., 1992; Katz et al., 1992). Therefore, activation of these G-proteins can potentially stimulate all of the activities that were attributed to the activation of $G_{q/11}\alpha$. In addition, the $G_i\alpha$ subunit of the heterotrimeric G-proteins is responsible for the inhibition of adenylate cyclase. Consequently, the GnRH receptor, by activating the $G_i\alpha$ protein, is able to turn off the adenylate cyclase stimulating effects of $G_s\alpha$. Paradoxically, the $\beta\gamma$ subunits released by activation of $G_i\alpha$ may stimulate the adenylate cyclase subtype II concomitantly with the activated $G_s\alpha$ that is required for such activation to produce cAMP. For such activation of adenylate cyclase II to occur, only a small amount of active $G_s\alpha$ is needed (Federman

et al., 1992). Consequently, in the gonadotrope, G_i may play a dual role by stimulating adenylate cyclase II to produce cAMP, and PLCβ2 isoform to produce IP_3 and DAG. Thus far, the subtypes of adenylate cyclase expressed in the gonadotrope are unknown. As a result, the presence or absence of βγ-mediated cAMP production is yet to be determined. The possible participation of βγ in the gonadotrope in mediating cAMP effects still does not explain the lack of detection of this second messenger. One possibility is that cAMP is produced at the site of action and therefore is localized, circumventing the need for elevated cAMP concentrations.

Effect of palmitoylation on G-protein availability and activity; a putative role for modulating multiple signal transduction pathways

G-proteins are thought to interact with the receptor at the level of the plasma membrane. Receptor G-protein interactions are partly dependent on their accessibility and availability to each other in the cellular environment. Thus, factors that improve membrane association of G-proteins could potentially increase their probability of activation. Palmitoylation of G-protein α-subunits, near the N-terminal cysteine, may increase the hydrophobicity of the protein, resulting in increased membrane avidity. Therefore, G-protein palmitoylation may have physiological significance. For instance, palmitoylation of G-proteins appears to be required for membrane association (Iri et al., 1996), and it correlates with $G_{q/11}\alpha$ redistribution to the membrane in response to GnRH, in rat gonadotropes (Cornea et al., 1998). G-protein activation is necessary for palmitic acid turnover, and GnRH receptor activation results in the palmitoylation of $G_{q/11}\alpha$, $G_i\alpha$ and $G_s\alpha$. Therefore, regulation of palmitic acid incorporation may help to control G-

protein abundance at the membrane, thereby controlling the probability of these G-proteins being activated.

It is now known that palmitoylation of $G_z\alpha$ and $G_i\alpha$ has an inhibitory effect on GTPase activating protein (GAP) for G_z, and other specific RGS-proteins (Regulators of G-protein Signaling; Tu et al., 1997). RGS-proteins are negative regulators of G-protein mediated signal transduction (Huang et al., 1997). These proteins mediate their action by accelerating the GTPase activity of G-proteins (Berman et al., 1996). Therefore, it is possible that signaling mediated by G-proteins would be turned off faster by increasing the GTPase activity of G-proteins. Recent findings show that the GTPase activating properties of RGS-proteins are inhibited when G-proteins are palmitoylated, and suggest that palmitoylation of G-proteins may play a role in prolonging or potentiating the signal mediated by the G-protein. RGS3 mRNA is present in gonadotrope-derived αT3-1 cell lines (Neill et al., 1997). These observations suggest that palmitoylation, in association with RGS-proteins, could play a role in regulating multiple signal transduction pathways in the gonadotrope.

Potential mechanisms for initiating multiple signal transduction pathways via a single class of receptors and multiple G-proteins

The mechanism(s) by which the GnRH receptor couples to multiple G-proteins is not known. It is generally accepted that receptor-ligand complexes initiate the signal transduction upon interaction with G-proteins. The earliest observable event, after the GnRH receptor is occupied by an agonist, is the microaggregation of the receptors on the cell surface (Janovick et al., 1996; Conn et al., 1982). The time course of this event

suggests that microaggregation precedes G-protein activation. Studies indicate that GnRH antagonists linked to one another with bivalent antibody will stimulate LH release from rat pituitary cell cultures. This suggests that receptor occupancy and aggregation is necessary for the initiation of signal transduction.

It is still not clear how multiple G-proteins may assemble with the microaggregated GnRH receptor to initiate signal transduction. Several possible permutations of GnRH receptor/G-protein complexes could exist that enable multiple G-protein mediated signal transduction in the gonadotrope (figure 2). 1) GnRH receptor microaggregation may result in the assembly with a single G-protein. 2) GnRH receptor microaggregation may favor the assembly with a homogeneous population of G-proteins at each receptor microaggregate. 3) The microaggregated receptors may result in the assembly of a heterogeneous population of G-proteins.

GnRH receptor microaggregation can potentially provide an intracellular surface to assemble one G-protein at a time. However, different G-proteins may assemble at different receptor complexes. If one type of G-protein is accessible and more abundant than others, the majority of the receptor/G-protein complexes will contain this particular G-protein, and therefore, the major signaling characteristics of the cell will be determined by this type of G-protein. This concept is true as long as the particular G-protein can interact with the receptor. However, GnRH receptor couples to multiple G-proteins, and as a result has the potential to initiate signal transduction through multiple G-proteins. Therefore, less abundant and lower affinity G-proteins may also be involved in signal transduction. Thus, this model would predict that the majority of the GnRH receptor microaggregates would assemble the most abundant and higher affinity G-proteins, and a

minority of receptor microaggregates would assemble the less abundant and lower affinity G-proteins.

Initiation of multiple signal transduction pathways in this manner may result in different physiological responses, and also may help to regulate the abundance and activity of different constituents in a signal transduction pathway. By changing the receptor and G-protein concentrations, and modulating the G-protein activity by regulating their palmitoylation and RGS-protein activity, multiple signal transduction pathways could be initiated in a time-dependent manner. Such a mechanism would provide the cell with a more dynamic and versatile way to use one-ligand/receptor model to regulate multiple physiological end points.

The second and third models (figure 2) suggest that microaggregation of GnRH receptors can result in the assembly of more than one G-protein at each receptor microaggregate. Microaggregation may provide the GnRH receptor with multiple intracellular surfaces capable of interacting with more than one G-protein. The next question is whether these multiple G-protein complexes, assembled at a GnRH receptor complex, are homogeneous or heterogeneous. It is possible that assembly of one G-protein at a receptor microaggregate may make it more or less likely for the assembly of similar G-proteins, thus giving rise to the assembly of either a homogeneous or a heterogeneous G-protein complex, respectively. It is also possible that depending on the initial G-protein interaction, receptor or loop conformation may change to favor a homologous or heterologous G-protein assembly at the receptor complex. Such mechanisms could play a role in the models we proposed. However cooperativity between the intracellular loops and the G-proteins would still be dependent on the

abundance of G-protein types and affinity of these proteins to GnRH receptor, because a higher abundant or higher affinity G-protein will have a higher chance of interacting with the receptor complex and affecting subsequent cooperativity with other G-proteins.

Studies remain to be performed to test these models. With the development of higher resolution laser confocal microscopy it should be possible to label G-proteins and receptors with specific dyes and examine co-localization of these tags. Furthermore, with the new GFP (green fluorescence protein) labels that use FRET (fluorescence resonance energy transfer) technology to transfer fluorescence between GFP proteins, it may be possible to detect the stoichiometry of receptor/G-protein interactions. These studies will be helpful in elucidating the underlying mechanisms of GnRH receptor coupling to multiple G-proteins.

Modulation of G-protein abundance and activity as a means of regulating multiple signal transduction pathways

The ability of a receptor to couple specific G-proteins is a complex function of several factors, including receptor affinity to specific G-proteins, G-protein availability, and receptor density. This in turn would reflect the signaling characteristics of the cell. Therefore the ability to regulate receptor affinity to G-proteins, G-protein abundance and activity, and receptor density could hypothetically provide the cell with a dynamic ability to change the signaling characteristics of the receptor/G-protein complex. This could result in different G-protein populations being activated to produce diverse effects in a time-dependent manner, enabling one ligand acting through one receptor to regulate multiple effects in a time-dependent manner.

G-protein phosphorylation may provide a mechanism by which signal transduction by this protein is terminated or diminished. For example, phosphorylation of rhodopsin greatly inhibits phosphodiesterase activity (Miller et al., 1986). Moreover, phosphorylation of the receptor may change its affinity from one type of G-protein to another. The β-adrenergic receptor couples predominantly to G_s proteins, however when phosphorylated it is able to couple G_i proteins (Daaka et al., 1997). Therefore, signal transduction pathways mediated through one G-protein has the potential to make the cognate receptors couple to other G-protein-mediated signal transduction pathways. Although similar mechanisms have not been identified in gonadotropes, they could play a role in mediating GnRH receptor coupling to multiple G-proteins.

Once activated, both low and high abundance G-proteins could modulate changes in G-protein abundance, accessibility and receptor density to bring about changes to the signal transduction pathway. For example, when a higher affinity G-protein is selectively down-regulated, a low affinity G-protein may be able to interact with the receptor and mediate signal transduction. For instance, the rat A3 adenosine receptor selectively downregulates the $G_{i3}\alpha$ proteins, but not other G-proteins it is capable of activating (Palmer et al., 1995). This would provide a situation where other G-proteins are able to interact with the receptor and mediate signal transduction, once the higher abundant and higher affinity G-protein is no longer available.

In the gonadotrope, GnRH receptor occupancy by an agonist results in a time-dependent movement of $G_{q/11}\alpha$ to a membrane compartment (Cornea et al., 1998). Furthermore, GnRH receptor activation results in a time-dependent increase in immunodetectable $G_{q/11}\alpha$ in gonadotropes. This may be the manifestation of a

mechanism where GnRH receptor activation results in an increase in $G_{q/11}\alpha$, thus enabling a higher incidence of activation of this protein, and consequently the signal transduction pathways mediated by this protein. We have not observed a similar modulation of either $G_i\alpha$ or $G_s\alpha$, although that does not mean such modulation does not occur.

It is conceivable that changing the activity of G-proteins could be achieved by regulating the factors that alter G-protein function and abundance. These factors could include RGS proteins, enzymes that catalyze palmitic acid incorporation, kinases that phosphorylate G-proteins and receptors, transcription factors that regulate G-protein synthesis and proteolytic enzymes that degrade G-proteins. These factors could be controlled by the signal transduction pathways that are activated by the receptor, thereby having a feedback effect on signal transduction.

Conclusions

The precise mechanism(s) by which the GnRH receptor regulates multiple cellular functions is not known. It is possible that regulation of GnRH receptor number enables the activation of different G-proteins which in turn initiates multiple signal transduction pathways to produce various physiological responses. The available data suggest that the dynamic interaction between the receptor and G-proteins involves several factors: such as G-protein abundance, receptor/G-proteins affinity and receptor density. The microaggregation of the GnRH receptor is the earliest detectable event following agonist binding. It is possible that following microaggregation of the GnRH receptor, a single G-protein, or a homogeneous or a heterogeneous population of G-proteins assembles at a

receptor complex to initiate signal transduction. Activation of these G-proteins can result in dynamic changes to these proteins and the receptors that activate them. Such changes to receptor number and G-protein abundance may lead to changes in the signal transduction pathway in a time-dependent manner. Therefore a model can be proposed where changes in GnRH pulse frequency result in changes in GnRH receptor number, which lead to changes in the G-proteins and the cognate signal transduction pathways (figure 3). Activation of multiple signal transduction pathways in a dynamic manner would provide the gonadotrope with the means of responding with multiple physiological effects to GnRH. Signal transduction cascades in the gonadotrope may initiate responses that have a feedback effect on the GnRH pulse generator, GnRH receptor number, G-protein abundance and activity, thus leading to an alteration in the gonadotrope response. These feedback effects may be direct or through other hormones such as gonadal steroids and peptide hormones. Such circuitous dynamic events may be a method for making the signal transduction pathways, initiated by a single receptor, more versatile and dynamic.

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Figure 7-1. The role of G_q, G_i, and G_s in the gonadotrope. A schematic diagram showing the putative functions of these three G-proteins in GnRH action in the gonadotrope. GnRH occupancy of the receptor causes microaggregation and subsequent G-protein activation and calcium mobilization. Receptor macroaggregation results in receptor down-regulation, down regulated receptors may recycle back to the cell surface. ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; PIP_2: phosphoinositol 4,5 bis-phosphate; IP_3: inositol 1,4,5 triphosphate; DAG: diacylglycerol; PKC: protein kinase C.

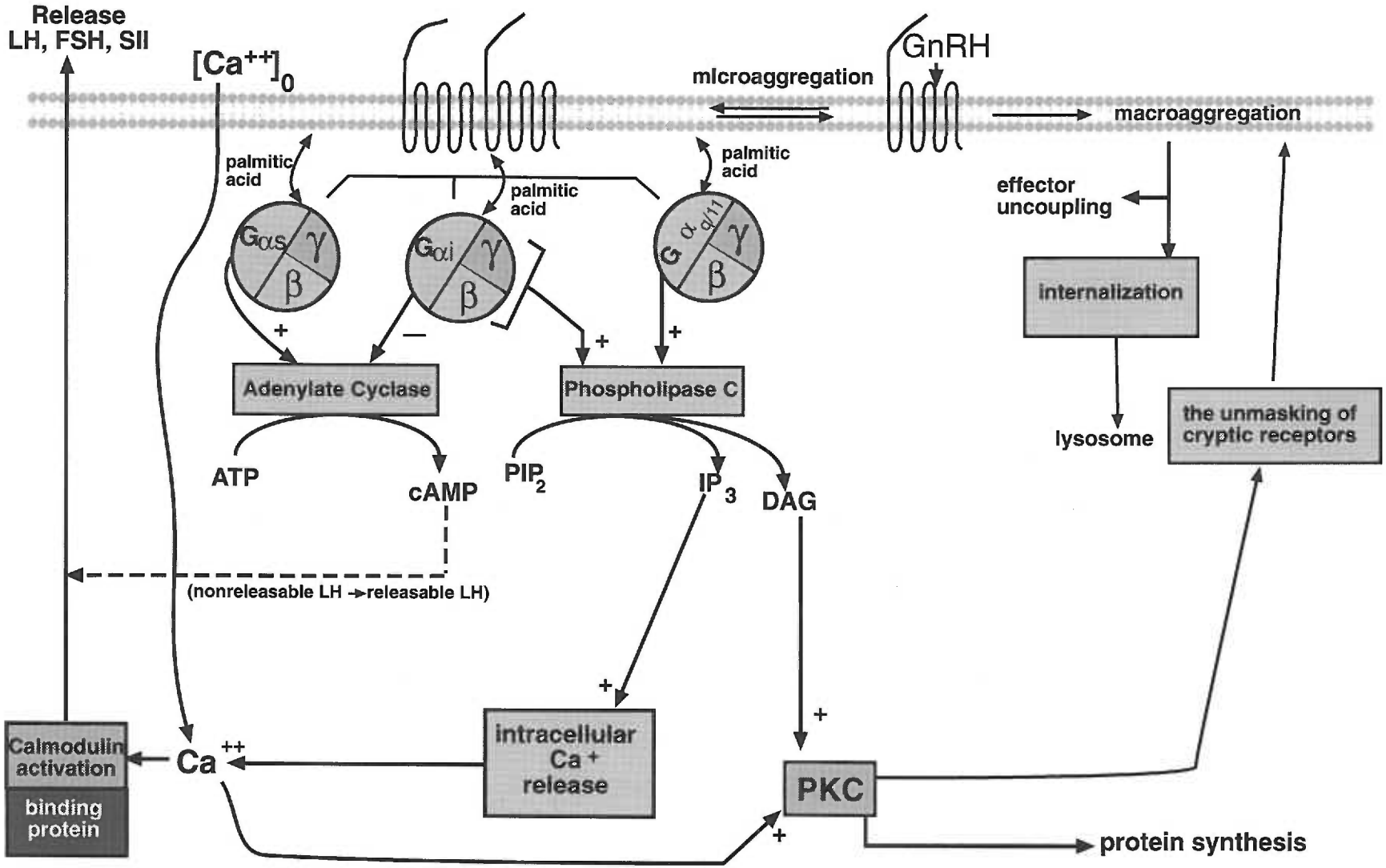
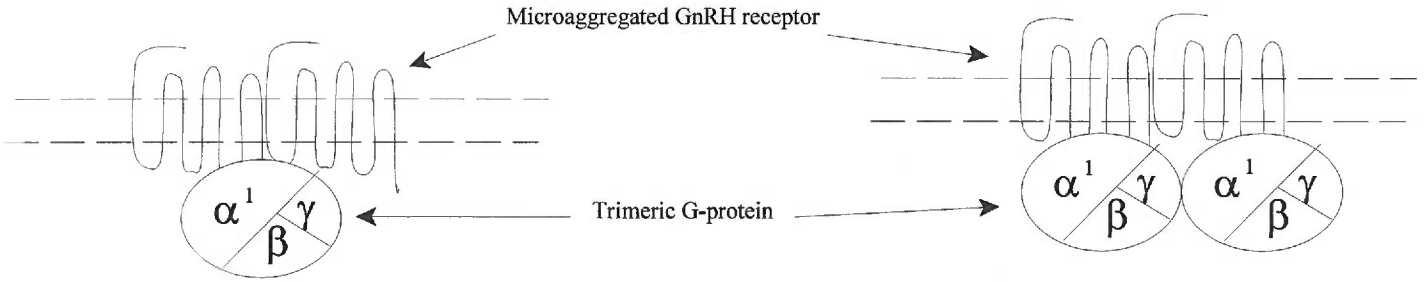

Release
LH, FSH, SII
[Ca++]0
microaggregation
GnRH
macroaggregation
palmitic acid
palmitic acid
palmitic acid
effector uncoupling
internalization
lysosome
the unmasking of cryptic receptors
Gαs
Gαi
Gαq/11
γ
β
+
—
+
+
Adenylate Cyclase
Phospholipase C
ATP
cAMP
PIP2
IP3
DAG
(nonreleasable LH →releasable LH)
+
+
Calmodulin activation
binding protein
Ca++
intracellular Ca+ release
PKC
protein synthesis

Figure 7-2. Models of possible permutations of multiple G-protein coupling to the microaggregated GnRH receptor. α^1 and α^2 indicate different G-proteins. β and γ may change depending on the type of α-subunit. As described in the text, a single trimeric G-protein or a group of trimeric G-proteins may couple to the receptor complex. These interactions may depend on the receptor specificity and the available G-proteins.

Model 1

Model 2



Model 3

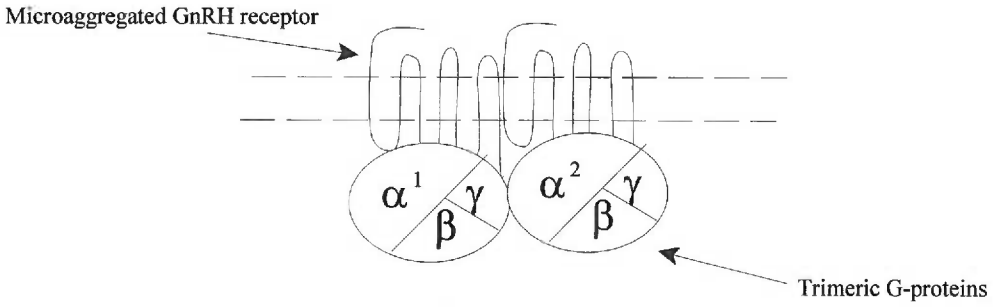
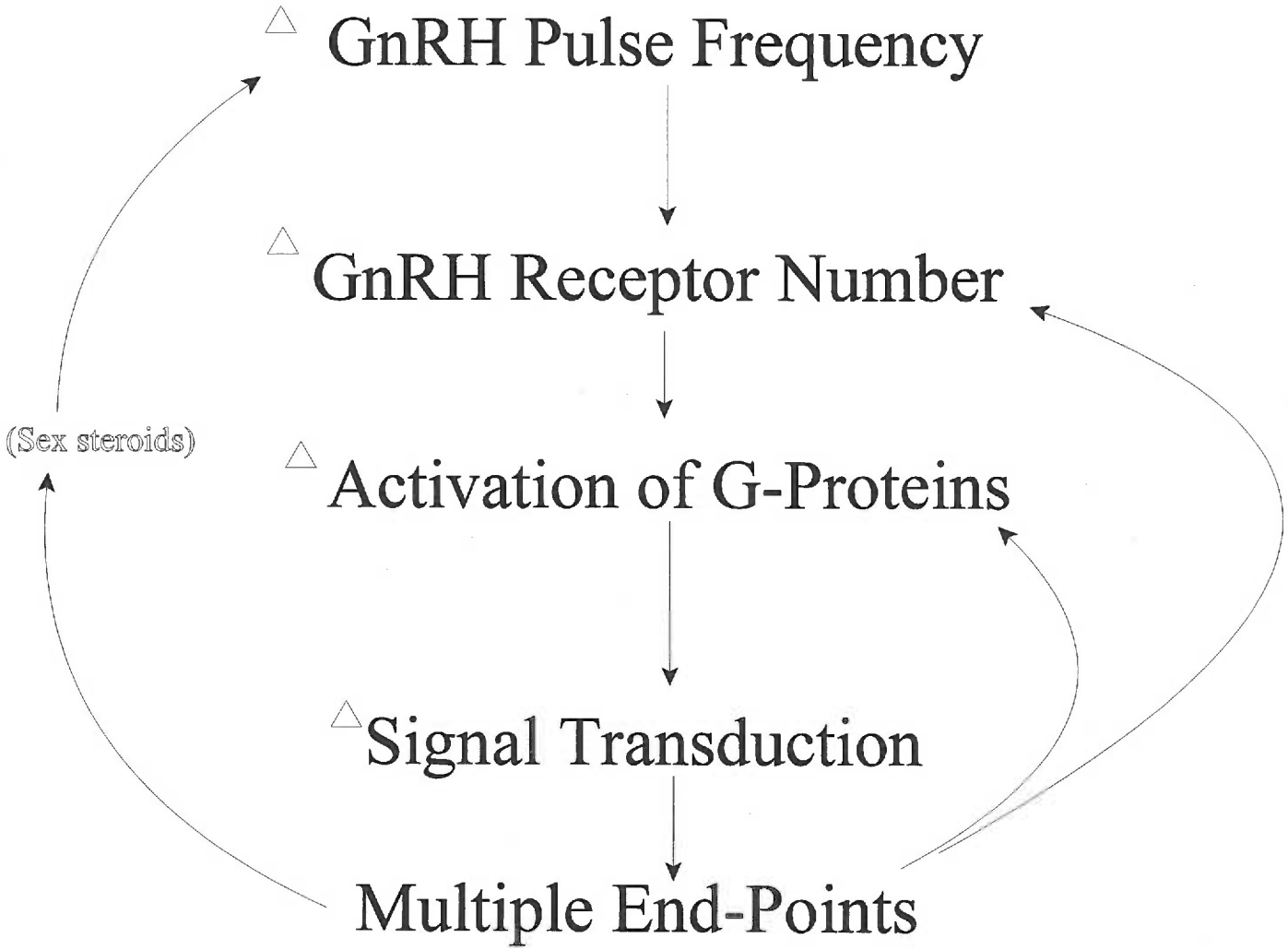


Figure 7-3. A schematic diagram of showing a model by which GnRH pulse frequency, GnRH receptor number, G-proteins, and signal transduction could coordinate responses to GnRH. The multiple end-points are the hormones such as LH and FSH, or GnRH receptor synthesis, or the modulation of the components of the signal transduction pathways. GnRH pulse frequency is affected by the gonadal steroids released in response to the gonadotropins.


△ GnRH Pulse Frequency
△ GnRH Receptor Number
△ Activation of G-Proteins
△ Signal Transduction
Multiple End-Points
(Sex steroids)

CONCLUSION

GH_3 cells were stably transfected with the cDNA for the GnRH receptor to obtain four clonally derived cell lines (GGH_3 cells). These cells respond to GnRH or GnRH agonist by synthesizing and releasing prolactin in a cyclic AMP dependent manner. GnRH stimulated prolactin release is synthesis dependent, one possible reason being the absence of secretory vesicles for the storage of hormone. Stimulation of the GnRH receptor also results in the dose- and time-dependent production of cyclic AMP and inositol phosphates. Although cyclic AMP is a second messenger for GnRH stimulated prolactin release, high intracellular concentrations of cyclic AMP inhibit GnRH agonist-stimulated hormone release.

This study also showed that GnRH stimulates dose- and time-dependent palmitoylation of $G_{q/11}$, G_i, and G_s proteins in rat pituitary cell cultures. Agonist bound receptors increase the palmitic acid turnover on G-proteins they activate. Therefore we used this phenomenon as a methodology to identify G-proteins that get activated in rat pituitary cell cultures. Results from the studies discussed here suggest that the GnRH receptor is able to couple to the G-proteins from $G_{q/11}$, G_i, and G_s family. Furthermore, overexpression of specific G-proteins in GGH_3 cells showed that $G_{q/11}$, G_i, and G_s proteins are able to couple to the GnRH receptor in a non-gonadotrope cell line. This indicates that the ability of the GnRH receptor to couple multiple G-proteins is not specific to the gonadotrope.

The role of G_{11} and G_q proteins in GnRH stimulated LH release was examined in mice that lacked these proteins. Studies done in these mice showed that these two proteins can substitute for each other in GnRH action.

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