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MULTIPLE G PROTEINS COMPETE FOR BINDING WITH THE HUMAN GONADOTROPIN RELEASING HORMONE RECEPTOR

Paul E. Knollman^{a,b} and P. Michael Conn^{a,b,c,*}

*a*Divisions of Neuroscience and Reproductive Biology, Oregon National Primate Research Center, Oregon Health and Science University

*b*Departments of Physiology and Pharmacology, Oregon Health and Science University

*c*Cell and Developmental Biology, Oregon Health and Science University

Abstract

The GnRH receptor is coupled to G proteins of the families G_q and G₁₁. G_q and G₁₁. Coupling leads to intracellular signaling through the phospholipase C pathway. GnRHR coupling to other G proteins is controversial. This study provides evidence that G protein families G_s, G_i, G_q and G₁₁ compete for binding with the GnRHR. We quantified interactions of over-expressed G proteins with GnRHR by a competitive binding approach, using measurements of second messengers, IP and cAMP. Transient co-transfection of HEK293 cells with human WT GnRHR and with stimulatory and inhibitory G proteins (G_q, G₁₁ and G_s, G_i) led to either production or inhibition of total inositol phosphate (IP) production, depending on the G protein that was over-expressed. Studies were conducted in different human (COS7, HeLa) and rodent-derived (CHO-K1, GH₃) cell lines in order to confirm that G-protein promiscuity observed with the GnRHR was not limited to a particular cell type.

Keywords

GnRHR; G Protein specificity; G protein coupled receptor; GPCR

INTRODUCTION

The heptahelical human gonadotropin-releasing hormone receptor (GnRHR) is a G protein-coupled receptor (GPCR) found in the plasma membrane of pituitary gonadotropes. The GnRHR is a key regulator in the hypothalamic-pituitary-gonadal axis and, consequently steroidogenesis, making it an attractive therapeutic target. The generation of second messengers by GnRH stimulation of its cognate receptor elicits multiple physiologically significant cellular responses (receptor up- and down regulation, sensitization and desensitization, synthesis and release of two gonadotropins and a secretogranin) [1]. The complexity of this regulation has made it attractive to consider involvement of multiple regulatory systems [2,3,4]. Further, it appears that this receptor transduces both frequency and

* Address correspondence and requests for reprints to: P. Michael Conn, ONPRC/OHSU, 505 NW 185th Avenue, Beaverton, Oregon, 97006, Ph. (503) 690 5297; Fax (503) 690 5569; Email: connm@ohsu.edu

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amplitude-modulated signals [5] which, in principle, could provide the basis for independent coupling to G proteins.

The GnRHR has been previously shown to be coupled to multiple G proteins during agonist activation, including G_q , G_{11} , G_s , G_i , G_{14} , G_{15} [3,4,6-8,25] although some laboratories have suggested that such regulation are all secondary to $G_{q/11}$ coupling [9]. Such a schema would limit the amount of information that the receptor might convey, of course. The pulsatile nature of GnRH and the complexity inherent in controlling multiple endpoints by way of signaling with a single hormone could conceptually support the notion of a promiscuous receptor. Receptor coupling to multiple G-proteins would provide regulation of different actions within the cell, and evidence for this is wanting. Indeed, debate has surrounded the G protein specificity of the GnRHR and promiscuity with families other than G_q/G_{11} . This study provides data which indicate that the receptor is able to bind, and presumably activate, G proteins of the families: $G_{q/11}$, $G_{s(long/short)}$, and $G_{i1/2/3}$.

In this study we employed pertussis and cholera toxin (PTX and CTX), PLC inhibitors (U73122), dibutyryl cyclic AMP (dBcAMP), forskolin, and the over-expression of G-proteins in both pre-mammalian and mammalian cell lines and measured second messenger responses to GnRHR agonist activation. Our study presents the possibility that promiscuity of the GnRHR is an important means of differentially regulating multiple physiological events by a single hormone interacting with a single receptor.

MATERIALS AND METHODS

Materials

Materials used in this study were obtained as indicated: DMEM, OPTI-MEM, lipofectamine, PBS, and pcDNA3.1 (Invitrogen, Carlsbad, CA); the GnRH analog, D-tert-butyl-Ser⁶-des-Gly¹⁰-Pro⁹-ethylamide-GnRH (Buserelin, Hoechst-Roussel Pharmaceuticals, Somerville, NJ); myo-[2-³H(N)]-inositol; sub-cloning reagents and competent cells (Promega Corp., Madison, WI); endofree maxi-prep kits (QIAGEN, Valencia, CA); cDNA for GnRHRs has been characterized in our laboratory [5] and other G protein cDNAs were obtained from Guthrie cDNA Resource Center (University of Missouri-Rolla, Rolla, MO); cholera and pertussis toxins were obtained from List Biological Laboratories (Campbell, CA). Other reagents were obtained from commercial sources and were of the highest degree of purity available.

Vector Construction

Human WT GnRHR and human WT G protein cDNAs were subcloned into pcDNA3.1+ at *KpnI* and *XbaI* restriction sites, as previously described [10].

Transient Transfection and Co-transfection

Cells were cultured and plated in either DMEM / 10% fetal calf serum / 20 µg/ml gentamicin (COS7, HEK293, GH3, HeLa) or F-12 / 10% fetal calf serum / 20µg/ml gentamicin (CHO-K1) growth media. Growth conditions were 37 C and 5% CO₂ in a humidified atmosphere; all medium was warmed to 37 C before being added to the cells, unless otherwise noted. For co-transfection of WT receptor and G proteins into HEK293 cells, 5×10^4 cells were plated in 0.25 ml growth medium in 48-well Costar cell culture plates. Twenty-four h after plating the cells were washed once with 0.5 ml OPTI-MEM and then transfected with 1 µl lipofectamine in 0.125 ml OPTIMEM (room temperature), according to manufacturer's instructions. For co-transfection experiments the cells were transfected with human WT hGnRHR (30 ng/well) and empty vector or human WT G proteins or G protein constructs (10-70 ng/well). The total amount of cDNA transfected remained constant, as complementary amounts of empty pcDNA3.1+ (empty vector), were included in the transfection mixture (to remain at 100ng

total). Five h after transfection, 0.125 ml DMEM with 20% fetal calf serum and 20 µg/ml gentamicin was added to the wells. Twenty-three h after transfection the medium was removed and replaced with 0.25 ml fresh growth medium.

Inositol Phosphate (IP) Assays

Cells were washed twice with 0.5 ml DMEM containing 0.1% BSA and 20 µg/ml gentamicin 27 h after transfection and then preloaded for 18 h with 0.25 ml of 4 µCi/ml myo-[2-³H(N)] inositol in DMEM (prepared without inositol). After preloading, cells were washed twice with 0.3 ml DMEM (without inositol) containing 5 mM LiCl (to prevent IP degradation), and then treated for 2 h with 0.25 ml of the indicated concentration of Buserelin (0 to 10⁻⁷M) in the same medium. The media was removed and the cells were frozen and thawed in the presence of 0.5 ml of 0.1 M formic acid (to rupture cells), and total IPs were determined as previously described [11].

CTX and PTX, PLC inhibitor U73122, dBcAMP, and Forskolin Studies

PTX and CTX lyophilized protein were dissolved in sterile sodium phosphate (10 mM) / PBS (pH 7.0) and sterile milli-Q water respectively. The PLC inhibitor (U73122 [12]) and Forskolin were dissolved in DMSO. The inhibitors (U73122, PTX) were added (in 25 µl to the media already on the cells) 17.5h after preloading (for 0.5 h) and again during stimulation (2 h in 0.25ml), at indicated concentrations. All other compounds (dBcAMP) were added in the stimulation media, with DMSO being additionally added to remain constant for all treatment media.

Quantification of cAMP release

Cells were washed twice with 0.5 ml DMEM containing 0.1% BSA and 20 µg/ml gentamicin 27 h after transfection, and incubated in the same medium for 18 h. Cells were subsequently stimulated with indicated Buserelin concentrations in 0.25 ml DBG containing 0.2 mM 3-isobutyl-1-methylxanthine (to inhibit phosphodiesterase activity) for 2 h at 37 C. After stimulation, the medium from each well was collected in tubes containing 25 µl of 10 mM 1,3-dimethylxanthine (theophylline, 1 mM final concentration). The samples were heated to 95 C for 5 min and RIA of cAMP was determined as previously described [11]. The data are presented as the mean ± SEM of triplicate transfections; each experiment was repeated three times with similar results. Added radioactivity from cAMP varied between experiments due to a complex function of preparation of the iodinated cAMP, its uptake, and minimal changes in the temperature, pH, and density of the cells, which makes it difficult to perform a statistical analysis between the individual experiments. The curve profiles from each experiment were similar, however.

Statistics

Data (n = 3) was analyzed with one-way ANOVA and then paired Student's t-test (SigmaStat 3.1, Jandel Scientific Software, Chicago, IL). P values ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

Results

Figure 1 shows dose response curves using the GnRHR agonist, Buserelin for the 5 cell lines used in the present study. EC₅₀s were similar for the cell lines, with values around 10⁻⁸M. The logistic shapes of the dose response curves varied as a function of IP counts per minute, and possibly indicate differential amounts of accessory proteins or altered ligand efficacy. These issues seem to be inherent in a particular cell type (lower in cells lines with low IP counts per minute) and support the strategy used in the present study of examining multiple cell types

from varying lineages. As shown in Figure 2, there is a clear increase in extra-cellular cAMP when $G_{s(long)}$ (or $G_{s(short)}$, data not shown) were co-transfected with the GnRHR in HEK293 cells, though this effect seems to be an agonist independent event. These effects also reach a maximum level at 3:1 G protein: receptor DNA.

Figure 3 shows Buserelin dose response curves for the 5 cell lines, indicating that elevated extra-cellular cAMP was not detectable by radioimmunoassay in a 2 h agonist stimulation time in cells expressing human WT GnRHR. Though we performed time course studies examining prolonged GnRHR stimulation, we included only 2 h data points since that is the optimal phosphoinositide (IP) release assay stimulation duration.

HEK293 cells are known to possess the WT G_s protein, and this cell line was used further in the current study to examine cAMP signaling [13]. Figure 4 shows the effects of various toxins, inhibitors, and stimulators on IP production after co-transfection of HEK293 cells with the human GnRHR and G proteins: $G_{s(short)}$, $G_{s(long)}$, G_{i1} , G_{i2} , or G_{i3} , G_q , and G_{11} . Interestingly, treatment of HEK293 cells with Forskolin (100 μ M), PTX (100 ng/ml), or dBcAMP (5mM), showed no alteration in total IPs produced, indicating that activation of PKA and cAMP seem to not have a direct effect on total IP production. However, when G_s proteins were co-transfected with the GnRHR, CTX treatment resulted in an increase in total IPs. The PLC inhibitor, U73122, blocked IP release in HEK293 cells transfected with families G_s and G_i , but maximally in WT and G_q or G_{11} cells. Control cells (data not shown), not exposed to Buserelin or other compounds examined in Figure 4, produced background-comparable IP counts (< 200 CPM).

Figure 5 shows transient co-transfection of HEK293 cells (along with 4 other cell lines) with human GnRHR and human G_q or G_{11} cDNA, resulting in an increase in total IPs (compared to hGnRHR and vector, $p < 0.05$) reaching a plateau at 3:1 (G protein cDNA:hGnRHR cDNA). Co-transfection with G-proteins of the families G_s and G_i show a reduction in total IPs, as increasing amounts of G protein are co-transfected ($p < 0.05$ at 7:3). COS7, HeLa, CHO-K1, and GH3 cells were additionally subjected to the same transfections, producing similar (though not as robust) G protein stimulation/inhibition curves found in the HEK293 data, likely due to the increased presence of multiple endogenous G-proteins.

Discussion

The present study utilizes a competitive binding approach to show that multiple G-proteins compete for binding with the hGnRHR. By transfecting a known amount of GnRHR, and increasing the amount of active G protein subunits, exogenous G protein subunits can compete for binding sites on activated GnRHRs and competition curves are generated (see figure 5). The approach was augmented by use of pertussis toxin (PTX) which promotes ADP ribosylation of the α -subunit of G_{α_i} , (uncoupling the receptor from the G protein and preventing inhibitory effects of adenylyl cyclase activity) and by cholera toxin (CTX, which inhibits GTPase activity, resulting in constitutive activation of $G_s\alpha$ -subunits, and thus adenylyl cyclase / PKA activity). We also utilized the specific PLC inhibitor U73122 which blocks phospholipase C catalytic activity and DBcAMP and forskolin were used to stimulate PKA activation.

The data suggest that the over-expression of established GnRHR coupled G proteins (G_q , G_{11}) results in an increase in PLC activity and thus in IP formation, an effect that can be blocked with a PLC inhibitor, U73122. This effect can be seen experimentally from the presence of "non-saturating" levels of endogenous G_q and G_{11} α -subunit protein, thus allowing transfection of additional amounts of protein to increase (or attenuate) Buserelin responses. Treatment of HEK293 cells with Forskolin (100 μ M), PTX (100 ng/ml), or dBcAMP (5 mM) indicates that in our 2 h stimulation period, PKC and PKA pathway crosstalk seem to have minimal impact on each other in terms of cAMP and IP release (see figure 4). The lack of an alteration in IP

formation by HEK293 cells in response to treatment with forskolin, dBcAMP, and PTX is consistent with the notion that direct competition for receptor binding is occurring between G proteins; this idea is additionally supported by the observation that over-expression of G proteins seems to have no effect or pathway crosstalk [14,15]. Interestingly, we also show that CTX treatment of cells co-transfected with hGnRHR and G_s results in an increase in IP production. Presumably, this effect is caused by a reduction in the number of trimeric G_s proteins available for GnRHR binding, and hence an increase in competition from endogenous G_q/G_{11} complexes. In previous work with pituitary cultures, increased LH release (through IP generation) in CTX pre-treated cells has been seen [30]. This data suggests that the GnRHR activates both the PLC and PKC messenger systems, however the cAMP amounts generated though G_s have presumably been too low to detect by radioimmunoassay for extracellular cAMP.

Interestingly, we noticed that $G_{s(short)}$, $G_{s(long)}$, G_{i1} , G_{i2} , or G_{i3} co-transfection with hGnRHR resulted in a decreased IP formation by HEK293 and COS7 cells. This suggests that $G_{s(short)}$, $G_{s(long)}$, and $G_{i1/2/3}$ compete for binding on the GnRHR by preventing endogenous G_q/G_{11} protein binding, and subsequent generation of the second messenger, as assessed by total IPs. Since receptors that bind but do not activate heterotrimeric G proteins would be selected against in nature, this data more likely supports the possibility that the hGnRHR possesses the ability to bind to the G_s and $G_{i1/2/3}$ regulatory classes of G proteins. In our hands, however, an accumulation of extra-cellular cAMP was not detected and could likely be attributed to a number of areas: receptor phosphorylation states from serine/threonine protein kinases, endogenous protein processing in the various cell lines, or affinity of the GnRHR to G proteins.

GnRHR signaling has remained elusive, however, numerous studies (in primary cultures, tumor cell lines, and immortalized cell lines) support the notion of coupling to G_s and G_i families of trimeric G proteins in addition to G_q/G_{11} and additionally confirm the presence of those regulatory G proteins in gonadotrope cells [31] thus making these findings physiologically relevant. Our data supports this body of evidence that the GnRHR *could* bind cAMP stimulatory (or inhibitory) G proteins depending on the microenvironment, prior exposure to ligand, and/or effector cross talk abilities of G protein coupled receptors (GPCRs) [16-22,25,30,31].

In L β T2 gonadotrope cells, GnRHR receptor stimulation was found to increase intracellular cAMP in a dose dependent fashion, and to increase a cAMP responsive reporter gene [23]. Also, in GH-secreting adenomas, GnRHR increased intracellular calcium levels, cAMP, and AC activity measured in membrane preparations [24]. However, chronic GnRH treatment of L β T2 cells led to receptor desensitization and down-regulation of PKC, cAMP, and Ca²⁺ dependent signaling. Additionally, the GnRHR has been shown to couple to G_{i1} in GI tract tumor cells [25,26] and G_i and G_s in lactotrope-derived cells [3].

The idea of the GnRHR signaling through multiple pathways has been controversial due to the use of multiple cells lines, stimulation durations and conditions, and pharmacological reagents used to elucidate signaling events. Moreover, the pulsatile nature of release of GnRH from the hypothalamus *in vivo* is hard to mimic *in vitro*. Oligomerization and interactions between receptor-G protein-effector networks might also determine specificity for G proteins, and depend on receptor regulation and plasma membrane expression [27-29].

Our results in multiple mammalian cell lines supports a view of the promiscuity of the GnRHR since G_s , G_i , and G_q/G_{11} appear to compete for binding with this GPCR; such an effect may assist this receptor in independent regulation of two gonadotropins (each two subunits), up- and down-regulation of the receptor itself and target cell sensitization.

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Figure 1.

Dose response curves with Buserelin in various cell lines: Cells were transfected with 30 ng of human WT GnRHR cDNA and stimulated for 2 h in various concentrations of Buserelin and total IP production measured. Each of the 5 panels represents a different cell line examined, as indicated by name and source at the top left of the graphs.

Figure 2.

Extra-cellular cAMP accumulation with exogenous Gs protein transfection: 10 ng of WT hGnRHR was co-transfected with the indicated amounts of WT G_{s(long)} cDNA into HEK 293 cells, which were stimulated for 1-5 h (top panel). Increasing amounts of G_{s(long)} cDNA was co-transfected with human WT GnRHR cDNA and stimulated for the indicated times (bottom panel).

Figure 3. Extra-cellular cAMP produced by the indicated cell lines: Cells were transfected with 30 ng of human WT GnRHR cDNA and stimulated for 2 h in various concentrations of Buserelin were assayed for cAMP by radioimmunoassay.

Figure 4.

PTX and CTX, PLC inhibitor, and cyclic AMP stimulatory effects on IP production: Cells were transfected with 30 ng of human WT GnRHR cDNA and 70 ng of the indicated G protein cDNA or empty vector to maintain total amount of transfected DNA constant. Cells were treated with the indicated compound (or control vehicle), and stimulated in a saturating dose (10^{-7} M) of Buserelin for 2 h and assayed for total IPs.

Figure 5.

Overexpression of G proteins and the effects on IP Production: Cells transfected with 30 ng of human WT GnRHR cDNA and 70 ng of the indicated G protein cDNA (total transfected DNA remained constant at 100 ng), then stimulated in a saturating dose (10^{-7} M) of Buserelin for 2h and assayed for total IP production. Asterisks in the figure legends indicate p values ≤ 0.05 , which were considered to be significant.