Lack of a C-terminal Tail in the Mammalian Gonadotropin-releasing Hormone Receptor Confers Resistance to Agonist-dependent Phosphorylation and Rapid Desensitization*

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The mammalian gonadotropin-releasing hormone receptor (GnRH-R) is, at present, the only G-protein-coupled receptor that activates phospholipase C and lacks a C-terminal tail. We have previously demonstrated that this unique structural feature is associated with resistance to rapid desensitization of phosphoinositide signaling in COS-7 and HEK-293 cells (Heding, A., Vrecl, M., Bogerd, J., McGregor, A., Sellar, R., Taylor, P. L., and Eidne, K. A. (1998) J. Biol. Chem. 273, 11472–11477). Using receptors tagged with a nonapeptide of the influenza hemagglutinin protein to enable immunoprecipitation, we now demonstrate that the mammalian GnRH-R is not phosphorylated in an agonist-dependent manner. In contrast, the mammalian thyrotropin-releasing hormone receptor and the African catfish GnRH-R, both of which have a C-terminal tail, are phosphorylated in response to agonist challenge. Furthermore, chimeras of the mammalian GnRH-R with the C-terminal tail of either the mammalian thyrotropin-releasing hormone receptor or the catfish GnRH-R are also phosphorylated in an agonist-dependent manner. Only those receptors having C-terminal tails showed desensitization of phosphoinositide responses within 5–10 min of agonist challenge. We also show that the internalization of all these receptors when expressed transiently in COS-7 cells is similar. This dissociates receptor internalization from rapid desensitization and demonstrates that the lack of a C-terminal tail in the mammalian GnRH-R results in an inability of the receptor to undergo agonist-dependent phosphorylation and that this results directly in a resistance to rapid desensitization.

Gonadotropin-releasing hormone (GnRH) is a decapptide released in a pulsatile manner from the hypothalamus, following which it acts upon pituitary gonadotropes to stimulate the exocytotic release of both luteinizing hormone and follicle-stimulating hormone. The receptor for GnRH (GnRH-R) is a member of the G-protein-coupled receptor (GPCR) family and the binding of GnRH activates phospholipase C (PLC) via G-proteins of the Gαq/11 family. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate both inositol 1,4,5-trisphosphate and diacylglycerol, and these second messengers are able to mobilize Ca2+ from intracellular stores and activate protein kinase C (PKC), respectively (1), thereby mediating, at least in part, the biological effects of GnRH.

At present the GnRH-R is unique among those GPCRs that couple preferentially to the activation of PLC, as it does not undergo rapid (seconds to minutes) desensitization following exposure to agonist. This is true of the GnRH-R expressed either as an endogenous protein in an immortalized mouse pituitary cell line (αT3–1) or as a recombinant protein in a number of cell types (2–5). Furthermore, we have recently demonstrated that, although GnRH-Rs of αT3–1 cells are not acutely regulated, muscarinic M3 receptors co-expressed as recombinant proteins in these cells are rapidly desensitized (6). Taken together, these data imply that structural features of the GnRH-R underlie its resistance to acute regulation.

The mechanism underlying acute regulation of GPCRs has been extensively investigated for the Gαq-coupled β2-adrenergic receptor, and it is accepted that receptor phosphorylation is responsible for receptor-G-protein uncoupling (7–12). Agonist-dependent phosphorylation has also been demonstrated for a number of PLC-coupled receptors, and this has been implicated in desensitization (13). Irrespective of the transduction pathway to which receptors are linked, these phosphorylation sites are often in the C-terminal tail or in some circumstances within the third intracellular loop (13). It is of interest, therefore, that mammalian GnRH-Rs have no C-terminal tail and only a relatively short third intracellular loop with relatively few serine/threonine residues as potential phosphorylation sites (14, 15).

We have recently reported that the lack of acute regulation of the GnRH-R is associated with the absence of a C-terminal tail (16) and the primary aim of the current study was to investigate whether the GnRH-R is resistant to agonist-dependent phosphorylation. In addition, we have explored the consequence of the lack of a C-terminal tail on both receptor phosphorylation and function by using the African catfish GnRH-R (cfGnRH-R) and the rat thyrotropin-releasing hormone receptor (rTRH-R) (both of which have C-terminal tails) and chimeras of the rGnRH-R having the C-terminal tail of either the cfGnRH-R or rTRH-R. Given the potential role of phosphorylation and/or the C-terminal tail in receptor internalization (17), we also compared the kinetics and extent of receptor internal-
ization with that of agonist-dependent phosphorylation and desensitization.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents of analytical grade were obtained from suppliers listed previously (6, 16, 18, 19) or from Sigma (Poole, United Kingdom). In addition, [32P]orthophosphate from Amersham Pharmacia Biotech (Little Chalfont, Bucks, UK), Ro-318220 was from Calbiochem (Nottingham, UK), and protein A-Sepharose from Amersham Pharmacia Biotech (St. Albans, UK). Molecular biology reagents were obtained from Amersham Pharmacia Biotech, Life Technologies (Paisley UK), or Qiagen Ltd. (Crawley, UK) unless otherwise stated.

**Derivation of Epitope-tagged Receptor Constructs**—To examine receptor phosphorylation, we incorporated an epitope tag sequence into that of the receptor to allow immunoprecipitation with a commercially available antibody. A double-stranded oligonucleotide fragment corresponding to a triple repeat of an epitope of the amino acid sequence of the influenza hemagglutinin (HA) protein (YPYDVPDYA) was synthesized and ligated in frame, into the N terminus of the rGnRH-R, rGnRH-R/TrH-R tail, and rGnRH-R/EgFrGnRH-R tail chimeric receptors in the vector pcDNA3. The eGnRH-R was constructed to contain a single HA epitope. The epitope-tagged rTrH-R cDNA, which contains a 3HA tag located after the translational initiation codon (ATG), was kindly provided by Dr. G. Milligan (University of Glasgow, Glasgow, UK). The inclusion of the epitope tag did not influence the ability of these receptors to bind agonist or to undergo agonist-dependent internalization (Ref. 19, data not shown).

**Cell Culture and Transfection**—COS-7 or HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, antibiotic (100 units/ml), and streptomycin (100 units/ml) at 37 °C in a humidified atmosphere of 5% fetal calf serum, glutamine (0.3 mg/ml), penicillin (100 units/ml), and 10% NaHCO3 containing 1% nonfat milk (pH 8.6) and then incubated with a 1:200 dilution of primary rat anti-HA monoclonal antibody (clone 3P10; Roche Molecular Biochemicals, Sussex, UK) in incubation buffer (PBS containing 0.1% bovine serum albumin, pH 7.4) overnight at 4 °C. Subsequently, cells were washed with PBS (three times) and incubated for 2 h at room temperature in a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rat IgG. After final washes in PBS (three times), the reaction was developed using the 3,3’,5,5’-tetramethylbenzidine (TMB) liquid substrate system. The enzymatic reaction was stopped after 30 min at room temperature with 0.5N H2SO4, and the absorbance at 450 nm was determined.

**Lack of GnRH Receptor Phosphorylation**

**ELISA**—Following transfection of COS-7 cells in 100-mm dishes, cells were trypsinized and plated into 48-well plates (5 × 104 cells/well) and the assays performed 48 h after transfection. ELISA for the measurement of surface expressed HA-tagged receptors was based on the method described previously (21). Briefly, cells were fixed with freshly prepared 4% paraformaldehyde for 10 min at room temperature. Following washing (three times in PBS), cells were blocked with 0.1% NaHCO3, containing 1% nonfat milk (pH 8.6) and then incubated with a 1:200 dilution of primary rat anti-HA monoclonal antibody (clone 3P10; Roche Molecular Biochemicals, Sussex, UK) in incubation buffer (PBS containing 0.1% bovine serum albumin, pH 7.4) overnight at 4 °C. Subsequently, cells were washed with PBS (three times) and incubated for 2 h at room temperature in a 1:100 dilution of horseradish peroxidase-conjugated goat anti-rat IgG. After final washes in PBS (three times), the reaction was developed using the 3,3’,5,5’-tetramethylbenzidine (TMB) liquid substrate system. The enzymatic reaction was stopped after 30 min at room temperature with 0.5N H2SO4, and the absorbance at 450 nm was determined.

**Lack of GnRH Receptor Phosphorylation**

**Immunoprecipitation and Western Blotting**—In order to examine the phosphorylation state of receptors, we had to ensure that the immunoprecipitations, as described above, were effective. To demonstrate this and to obtain an indication of the relative efficiencies of immunoprecipitation for the different receptors, we carried out immunoprecipitations followed by Western blots. Immunoprecipitations were performed exactly as described above, with the exception that cells were not labeled with [32P]orthophosphate, and the buffer contained 1.2 mCi KH2PO4. Following resolution by 8% SDS-PAGE, proteins were transferred to nitrocellulose. This was blocked overnight at 4 °C (20 mCi Tris, 500 mCi NaCl, 0.05% Tween 20, 5% dried milk) and then probed with 1 µg/ml mouse monoclonal antibody raised against the HA epitope tag (clone 12CA5; Roche Molecular Biochemicals, Sussex, UK). This antibody was visualized using a peroxidase-conjugated anti-mouse IgG and ECL reagents (Amersham Pharmacia Biotech). All blots shown are representative examples of at least three independent experiments. ELISA—Following transfection of COS-7 cells in 100-mm dishes, cells were trypsinized and plated into 48-well plates (5 × 104 cells/well) and the assays performed 48 h after transfection. ELISA for the measurement of surface expressed HA-tagged receptors was based on the method described previously (21). Briefly, cells were fixed with freshly prepared 4% paraformaldehyde for 10 min at room temperature. Following washing (three times in PBS), cells were blocked with 0.1% NaHCO3, containing 1% nonfat milk (pH 8.6) and then incubated with a 1:200 dilution of primary rat anti-HA monoclonal antibody (clone 3P10; Roche Molecular Biochemicals, Sussex, UK) in incubation buffer (PBS containing 0.1% bovine serum albumin, pH 7.4) overnight at 4 °C. Subsequently, cells were washed with PBS (three times) and incubated for 2 h at room temperature in a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rat IgG. After final washes in PBS (three times), the reaction was developed using the 3,3’,5,5’-tetramethylbenzidine (TMB) liquid substrate system. The enzymatic reaction was stopped after 30 min at room temperature with 0.5N H2SO4, and the absorbance at 450 nm was determined.
that cells were prelabeled with $^{32}$P orthophosphate. Following SDS-immunoprecipitation and resolved as described above with the exception or following short term (5–15 min) treatment with 1 mM agents. Prior to solubilization, cells were either challenged or not with a monoclonal antibody against the HA epitope. This antibody was visualized using a peroxidase-conjugated anti-mouse IgG and ECL reagents. Prior to solubilization, cells were either challenged or not with 1 mM GnRH for 5 min (+, −). B, cells were solubilized and receptors immunoprecipitated and resolved as described above with the exception that cells were prelabeled with $^{32}$P orthophosphate. Following SDS-PAGE, the gels were dried and exposed to photographic film for 3 days. Cells were either untreated (−), challenged for 5 or 15 min with 1 mM GnRH (+5, +15), or challenged with 1 μM PDBu for 5 min. Molecular size markers in kDa are indicated to the left.

FIG. 1. Immunoprecipitation (A) and absence of agonist-mediated phosphorylation (B) of GnRH-Rs stably expressed in HEK-293 cells. Cells were transfected with the cDNA encoding the HA-epitope-tagged rGnRH-R and stable cell lines selected as described under “Experimental Procedures.” A, parental (wild-type) cells or cells expressing the HA-epitope-tagged rGnRH-R were solubilized and receptors immunoprecipitated using a rabbit polyclonal IgG raised against the HA epitope. Following isolation using protein A-Sepharose, the immunoprecipitated proteins were resolved by 8% SDS-PAGE and transferred to nitrocellulose, which was then probed with a mouse monoclonal antibody against the HA epitope. This antibody was visualized using a peroxidase-conjugated anti-mouse IgG and ECL reagents. Prior to solubilization, cells were either challenged or not with 1 mM GnRH for 5 min (+, −). B, cells were solubilized and receptors immunoprecipitated and resolved as described above with the exception that cells were prelabeled with $^{32}$P orthophosphate. Following SDS-PAGE, the gels were dried and exposed to photographic film for 3 days. Cells were either untreated (−), challenged for 5 or 15 min with 1 mM GnRH (+5, +15), or challenged with 1 μM PDBu for 5 min. Molecular size markers in kDa are indicated to the left.

RESULTS

Lack of Agonist-dependent Phosphorylation of the rGnRH-R Stably Expressed in HEK-293 Cells—Using immunoprecipitation followed by Western blotting, we were able to demonstrate that the HA epitope-tagged rGnRH-R (HA-rGnRH-R), when expressed stably in HEK-293 cells, could be immunoprecipitated effectively by a rabbit polyclonal antibody raised against the epitope tag. Western blotting of the immunoprecipitate with a monoclonal antibody raised against the epitope tag revealed a band of 70 kDa present only in the transfected cells and not the parental cell line (Fig. 1A). There was some cross-reactivity between the peroxidase-conjugated anti-mouse IgG (used as the secondary antibody in the Western blot) and the IgG of the rabbit polyclonal antibody used for the immunoprecipitation. This appeared as a band of approximately 56 kDa irrespective of whether or not the cells were expressing the epitope-tagged receptor. Short term (5 min) treatment of cells with 1 μM GnRH did not affect our ability to detect the HA-rGnRH-R (Fig. 1A). In studies examining the potential phosphorylation of the HA-rGnRH-R, we were unable to detect any additional phosphorylated bands in transfected compared with non-transfected cells at 70 kDa or at any other point on the autoradiograph either under basal (non-stimulated) conditions or following short term (5–15 min) treatment with 1 μM GnRH or following the activation of PKC with 1 μM phorbol dibutyrate (PDBu) (Fig. 1B and data not shown).

Consequence of the Lack of a C-terminal Tail of the GnRH-R on Receptor Phosphorylation—We next examined whether the absence of agonist-dependent phosphorylation of the rGnRH-R was a consequence of the lack of a C-terminal tail. Initial experiments were performed using the cfGnRH-R, which, in contrast to the rGnRH-R, does possess a C-terminal tail. In COS-7 cells transiently transfected with either the HA-rGnRH-R or HA-cfGnRH-R, we were able to detect a number of specific bands by Western blotting following immunoprecipitation. Blots of the HA-cfGnRH-R revealed a major band of immunoreactivity at 83 kDa along with other bands at 44, 130, and 190 kDa. Blots of the HA-rGnRH-R revealed an immunoreactive band at 78 kDa. In addition, there was a faint band at 120 kDa and an intense band at 42 kDa (Fig. 2A). Although untransfected cells also have an immunoreactive band at approximately this molecular mass, the high intensity of the band in blots from HA-rGnRH-R-transfected cells and the presence of a similar band at the slightly lower molecular mass of 44 kDa in HA-cfGnRH-R-transfected cells leads us to believe that this is also a form of the HA-rGnRH-R. In phosphorylation studies on the HA-cfGnRH-R, we were able to detect basal
phosphorylation of the 83-kDa band. Furthermore, challenge of the cells for 5 min with 1 μM chicken II GnRH (the preferred agonist of the GnRH-R; Ref. 22) resulted in an increase in the level of phosphorylation of this band but no clear evidence of an agonist-dependent phosphorylation of any other bands (Fig. 2B). In addition there was a smaller increase in the level of phosphorylation of the 83-kDa band following challenge of the cells for 5 min with 1 μM PDBu, an activator of PKC (Fig. 2B).

In COS-7 cells transiently transfected with the HA-rGnRH-R, we were not, however, able to detect basal phosphorylation nor any increases in the phosphorylation of bands at any molecular size following challenge with either GnRH (1 μM, 5 min) or PDBu (1 μM, 5 min) (Fig. 2B).

Our Western blotting following immunoprecipitation demonstrated that we were able to immunoprecipitate far more of the 83-kDa band of the HA-cfGnRH-R than the 78-kDa band of the HA-rGnRH-R (see above). Given that both of these receptor constructs are recognized by the same HA epitope tag, it is likely that this difference in immunoprecipitation is a consequence of different levels of receptor expression. Indeed, although measuring only cell-surface expression, the ELISA data suggest that the HA-cfGnRH-R is expressed at much higher levels than the HA-rGnRH-R (see below). It was possible, therefore, that our inability to detect phosphorylation of the HA-rGnRH-R was a consequence of a relatively low level of expression and immunoprecipitation compared with that of the HA-cfGnRH-R. We therefore reduced the loading onto the gels of immunoprecipitated HA-cfGnRH-R by 90% to produce an 83-kDa band in the Western blot following immunoprecipitation that was equivalent in intensity to the 78-kDa band of the HA-rGnRH-R (Fig. 2C). Under these circumstances we were still able to see phosphorylation of the 83-kDa band of the HA-cfGnRH-R under basal conditions and an increase in the level of phosphorylation following challenge of the cells with either chicken II GnRH (1 μM, 5 min) or PDBu (1 μM, 5 min) (Fig. 2D).

Agonist-dependent phosphorylation of the 83-kDa band of the HA-cfGnRH-R occurred in a time-dependent manner and was maximal by approximately 1 min (Fig. 3). Preincubation of cells with an inhibitor of PKC (Ro-318220; 10 min, 10 μM) did not reduce the agonist-dependent (chicken II GnRH; 1 μM, 5 min) phosphorylation of this 83-kDa band. The efficacy of Ro-318220 was demonstrated by its ability to block phosphorylation mediated by challenge of the cells with PDBu (1 μM, 5 min) (Fig. 3).

Phosphorylation experiments were also conducted on the HA-rTRH-R and the chimeric HA-rGnRH-R/cfGnRH-R-tail and HA-rGnRH-R/rTRH-R-tail. Immunoprecipitation followed by Western blotting demonstrated bands for the HA-rTRH-R at 85 and 44 kDa with a fainter band at 110 kDa, for the HA-rGnRH-R/cfGnRH-R-tail at 83 and 44 kDa with a fainter band at 108 kDa, and for the HA-rGnRH-R/rTRH-R-tail at 83 and 44 kDa with bands also at 108 and 158 kDa (Fig. 4). Although bands were also present in untransfected cells in the region of the lower molecular mass (~44 kDa) bands of all the receptor types, the greater intensity in transfected cells leads us to believe that this immunoreactivity is indeed associated with the receptor.

Although we were able to visualize all of the HA-epitope-tagged receptors when transiently expressed in COS-7 cells in the molecular mass region of the 83-kDa phosphorylated band of the HA-cfGnRH-R (HA-rGnRH-R, 78 kDa; HA-rTRH-R, 85 kDa; HA-rGnRH-R/cfGnRH-R-tail, 83 kDa; and HA-rGnRH-R/rTRH-R-tail, 80 kDa), there were marked differences in the band intensities between receptor types. This implies marked differences in our ability to immunoprecipitate the various HA-tagged receptors, which again may be related to the levels of receptor expression (see below). Based on the intensity of the 78–85 kDa bands for each receptor, we could immunoprecipitate the HA-cfGnRH-R > HA-rTRH-R > HA-rGnRH-R > HA-rGnRH-R/cfGnRH-R tail > HA-rGnRH-R/rTRH-R tail. The relatively poor detection of the chimeric receptors by immunoprecipitation and Western blotting is emphasized in Fig. 4, in which the cfGnRH-R bands are overexposed (Fig. 4, ii, exposure 1; and iii, exposure 2) in order to allow visualization of the chimeric receptors.
transiently expressed in COS-7 cells. (rGnRH-R/cfGnRH tail, rGnRH-R/rTRH-R tail) and the rTRH-R as described under "Experimental Procedures." After 48 h, cells were the cDNA encoding the HA-epitope-tagged receptors or the vector alone for 5 min with either 1 mM TRH, 1 mM GnRH, or 1 mM PDBu. Shown for comparison from the same experiments is the lack of phosphorylation of the rGnRH-R following stimulation with either 1 mM GnRH or 1 mM PDBu. Molecular size markers in kDa are indicated to the left.

TRH (1 mM, 5 min), there was an increase in the level of phosphorylation associated with the 85-kDa band (Fig. 5, i). Furthermore, despite the very low level of immunoprecipitation of the chimeric receptors, we were able to demonstrate agonist-dependent (GnRH; 1 mM, 5 min) phosphorylation of each of them (Fig. 5, ii and iii). This enhanced phosphorylation was associated with bands on the autoradiograms that coincided with the molecular mass bands of 83–85 kDa detected by Western blotting following immunoprecipitation (see above). These phosphorylated bands were broad but enhanced following agonist stimulation, and our ability to detect agonist-dependent phosphorylation of these poorly expressed immunoprecipitated receptors further supports our argument that the rGnRH-R is not phosphorylated following exposure to agonist. With the possible exception of the rGnRH-R/rTRH-R tail chimera, PDBu had relatively little effect on the level of phosphorylation (Fig. 5), suggesting that agonist-dependent phosphorylation is unlikely to be PKC-mediated. This is in agreement with experiments on the cfGnRH-R (see above).

Cell-surface Receptor Expression Determined Using ELISA—ELISA assays were performed on intact cells to examine the membrane expression of receptors. Based on the absorbance at 450 nm, the extents of expression for the different constructs relative to the rGnRH-R were: cfGnRH-R, 8.55 ± 3.39; rGnRH-R/cfGnRH-R tail, 2.86 ± 0.88; rTRH-R, 6.12 ± 2.73; rGnRH-R/rTRH-R tail, 0.97 ± 0.19 (fold over rGnRH-R, mean ± S.E., n = 4 for all experiments) (see also Fig. 6).

Receptor Desensitization—Previous studies have demonstrated that the functional desensitization of PLC-activating GPCRs can be demonstrated experimentally by showing the lack of agonist stimulation to maintain the initial rate of \[^{[H]}\text{Ina}_{3P}\] accumulation against a Li⁺ block of inositol mono-phosphatase (which provides an index of total PLC activity) (6, 23). In the current study we show that challenge of COS-7 cells transiently expressing the rGnRH-R with 1 mM GnRH did, however, result in an accumulation of \[^{[H]}\text{Ina}_{3P}\], that was approximately linear for at least 45 min (Fig. 7). Thus, the initial rate of phosphoinositide hydrolysis was sustained throughout the experimental period, indicating a lack of functional desensitization. In contrast, the accumulation of \[^{[H]}\text{Ina}_{3P}\] in response to challenge of all other receptors (rTRH-R, cfGnRH-R, rGnRH-R/rTRH-R tail, and rGnRH-R/cfGnRH-R tail) demonstrated marked reductions after 5–10 min of receptor stimulation (Fig. 7), indicating desensitization of the phosphoinositide responses. Interestingly, the rate of accumulation of \[^{[H]}\text{Ins}_{3P}\] following agonist activation of the rGnRH-R appeared less than that of the initial rates of the other receptors. Given the likely differences in the levels of plasma membrane expression of the various receptor constructs (see elsewhere), we are unable to comment directly on the relative coupling efficiencies of the different receptor constructs. However, the resistance to rapid desensitization of mammalian GnRH receptors is a receptor-specific phenomenon (2–6) and is not accounted for by the relatively low rate of \[^{[H]}\text{Ins}_{3P}\] accumulation following agonist challenge of the rGnRH-R observed in the current studies. For example, our recent work with the endogenously expressed murine GnRH-R of αT3–1 cells demonstrated that this receptor (which is expressed at relatively high levels (>1 pmol/mg of protein) in this cell line) is resistant to rapid desensitization, while a recombinant human muscarinic M₃ receptor expressed in the same cells at lower levels (~300 fmol/mg of protein), and giving smaller responses, does desensitize (6).

Receptor Internalization—Agonist-induced internalization for the different receptor constructs was determined in COS-7 cells. The rGnRH-R, cfGnRH-R, and rGnRH-R/cfGnRH-R tail were internalized by approximately 20% over a 60-min period and showed comparable levels of internalization throughout this experimental period (Fig. 8). In contrast, the rTRH-R and rGnRH-R/rTRH-R tail were internalized by approximately 40% over this period.

**DISCUSSION**

This study demonstrates that the mammalian GnRH-R does not undergo agonist-dependent phosphorylation and that this correlates with the inability of the receptor to undergo rapid desensitization. We demonstrate that these features are associated with the lack of a cytoplasmic C-terminal tail by the use of chimeras of the rGnRH-R with the C-terminal tail of either the cfGnRH-R or the rTRH-R. These chimeric receptors desensitize within minutes of agonist addition and undergo agonist-
dependent phosphorylation. These data are consistent with our observations that the receptors from which the tails were derived are also subject to rapid desensitization and undergo agonist-dependent phosphorylation. Furthermore, the comparatively slow internalization kinetics of the receptor constructs in COS-7 cells and particularly the similarity between the rGnRH-R and the other receptors over the first 5–10 min suggests that differences in internalization are not the cause of the differences in the desensitization profiles. While studies involving the mutation of PLC-coupled receptors with a C-terminal tail have suggested a role for this region in promoting desensitization through phosphorylation (24–27), we now demonstrate this association by the use of a naturally occurring "tail-less" GnRH-R and chimeras of this receptor constructed to contain a tail.

Of the receptors that were demonstrated to undergo agonist-dependent phosphorylation, this was associated with a band coincident with that detected by Western blotting following immunoprecipitation of apparent molecular mass of 83–85 kDa. For all of the receptor constructs, Western blotting following immunoprecipitation also revealed bands with molecular masses both greater and smaller than this and indeed the relative intensities of the bands (particularly those at 40–48 and 83–85 kDa) differed between receptors (e.g. HA-rGnRH-R and HA-cfGnRH-R). However, the current data indicate that, for each receptor, at least the band of 83–85 kDa must be inserted into the plasma membrane to enable agonist binding and subsequent phosphorylation. Although these bands do not reflect the predicted molecular size of the receptors from sequence data (40 kDa), GPCRs invariably have a higher molecular mass due to post-translational modification. For example, the angiotensin AT1A receptor has a predicted molecular mass of 42 kDa but migrates in an SDS-PAGE gel with an apparent molecular mass of 81 kDa predominantly due to glycosylation (28). In the current study, it is likely, therefore, that the bands of lower molecular mass (40–48 kDa) represent unmodified forms of the receptor. It is possible that the bands observed at 83–85 kDa and higher could reflect either receptor dimers/multimers or protein-protein interactions that occur.

Fig. 7. Receptor-mediated total PLC activity. Cells were transfected with the cDNA encoding the different receptor constructs and transferred to 24-well plates after 24 h. Cells were then labeled with [3H]inositol for 48 h and challenged with agonist in the presence of a Li+ block of inositol monophosphatase activity. Reactions were terminated after the indicated times and the inositol phosphates extracted and determined as described under *Experimental Procedures.* [3H]InsP3 are expressed as a percentage of the total cell incorporated radioactivity. Receptor constructs and agonists were: A, rGnRH-R, 1 μM GnRH; B, cfGnRH-R, 1 μM chicken II GnRH; C, rGnRH-R/cfGnRH-R tail, 1 μM GnRH; D, rTRH-R, 1 μM TRH; E, rGnRH-R/rTRH-R tail, 1 μM GnRH. Data shown are mean ± S.E. of triplicate observations from a single experiment representative of at least three. As a percentage of the rate of [3H]InsP3 accumulation over the first 5 min of agonist stimulation, the rates over the period between 30 and 45 min were: rGnRH-R, 104.3%; cfGnRH-R, 1.7%; rGnRH-R/cfGnRH-R tail, 20.7%; rTRH-R, 11.3%; rGnRH-R/rTRH-R tail, 7.4%.

Fig. 8. Agonist-mediated receptor internalization. Cells were transfected with the cDNA encoding the different receptor constructs as described under *Experimental Procedures* and transferred to 24-well plates after 24 h. An additional 48 h, cells were incubated with 125I-labeled GnRH agonist (rGnRH-R (□), rGnRH-R/cfGnRH-R tail (●), rGnRH-R/rTRH-R tail (▲), 125I-labeled chicken II GnRH (cfGnRH-R (○)), or 125I/TRH-(3-Me-His2) (rTRH-R (△)) for the indicated time at 37 °C. The extracellular ligand was removed by acid wash and the radioactivity determined. Internalized radioactivity was determined after solubilizing the cells. Nonspecific binding for each time point was determined in the presence of 1 μM unlabeled agonist. After subtraction of nonspecific binding, the internalized radioactivity was expressed as a percentage of the total binding at that time interval. All time points were performed in triplicate in at least three separate experiments.

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naturally or are formed during the preparative process (despite strongly reducing conditions). We are confident that these bands do reflect immunoreactivity associated with receptors, as there were differences in the mobilities of these bands between receptors of the magnitudes expected. The formation of such complexes is not, however, agonist-dependent, as short term (5 min) agonist treatment of the HA-rGnRH-R or the HA-cf-GnRH-R did not influence the relative intensities of the different bands (data not shown).

The current study demonstrates that the rGnRH-R does not undergo agonist-dependent phosphorylation whether expressed stably in HEK-293 cells or transiently in COS-7 cells. This is in agreement with a previous study, which suggested that this receptor transiently expressed in COS-1 or SF9 cells was not phosphorylated (29). However, in the current study, we are now able to demonstrate that, under the assay conditions used and with approximately equivalent (or lower) levels of immunoprecipitation, we are able to detect phosphorylation of a number of receptors containing the same antigenic sequence as the rGnRH-R. Furthermore, we demonstrate that this absence of agonist-dependent phosphorylation is associated with the lack of a C-terminal tail, as we were able to recover phosphorylation by the addition of the C-terminal tail of either the cfGnRH-R or rTRH-R to the rGnRH-R.

It is widely acknowledged that phosphorylation of the β<sub>2</sub>-adrenergic receptor at sites within the C-terminal tail is able to uncouple the receptor from G<sub>α<sub>q</sub></sub>, through which it is able to activate adenylyl cyclase (7–12). The literature now also contains many reports of agonist-dependent phosphorylation of phospholipase C-coupled receptors, and there is accumulating evidence that this is linked to receptor desensitization (see Ref. 13). Furthermore, a number of studies (e.g., Ref. 26), including a recent one examining the rTRH-R (30) have demonstrated a role for the C-terminal tail in rapid desensitization. The mammalian GnRH-R is at present unique among those receptors coupled to phospholipase C not undergoing rapid desensitization and in not having a C-terminal tail. Within the present study, we demonstrate that these two features are most likely associated through the lack of appropriate regulatory phospho-acceptor sites. This is in stark contrast to a previous report suggesting that, despite a lack of agonist-dependent phosphorylation, the rGnRH receptor is acutely regulated (29). The reason for such discrepancy is unclear, but the current data are in accord with several studies demonstrating the inability of the mammalian GnRH receptor to undergo rapid desensitization when expressed as an endogenous or recombinant protein in a number of cell types (2–6). Furthermore, we have previously reported evidence that this lack of rapid desensitization is attributable to a lack of the C-terminal tail (16). That the phosphorylation of the C-terminal tail underlies desensitization is supported in the current study by the observation that phosphorylation of the cfGnRH-R is temporally compatible with that of functional desensitization. Such phosphorylation of the cfGnRH-R is not mediated by PKC, given that agonist-dependent phosphorylation is insensitive to inhibition of PKC. Furthermore, this receptor is a poor substrate for PKC activated by phorbol ester, suggesting that other kinases such as members of the G-protein-coupled receptor kinase family (31) or more novel receptor kinases such as casein kinase 1α (32) may be responsible. The C-terminal tail of several GPCRs has also been implicated in the regulation of internalization (17). However, the association between the internalization of PLC-coupled receptors and their desensitization is equivocal and may show some receptor (and possibly cellular) specificity. Thus, although the two events have been linked with, for example, the substance P receptor (33) and somatostatin<sub>25</sub> receptor (34), others have demonstrated a dissociation using, for example, the P2Y2 receptor (35) and muscarinic M<sub>3</sub> receptor (36–38). Furthermore, receptor mutations or experimental manipulations designed to reduce receptor internalization have failed to affect the rate or extent of rapid desensitization of the neurokinin<sub>1</sub> receptor (39) or the TRH-R (30). Indeed, in the current study we have demonstrated an agonist-dependent phosphorylation of the rTRH-R, suggesting that phosphorylation of this receptor type might also underlie its rapid desensitization. Furthermore, we show that in COS-7 cells, although the rTRH-R and chimeric rGnRH-R/rTRH-R tail show somewhat enhanced internalization compared with the other receptor constructs, the slow and limited internalization of all receptor constructs indicates a dissociation between desensitization and internalization. This is also supported by the observation that addition of the C-terminal tail of the cfGnRH-R to the rGnRH-R enhanced its desensitization but not its internalization. Thus, rapid desensitization of the rTRH-R, cfGnRH-R, and the chimeric rGnRH-R containing the C-terminal tail of either of these two receptors is strongly associated with receptor phosphorylation.

In a recent study we have demonstrated that internalization of the rGnRH-R is clathrin-dependent but β-arrestin-independent (19), whereas internalization of the rTRH-R is both clathrin-dependent (40) and β-arrestin-dependent (19). Those data would fit with the lack of phosphorylation of the rGnRH-R and the inability, therefore, of agonist-binding to increase receptor affinity for β-arrestin. The similarity of the rate and extent of internalization among the receptor constructs in the current study is most likely a result of the low level of β-arrestin expression in COS-7 cells (41). Indeed, overexpression of β-arrestin is able to enhance the internalization of the rTRH-R but not the rGnRH-R in these cells (19). Furthermore, we have demonstrated (16) that the C-terminal tail in the cfGnRH-R, rTRH-R, and rGnRH-R/rTRH-R tail is associated with markedly enhanced internalization kinetics compared with the rGnRH-R when expressed in HEK-293 cells, which express higher levels of β-arrestin than COS-7 cells. Such differences in the expression of β-arrestin, or indeed other regulatory components, may also explain the markedly faster rates of receptor desensitization in HEK-293 cells compared with COS-7 cells. Thus, although the current data are entirely consistent with the patterns of desensitization of the rGnRH-R, rTRH-R, cfGnRH-R, and rGnRH-R/rTRH-R tail expressed in HEK-293 cells, where desensitization occurred it did so within 1–2 min of agonist addition (16).

The absence of rapid desensitization of the GnRH receptor along with slow internalization kinetics may serve to maintain receptors at the cell surface in a fully agonist-sensitive state. This may not only allow the mammalian GnRH-R to maintain signaling during sustained agonist exposure but may also prevent functional desensitization in circumstances where the receptors are repetitively challenged with agonist. These features may serve to maintain cellular sensitivity and responsiveness during events such as the GnRH surge that precedes the pre-ovulatory luteinizing hormone surge (42) and allow the frequency encoded pattern of the hypothalamic pulsatile GnRH release (43) to be faithfully maintained at the level of the pituitary gonadotropes.

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