β-Arrestin-dependent Desensitization of Luteinizing Hormone/Choriogonadotropin Receptor Is Prevented by a Synthetic Peptide Corresponding to the Third Intracellular Loop of the Receptor*

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Sutapa Mukherjee‡, Krzysztof Palczewski§, Vsevolod V. Gurevich¶, and Mary Hunzicker-Dunn‡

From the §Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, Illinois 60611, the ¶Department of Ophthalmology, Pharmacology, and Chemistry, University of Washington School of Medicine, Seattle, Washington 98195-6485, and the Ralph and Muriel Roberts Laboratory for Vision Research, Sun Health Research Institute, Sun City, Arizona 85372

Desensitization is a ubiquitous response of guanine nucleotide-binding protein-coupled receptors (GPCRs) characterized by the waning of effector activity despite continued presence of agonist. Binding of an arrestin to the activated, often phosphorylated GPCR triggers desensitization. We reported for the luteinizing hormone/choriogonadotropin receptor (LH/CG R) that β-arrestin tightly bound to porcine ovarian follicular membranes mediates agonist-dependent desensitization of LH/CG R-stimulated adenyl cyclase (AC) activity (Mukherjee, S., Palczewski, K., Gurevich, V. V., Benovic, J. L., Banga, J. P., and Hunzicker-Dunn, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 493–498). We now show that addition of a synthetic peptide corresponding to the entire third intracellular loop (3i) of the LH/CG R completely and specifically reverses desensitization of AC activity, with an ED50 of 10 μM but does not modulate basal, hCG-stimulated, or forskolin-stimulated AC activities. β-Arrestin binds selectively to the 3i peptide coupled to activated Sepharose. Desensitization of LH/CG R-stimulated AC activity is rescued when the 3i peptide is preincubated with exogenous β-arrestin. These results show that endogenous β-arrestin participates in cell-free desensitization of agonist-dependent LH/CG R-stimulated AC activity in follicular membranes by interacting directly with the 3i loop of the receptor, thereby preventing Gs activation.

The luteinizing hormone/choriogonadotropin receptor (LH/CG R) is a guanine nucleotide-binding protein-coupled receptor (GPCR) localized on the cell surface predominately of ovarian theca, granulosa and luteal cells and testicular Leydig cells (1, 2). Agonist activation of the LH/CG R leads to activation of adenyl cyclase (AC) via Gs (3) and, in some cells, to activation of phospholipase C (4–6). Like the responsiveness of most GPCRs (7), agonist-stimulated AC activity wanes in response to persistent stimulation of LH/CG R with saturating agonist concentrations (8–10). Desensitization of LH/CG R-stimulated AC activity occurs physiologically in preovulatory ovarian follicles in response to the mid-cycle surge of luteinizing hormone, which induces ovulation and corpus luteum formation (8, 11–13). The initial 60 min of follicular desensitization in which receptor-stimulated AC activity is reduced ~50% (14–17) without a concomitant reduction in LH/CG R numbers (18–22) can be mimicked in cell-free follicular membrane preparations (23–30). Desensitization of LH/CG R-stimulated AC activity in follicular membranes requires GTP (Km = ~70 mM) (23, 25–27) and is reversed by the GDP analog GDPβS (29) that prevents G protein activation. A hyperdesensitized state characterized by ~80% desensitization can be achieved by preincubating follicular membranes in the presence of 8% ethanol (31). This hyperdesensitized state of the LH/CG R retains dependence on GTP and agonist for development as well as its ability to be reversed by GDPβS, and like LH/CG R desensitization in the absence of ethanol is unaffected by addition of exogenous Gβγ or by sequestration of endogenous Gβγ (32, 33).

LH/CG R desensitization in follicular membranes is, however, dependent upon an endogenous arrestin, likely β-arrestin (34). Disruption of β-arrestin binding to the LH/CG R with monoclonal arrestin antibodies that recognize common epitopes on all arrestins specifically blocks agonist-dependent LH/CG R desensitization, and this effect is reversed by a synthetic peptide corresponding to the antibody-binding site on β-arrestin (34). Moreover, addition of purified recombinant β-arrestin to follicular membranes mimics desensitization by reducing agonist-dependent AC activity to nearly basal levels, with an ED50 value of 0.1 mM (34). The sites on the ligand-activated, phosphorylated GPCRs with which arrestins interact have been investigated using purified light-activated phosphorylated rhodopsin and synthetic peptides to cytoplasmic domains of rhodopsin (35). Results showed that arrestin binding with highest affinity to the third intracellular (3i) loop as well as to portions of the second intracellular (2i) loop and the C-terminal tail of rhodopsin. Consistent with this result, arrestins were shown to bind with high affinity to recombinant 3i subdomains of the m2- and m3-muscarinic and α2A/D-adrenergic receptors fused to glutathione S-transferase (36). Based on our

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† To whom correspondence should be addressed: Dept. of Cell and Molecular Biology, Northwestern University Medical School, 303 East Chicago Ave., Chicago, IL 60611. Tel.: 312-503-8940; Fax: 312-503-0566; E-mail: mhd@nwu.edu.

‡ The abbreviations used are: LH/CG R, luteinizing hormone/choriogonadotropin receptor; GPCR, guanine nucleotide-binding protein-coupled receptor; AC, adenyl cyclase; AMP-PNP, adenylyl-imidodiphosphate; BSA, bovine serum albumin; TM, transmembrane domain; GDPβS, guanosine 5′-O-(2-thiodiphosphate).

§ R. and M. Roberts Laboratory for Vision Research, Sun Health Research Institute, Sun City, Arizona 85372

¶ Ralph and Muriel Roberts Laboratory for Vision Research, Sun Health Research Institute, Sun City, Arizona 85372

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evidence that endogenous \( \beta \)-arrestin participates in desensitization of the LH/CG R, in this report we sought to determine whether the putative 3i loop of the LH/CG R could compete with LH/CG R for endogenous \( \beta \)-arrestin and reverse agonist-dependent LH/CG R desensitization.

**EXPERIMENTAL PROCEDURES**

*Materials—* Visual arrestin was purified from bovine retinas (37); recombinant \( \beta \)-arrestin was expressed and purified (38). \( \beta \)-Arrestin peptide (VFEDFARQRLKG) and indicated LH/CG R peptides (see Fig. 1) were synthesized and purified to 95% purity by high pressure liquid chromatography by the Protein Chemistry Core Facility at Baylor College of Medicine (Houston, TX), dissolved in water, and neutralized. CNBr-activated Sepharose 4B was purchased from Amersham Pharmacia Biotech; remaining sources are as described previously (34).

**Desensitization and AC Assay—** A partially purified membrane fraction enriched in AC activity was isolated from porcine ovarian follicles (34) and stored at \(-70^\circ C\). Protein concentrations were determined (39) using BSA as standard. For the two-stage desensitization reaction, reagents (in 20 \( \mu l \)) for stage 1 desensitization reaction (BSA or hCG at 10 \( \mu g/ml \) in 8% ethanol, 25 \( \mu M \) 3 bi-tris(hydroxymethyl)-methylamino)propane, pH 7.2, 10 \( \mu M \) EDTA, 1 \( \mu M \) EGTA, 0.2 mg/ml creatine phosphokinase, 20 mM phosphocreatine, 5 mM MgCl\(_2\), 1 mM ATP (or if indicated AMP-PNP), and 1 \( \mu M \) [\(^3\)H]cAMP (\(-20,000\) cpm) added to follicular membranes (\(-30\) \( \mu l \) in 20 \( \mu l \), preincubated with water or indicated synthetic peptide), and incubated for 40 min at 30 \( ^\circ C \). An assay for AC activity (stage 2) was immediately performed at 30 \( ^\circ C \) for 5 min with addition of a 10-\( \mu l \) volume containing 100 \( \mu M \) GTP, [\( \alpha ^{32}\)P]ATP (\(-5 \mu Ci, 100-200\) cpm/\( \mu l \)), and 10 \( \mu M \) hCG or BSA. When only the AC assay was conducted (and stage 1 was omitted), complete reaction mix was added to membranes (in a 50-\( \mu l \) volume, such that final incubation volume was 50 \( \mu l \)). Reaction was stopped (23), and [\(^3\)P]cAMP was purified and quantified (24, 40). Final concentrations of reagents in a 50-\( \mu l \) reaction volume are indicated throughout.

**Binding of \( \beta \)-Arrestin to LH/CG R Peptides—** 50 mg of CNBr-activated Sepharose 4B was swollen in 1 mM HCl, coupled overnight (4 \( ^\circ C \)) to 0.1 mg/ml indicated synthetic LH/CG peptide in 0.1 \( M \) NaHCO\(_3\), (pH 8.3) containing 0.5 \( M \) NaCl, then washed to remove unbound peptide, incubated 2 \( h \) in 0.1 \( M \) Tris-HCl (pH 8.0), washed with 0.1 \( M \) acetate buffer (pH 4.0) containing 0.5 \( M \) NaCl, washed with 0.1 \( M \) Tris-HCl (pH 8.0) containing 0.5 \( M \) NaCl, equilibrated in 50 mM Tris-HCl (pH 7.4) containing 100 \( mM \) NaCl (buffer A), and incubated with 0.2 ml of 0.2 \( mg/ml \) recombinant \( \beta \)-arrestin 1 \( h \) at room temperature. Sepharose was then washed with buffer A; proteins were eluted with 0.25 \( M \) NaOH sample buffer, separated by SDS-polyacrylamide gel electrophoresis, and transferred to Hybond for Western blot analysis using arrestin antibody (Transduction Laboratory) (34).

**Statistics—** Results (means \pm S.E.) were analyzed using Student’s \( t \) test (\( p < 0.05 \)) (41).

**RESULTS**

Experiments were conducted to determine whether synthetic peptides (Fig. 1) corresponding to the entire 2i and 3i cytoplasmic loops or the N-terminal portion of the cytoplasmic tail (4i) of the porcine LH/CG R (1) could competitively disrupt agonist-dependent desensitization of AC activity in porcine ovarian follicular membranes. To this end, membranes were first preincubated (30 min at 4 \( ^\circ C \)) with/without synthetic LH/CG R peptides and then subjected to the stage 1 incubation (40 min at 30 \( ^\circ C \)) under conditions which do promote (plus hCG) or do not promote (minus hCG) desensitization, followed by a 5-min AC assay. Agonist-dependent hyperdesensitization of LH/CG R-stimulated AC activity was readily demonstrated in membranes incubated with hCG, GTP, and 8% ethanol during the stage 1 desensitization incubation (Fig. 2A, compare solid bars). Preincubation of follicular membranes with a synthetic peptide corresponding to the LH/CG R 3i loop resulted in a concentration-dependent rise in hCG-stimulated AC activity when hCG was present in stage 1 of the desensitization reaction (Fig. 2A, compare hatched bars) and a corresponding reduction in the percentage of desensitization of hCG-stimulated AC activity (Fig. 2B). The ED\(_{50}\) for LH/CG R 3i peptide to increase hCG-stimulated AC activity was 10 \( \mu M \). Concentrations of LH/CG R 3i greater than 20 \( \mu M \) were less effective and, at 114 \( \mu M \), no longer reversed desensitization (Fig. 2C). In contrast to its effect on desensitization of agonist-dependent AC activity, when the desensitization (stage 1) incubation was omitted, the LH/CG R 3i peptide over the same concentration range did not affect basal or hCG-stimulated AC activities and only minimally (\(-10%) reduced forskolin-stimulated AC activities (Fig. 2D). Like the entire 3i loop of the LH/CG R, a peptide (3iTM6) corresponding to the C-terminal 12 residues of 3i through the N-terminal 8 residues of transmembrane domain (TM) 6 also reversed desensitization by preventing the hCG-dependent fall in hCG-stimulated AC activity (Fig. 2E). The concentration dependence for LH/CG R 3iTM6 was very similar to that of LH/CG R 3i. The maximum effect of LH/CG R 3iTM6 was seen at 20 \( \mu M \), and 3iTM6 peptide was ineffective at 114 \( \mu M \) (not shown). LH/CG R 3iTM6 did not significantly (\( p > 0.05 \)) affect basal or hCG-stimulated AC activities in a 5-min AC assay (Fig. 2F).

To determine whether the effect of LH/CG R 3i peptide on agonist-dependent LH/CG R desensitization was specific, the effects of LH/CG R peptides corresponding to the entire 2i loop or the N terminus of 4i were tested. A synthetic peptide containing the 20 amino acids of 3i randomly scrambled was also tested. Neither LH/CG R loops 2i (Fig. 2G) nor the N terminus of 4i...
FIG. 2. Effect of synthetic peptides corresponding to indicated regions of the porcine LH/CG R on agonist-dependent desensitization of LH/CG R-stimulated AC activity in porcine ovarian follicular membranes. A, preincubation of membranes with a synthetic
of 4i (Fig. 2E) nor follicle-stimulating hormone receptor 3i loop (not shown) at 15 μM affected agonist-dependent LH/CG R desensitization. Higher concentrations of LH/CG R 2i also did not modulate hCG-desensitized AC activity (Fig. 2C). A peptide corresponding to N-terminal 20 amino acids of the LH/CG R (located in the extracellular domain) also did not modulate agonist-dependent LH/CG R desensitization (Fig. 2F). Scrambled 3i peptide did not modulate agonist-dependent LH/CG R desensitization (Fig. 2F) and did not affect basal or hCG-stimulated AC activities in a 5-min AC assay (not shown). Based on our previous evidence that agonist-dependent desensitization of the LH/CG R is mediated, at least in part, by the binding of endogenous β-arrestin to the LH/CG R (34), these results are consistent with the hypothesis that the endogenous β-arrestin is binding primarily to the 3i loop of the LH/CG R.

To determine whether β-arrestin could bind directly to the 3i loop of the LH/CG R, synthetic peptides corresponding to the LH/CG R 3i or 2i loops or a peptide containing amino acids of the 3i loop randomly scrambled were coupled to CNBr-activated Sepharose. The ability of recombinant β-arrestin to bind to Sepharose-coupled peptides was then evaluated. β-Arrestin bound only to authentic LH/CG R 3i synthetic peptide and not to LH/CG R 2i or a 3i scrambled peptide (Fig. 3).

If the synthetic peptide corresponding to the 3i loop of the LH/CG R is blocking agonist-dependent LH/CG R desensitization by binding to the endogenous membrane-associated β-arrestin, then saturation of the LH/CG R 3i synthetic peptide with an exogenous arrestin should free endogenous β-arrestin and thereby revive agonist-dependent LH/CG R desensitization. Results in Fig. 4A show that when the 3i LH/CG R peptide (at 7.5 μM) was incubated with 0.2 mM visual arrestin (4C, 30 min) and then membranes were added and preincubation was continued for 30 min at 4 °C, followed by the two-stage desensitization reaction, agonist-dependent desensitization of LH/CG R was rescued to levels seen in the water control (Fig. 4A, compare hatched bars). Equivalent results were obtained when 3i LH/CG R peptide was incubated with 40 μM β-arrestin (Fig. 4B). In agreement with previous results (34), addition of unopposed β-arrestin reduced full hCG-stimulated AC activity by 20% (Fig. 4B, solid bars) to levels seen with hCG in stage 1 (Fig. 4B, hatched bars).

**DISCUSSION**

These results comprise the first report, to our knowledge, of the ability of a synthetic peptide corresponding to the 3i loop of a GPCR to reverse completely agonist-dependent desensitization of effector AC activity. This result was anticipated, based on evidence that arrestin binds primarily to the 3i loop of phosphorylated, light-activated rhodopsin (35) to quench receptor signaling (42); that ligand-activated, phosphorylated β2-adrenergic receptor requires binding of an arrestin to uncouple the phosphorylated receptor from Gs (43–45), resulting in receptor-dependent effector desensitization; and that apparent peptide corresponding to the 3i loop of the LH/CG R resulted in a concentration-dependent rise in hCG-stimulated AC activity when membranes were incubated in stage 1 of the desensitization with hCG. Membranes were desensitized in the presence of synthetic peptide or water, as indicated, at 4 °C for 30 min. Following preincubation, the two-stage desensitization incubation was conducted, consisting of a 40-min stage 1 reaction under conditions that promote development of desensitization of hCG-stimulated AC activity (+ hCG) or do not promote desensitization (+ BSAs) followed by a 5-min AC assay (± hCG; stage 2) as described under “Experimental Procedures.” The presence of BSAs in stages 1 and 2 measured basal AC activity; BSAs in stage 1 and hCG in stage 2 measured full hCG-stimulated AC activity; hCG in stages 1 and 2 measured hCG-induced desensitization of AC activity. The percentage of reduction of full hCG-stimulated AC activity above basal AC activity, expressed as the percentage of desensitization, was used as a measure of the extent of LH/CG R desensitization. Peptide concentrations refer to final concentrations in 50-μl reaction volume. Results are the means ± S.E. of quadruplicate determinations from a single experiment and are representative of one to four separate experiments. Equivalent results were obtained when desensitization reaction contained 1 mM AMP-PNP instead of ATP. B, composite effect of indicated concentrations of 5i LH/CG R peptide on the percentage of desensitization of hCG-stimulated AC activity. Results are the means ± S.E. of four (water, 15 μM peptide) or two (6.5 μM peptide) separate experiments and include the experiment in which AMP-PNP was substituted for ATP. *, differences between the percentage of desensitization values with 3i peptide are significantly different from water control (p < 0.05). C, concentration-dependent effect of synthetic peptides corresponding to LH/CG R loops 3i and 2i on the AC activity of membranes preincubated with the indicated concentrations of peptides then subjected to a 40-min desensitization incubation and a 5-min AC assay both in the presence of hCG (i.e. hCG/hCG in stage 1/stage 2). Results are the means ± S.E. of quadruplicate determinations and are representative of two experiments. D, effect of synthetic peptides corresponding to 3i loop of the LH/CG R on basal, hCG-stimulated, and forskolin-stimulated AC activities in follicular membranes. Membranes were preincubated as described in A in the presence of indicated concentrations (in final reaction assay volume) of synthetic peptide corresponding to LH/CG R loop 3i and then subjected to a 5-min AC assay (omitting the 40-min desensitization reaction) in the presence of 10 μg/mL BSA or hCG or 10 μM forskolin. Results are the means ± S.E. of quadruplicate determinations and are representative of two separate experiments. E, effect of synthetic peptides corresponding to 4i and 3iTM6 loops of the LH/CG R on agonist-dependent desensitization of LH/CG R-stimulated AC activity in follicular membranes. Membranes were incubated as in A. Results are the means ± S.E. of quadruplicate determinations and are representative of three separate experiments. F, effect of synthetic peptides corresponding to 3iTM6 loop of the LH/CG R on basal, hCG-stimulated, and forskolin-stimulated AC activities in follicular membranes. For details see D. Results are the means ± S.E. of quadruplicate determinations and are representative of two separate experiments. Forskolin-stimulated AC activities in the presence of 0.1 and 114 μM 3iTM6 are significantly different (p < 0.05). G, effect of synthetic peptides corresponding to the 2i loop of the LE of LH/CG R on agonist-dependent desensitization of LH/CG R-stimulated AC activity in follicular membranes. For details see A. Results are the means ± S.E. of quadruplicate determinations and are representative of two separate experiments. H, effect of synthetic peptides corresponding to the N1–20 of the LH/CG R on agonist-dependent desensitization of LH/CG R-stimulated AC activity in follicular membranes. For details see A. Results are the means ± S.E. of quadruplicate determinations and are representative of two separate experiments.
LH/CG R Peptide Inhibits LH/CG R Desensitization

arrestin-dependent internalization of the muscarinic m2 receptor is lost with deletion of the 3i loop (46), which includes the G protein receptor kinase 2 phosphorylation sites (47). However, receptor is lost with deletion of the 3i loop (46), which includes the G

hyperdesensitization, the ability of the poorly hydrolyzable AMP-PNP (34) to completely reverse desensitization (29), and the absence of detectable phosphate incorporation into immunoprecipitated LH/CG R under experimental conditions that support desensitization (30) and hyperdesensitization, the ability of the poorly hydrolyzable GDP analog GDPβS to completely reverse desensitization (29), and the ability to promote or abolish agonist-dependent desensitization of the LH/CG R by exogenous β-arrestin or by β-arrestin antibody, respectively, in the presence of AMP-PNP (34).

Recombinant β-arrestin binds directly to a synthetic peptide corresponding to the 3i loop of the LH/CG R and not to peptides corresponding to the 3i loop or scrambled 3i. Moreover, reversal of agonist-dependent LH/CG R desensitization is specific for the 3i loop of the LH/CG R. These results suggest that membrane-bound follicular β-arrestin binds predominately to the 3i loop of the LH/CG R to functionally uncouple the activated LH/CG R from Gs, perhaps by displacing Gs. Although it is well established for a number of GPCRs that the 3i loop comprises the primary binding site for Gs (48–51), sites of Gs contact with the active LH/CG R are less clear. Synthetic peptides corresponding to the lower portion of TM6 and to C-terminally extended TM6 peptides containing point mutations that promote constitutive LH/CG R activation (5, 52) can partially activate Gs to stimulate AC activity (53, 54), consistent with the possibility that critical Gs-binding sites in this region of TM6 become exposed on LH/CG R activation and are displaced by β-arrestin binding to the juxtaposed 3i loop of the receptor. In summary, our results show that endogenous β-arrestin binding to the C-terminal portion of the 3i loop of the LH/CG R promotes desensitization of LH/CG R-stimulated AC activity by blocking receptor activation of Gs in a physiological membrane model. A synthetic peptide corresponding to the 3i loop of the LH/CG R prevented development of desensitization of AC activity apparently by displacing β-arrestin from the receptor but did not uncouple receptor-stimulated activation of Gs. These results suggest that a synthetic peptide corresponding to the putative 3i loop of the LH/CG R may be used to enhance signaling of LH/CG R by preventing desensitization, whereas β-arrestin replacement has the potential to decrease signaling of overactive LH/CG Rs. Synthetic peptides to other GPCRs that interfere with arrestin binding are therefore predicted to block desensitization of effector activities as well as other receptor responses to arrestins, including receptor sequestration and possibly down-regulation.

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