Endocrine, *vol. 25, no. 1, 49–54, October 2004* 0969–711X/04/25:49–54/$25.00

Regulation of Follicle-Stimulating and Luteinizing Hormone Receptor Signaling by "Regulator of G Protein Signaling" Proteins

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Follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) belong to the superfamily of G protein–coupled receptors (GPCR); GPCRs are negatively regulated by RGS ("regulators of G protein signaling") proteins. In this study we evaluated the effects of RGS3 and RGS10 on FSHR and LHR ligand binding and effector coupling. FSHR and LHR ligand binding were unchanged in the presence of RGS3 or RGS10. However, signaling by FSHR and LHR was diminished by RGS3 but not by RGS10. This constitutes the first demonstration of an interaction between RGS proteins and LH and FSH signaling pathways and identifies a mechanism for negative regulation of RGS3 on FSHR and LHR signaling.

Key Words: RGS proteins; FSH receptor; LH receptor.

Introduction

The follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) belong to the G protein–coupled receptor (GPCR) family *(1,2)*. These receptors are coupled to $G_s\alpha$, which activates adenylyl cyclase in order to increase the synthesis of the second messenger cyclic AMP (cAMP) *(3,4)*, and to $G_{q/11}\alpha$, which activates phospholipase-C and results in production of the second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol; it has also been suggested that FSHR and LHR couple to $G_{i/0}\alpha$ that inhibits the activation of the adenylyl cyclase *(1,3)*.

The selectivity and intensity of the GPCR signals are regulated by several mechanisms. A family of more than 20 known proteins, the "regulators of G protein signaling" (RGS) proteins, have been identified as components of signal transduction cascades generated by GPCR-G proteins. The main function of these proteins is to negatively regulate G proteins, by acting as GTPase-activating proteins (GAPs), thereby accelerating G protein–GTP hydrolysis *(5–8)* and decreasing the length of time the G protein is in the activated state *(9)*. RGS proteins share a highly conserved domain of about 120 amino acids, which mediates the interaction of RGS proteins with the Gα subunit, considered to be the main contributor to their intrinsic GAP activity *(7,10)*. It has also been shown that RGS proteins can have regulatory functions other than as GAPs, they can increase the signaling of several receptors and regulate gene transcription *(10–12)*. RGS proteins have been shown to mainly interact with $G_q\alpha$ and $G_i\alpha$ subunits *(13,14)*; nevertheless, there is evidence of an effect of RGS proteins on the $G_s\alpha$ subunit *(15–18)*.

In this study, we analyzed the effect of the RGS3 and RGS10 proteins on FSHR and LHR expression, ligand binding, and signaling. Our observations show a previously unappreciated regulation of FSHR and LHR by RGS proteins.

Results

The expression of RGS3 and RGS10 in HEK-293 cells after transfection of DNA for these proteins was verified by Western blot. Expression of RGS3 or RGS10 proteins resulted in bands of similar density for both proteins, and when cells were transfected with the control vector, there were no observable bands (Fig. 1).

Scatchard binding analysis of the FSHR and LHR in the presence of RGS3 or RGS10 are shown in Figs. 2 and 3. Cells that transiently express the FSHR or LHR and RGS3 or RGS10 had a similar receptor affinity and similar average number of receptors/cell compared to those cells expressing the receptors and the control vector, pcDNA3.1 (FSHR/pcDNA3.1 K_d = 1.3 ± 0.1 n*M*, receptors/cell = 29,781 ± 1,523; FSHR/RGS3 K_d = 1.3 ± 0.2 n*M*, receptors/cell = 34,626 ± 2,114; FSHR/RGS10 K_d = 1.2 ± 0.2 n*M*, receptors/cell = 32,692 ± 2,012; LHR/pcDNA3.1 K_d = 1.1 ± 0.3 n*M*, receptors/cell = 30,315 ± 2,617; LHR/RGS3 K_d = 0.8 ± 0.2 n*M*, receptors/cell = 26,400 ± 1,985; LHR/RGS10 K_d = 1.0 ± 0.2 n*M*, receptors/cell= 26,809 ± 1,879).

Received July 19, 2004; Revised August 25, 2004; Accepted September 17, 2004.

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Fig. 1. Expression of RGS3 and RGS10. Expression of (**A**) RGS3 and (**B**) RGS10 expressed in HEK-293 cells was confirmed by Western blot, assayed as described in Materials and Methods. Three different experiments were performed with similar results to the representative one shown.

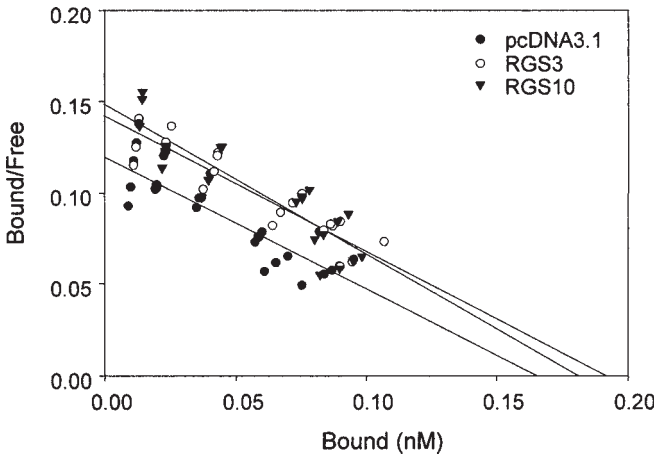

Fig. 2. Scatchard binding plots of FSHR and RGS10 or RGS3 coexpressing cells. Seventy-two hours after transfection, HEK-293 cells co-expressing the FSHR and pcDNA3.1, RGS3, or RGS10 were exposed to increasing concentrations of [^{125}I]FSH for 3 h and binding was determined. Three different experiments were performed with similar results.

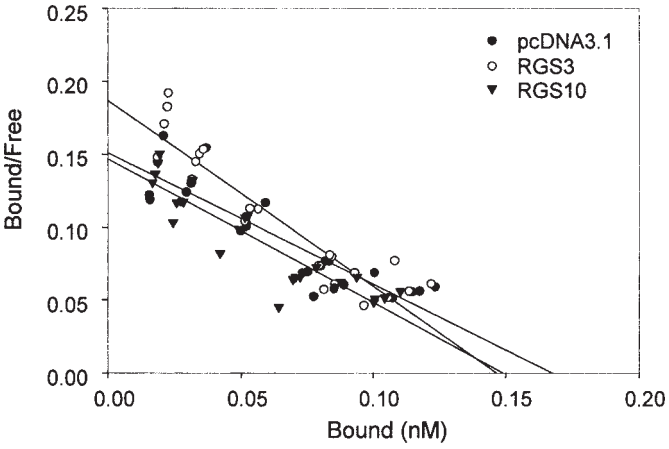

Fig. 3. Scatchard binding plots of LHR and RGS10 or RGS3 coexpressing cells. Seventy-two hours after transfection, HEK-293 cells co-expressing the LHR and pcDNA3.1, RGS3, or RGS10 were exposed to increasing concentrations of [^{125}I]LH for 3 h and binding was determined. Three different experiments were performed with similar results.

We analyzed the effect of RGS3 and RGS10 on cAMP release from HEK-293 cells, transiently expressing the FSHR or LHR, in response to increasing concentrations of the agonists (0, 12.5, 25, 50 ng/500 µL of FSH or hCG, as noted) (Figs. 4 and 5). RGS10 had no effect on the release of cAMP from FSHR or LHR expressing cells. In contrast, RGS3 had a significant inhibition on cAMP released from FSHR expressing cells. At the highest concentration of agonist studied, there was an approx 50% inhibition, compared to the control values (Fig. 4A). RGS3 also had a significant inhibition (approx 43% at the highest agonist concentration) on cAMP released mediated through the LHR (Fig. 4B).

Finally, we examined the effect of RGS3 and RGS10 on IP production from cells transiently expressing the FSHR or LHR in response to increasing concentrations of the agonists (0, 12.5, 25, 50 ng/500 µL of FSH or hCG, as noted) (Fig. 5). When RGS10 was expressed with either FSHR or LHR there was no difference in IP response from the control values; in contrast, RGS3 exhibited a significant inhibition of IP production with all concentrations of agonists

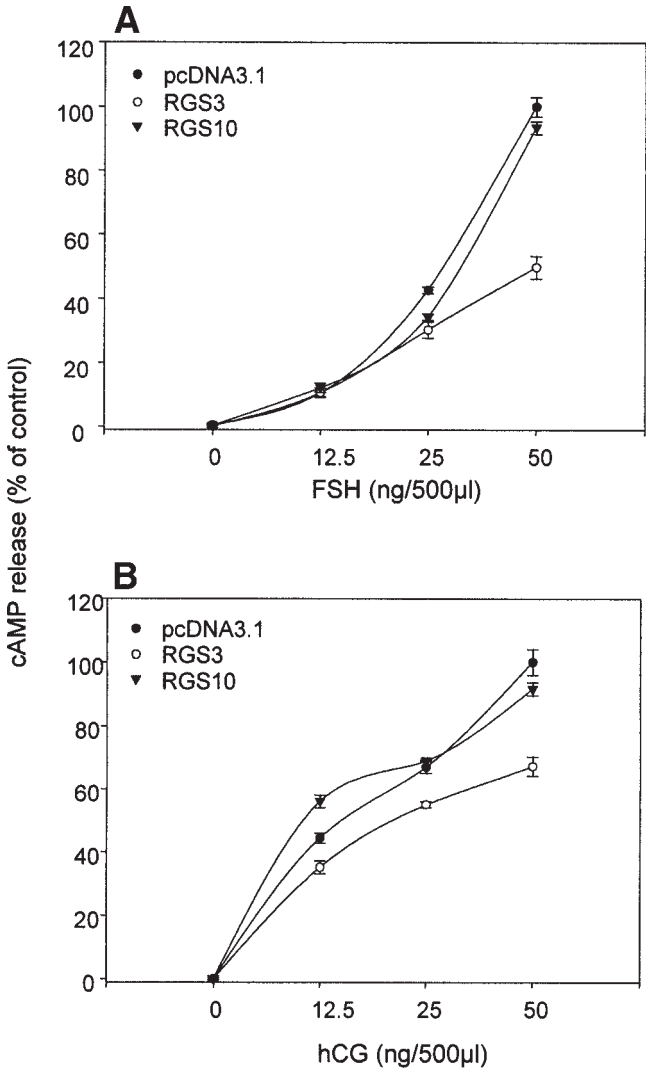

Fig. 4. Effect of RGS3 or RGS10 on cAMP release in HEK-293 cells expressing FSHR or LHR. Concentration–response curves for (**A**) FSH- or (**B**) hCG-stimulated cAMP release from HEK-293 cells transiently expressing FSHR or LHR and pcDNA3.1, RGS3, or RGS10 were assayed. Forty-eight hours after transfection, cells were stimulated for 24 h with the indicated concentrations of FSH or hCG and cAMP release was measured by RIA. Data are presented as the mean ± SE of triplicate transfections. Three different experiments showed similar results.

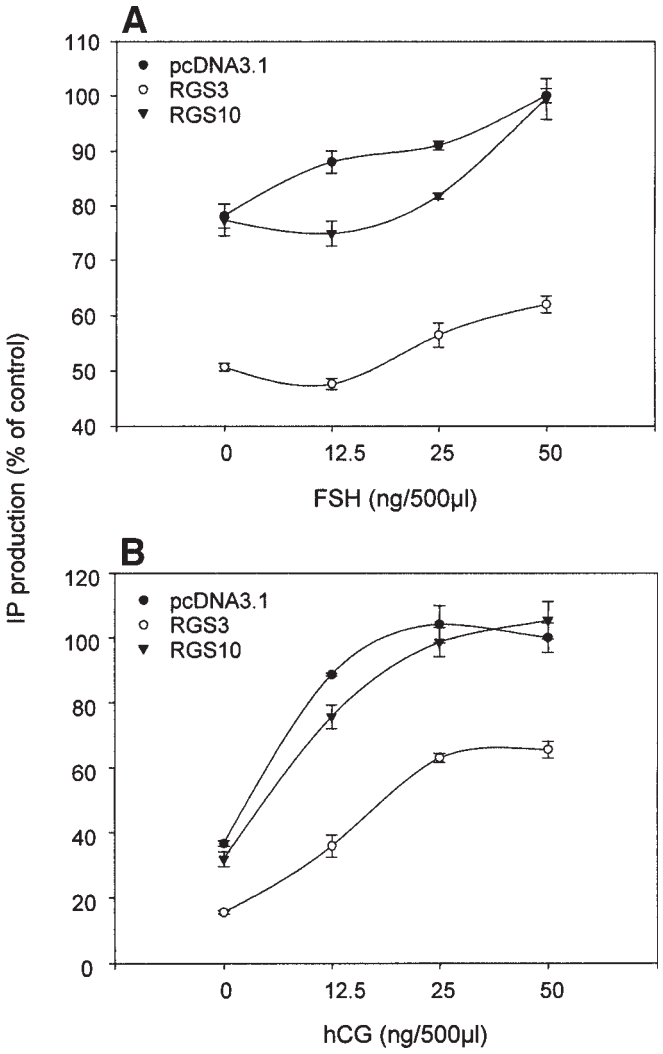

Fig. 5. Effect of RGS3 or RGS10 on IP production of HEK-293 cells expressing FSHR or LHR. Concentration–response curves for (**A**) FSH- or (**B**) hCG-stimulated IP production from HEK-293 cells transiently expressing FSHR or LHR and pcDNA3.1, RGS3, or RGS10 were assayed. Seventy-two hours after transfection, cells were stimulated for 2 h with the indicated concentrations of FSH or hCG, and IP production was measured. Data are presented as the mean ± SE of triplicate transfections. Three different experiments showed similar results.

studied, including those values obtained when the receptor was not stimulated.

Discussion

In this study, we observed that RGS3 negatively regulates FSHR and LHR-mediated G protein signaling. The expression of RGS3 and RGS10 in HEK-293 cells was verified by Western blot. The results observed suggest that both RGS proteins express in HEK-293 cells after transfection; however, these proteins are not expressed in these cells when transfection of RGS proteins is lacking. These results are similar from some previously reported where they expressed RGS3, RGS4, or RGS10 in HEK-293 cells *(19)*.

When cells co-expressed either the FSHR or LHR and RGS3 or RGS10, receptor–ligand affinity is unchanged, suggesting that the RGS proteins are not involved in ligand binding or expression of these receptors.

RGS3 inhibits cAMP response by negatively regulating the FSHR and LHR signaling, while RGS10 does not have

an effect on cAMP release. There is little previous evidence of the interaction of these proteins with the $G_s\alpha$ subunits. It has been demonstrated that RGS2 inhibits $G_s\alpha$-mediated cAMP release *(15–17)* and it interacts directly with $G_s\alpha$ in vitro *(15)*. It has also been observed that a truncated form of RGS3 (RGS3T) inhibits the $G_s\alpha$ subunit *(18)*. In previous studies, we observed that expression of RGS10 resulted in a diminished cAMP release compared to the control cells in cells expressing the gonadotropin-releasing hormone receptor (GnRHR); however, this decrease seemed to be independent of GnRHR stimulation since there was a decrease of cAMP release even when the agonist was absent *(20)*. In the present study, we suggest that RGS3 might be acting as a GTPase enhancer of $G_s\alpha$, thereby inhibiting cAMP release. However, this effect could not be due to a direct interaction of RGS proteins with $G_s\alpha$ subunits but rather regulate the receptors signaling by secondary mechanisms.

We also analyzed the effect of RGS3 and RGS10 on IP release. We observed that RGS10 has no effect on FSHR or LHR signaling because there is no differential IP release compared to the control values. In contrast, we saw that in the presence of RGS3, there is a significant inhibition of IP release with all concentrations of agonist studied for both receptors, and even without agonist stimulation. In the case of LHR, RGS3 greatly inhibited the IP response, but did not alter the concentration–response characteristics; however, for FSHR we see that the response is significantly inhibited in agonist absence and with all the agonist concentrations used. These results suggest that, although there is a probable action of RGS3 on $G_q\alpha$-mediated signaling via these receptors, RGS3 inhibits the FSH signaling pathway with only a modest effect on receptor activation, because there is attenuation of cAMP released even in basal levels. This suggests that RGS3 might be interacting with this subunit by mechanisms independent of receptor activation.

One possible explanation for the difference in the signaling regulation of both RGS proteins could be that RGS10 has been shown to be primarily localized to the nucleus, while RGS3 is found in the cytoplasm *(21)*, accessible to the G protein subunits, effectors, and GPCRs. The differential cellular localization of RGS proteins can provide functional heterogeneity to the RGS proteins *(22)*. It could also be that these differential effects were a consequence of modified interactions via diverse domains between the receptors and RGS10 compared to RGS3, serving dissimilar regulatory functions *(10,11,23,24)* or differential recognition of conformational states of $G_s\alpha$ and $G_q\alpha$ exposed by RGS10 or RGS3. Differential recognition has been observed on serotonin 5-HT1A, 5-HT2A, and dopamine D2 receptors, where it was demonstrated that RGSZ1, RGS2, RGS4, RGS7, and RGS10 modulate diverse signaling pathways in a different manner depending on the receptor on which its action is taking place. They observed that domains which are distinct from the conserved domain of RGS proteins confer selectivity to the RGS protein toward some GPCRs, such as the RGS4 N-terminal domain that discriminates between GPCR signaling complexes *(25)*.

The present study provides the first evidence of the interaction between any RGS protein and FSHR or LHR signaling. FSHR or LHR signaling has been demonstrated to be negatively regulated by RGS3 but not RGS10, suggesting a different effect between RGS proteins on the diverse systems and on the different Gα proteins involved. The regulatory mechanisms of the RGS proteins have been observed to be diverse; they have a positive or negative regulation on the GPCRs-mediated signaling, they directly regulate the effectors, or they act as scaffolds by assembling protein complexes and modulating their signaling *(10,11,23)*. It is well known that many RGS proteins can turn off signaling of some GPCRs *(7)*, and this is likely the case with the FSHR and LHR. This negative regulation may play an important role in the action of these receptors by modulating their responses when a modified environment is present. These regulatory mechanisms are important to further understand the physiological role of the RGS proteins on GPCRs actions.

Materials and Methods

Materials

The RGS3 and RGS10 cDNAs and the RGS3 polyclonal antibody were generously provided by P. Michael Conn (Oregon National Primate Research Center, Oregon Health and Science University, Beaverton, OR). The RGS10 polyclonal antibody RGS10 (C-20) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The agonists and iodination grade LH and FSH were obtained from Calbiochem (San Diego, CA). TRIZol, pcDNA3.1/V5-His-TOPO-TA, JM109 competent cells, PCR reagents, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), lipofectamine reagent, OPTI-MEM, and bovine serum albumin (BSA) were obtained from Invitrogen Life Technologies (Grand Island, NY). Myo-[2-^{3}H(N)]-inositol was obtained from Perkin Elmer (Boston, MA). Iodine-125 was purchased from Amersham Biosciences (Piscataway, NJ).

FSH and LH Receptor Cloning

Female Sprague-Dawley rats of 28 d of age were used in agreement with the ethical committee guides from the Hospital de Pediatría del Centro Médico Nacional Siglo XXI, Instituto Mexicano del Seguro Social. The brain and ovaries were extracted and homogenized using 5 mL of TRIZol Reagent for every 250 mg of tissue. Total RNA was obtained and purified, cDNA was synthesized, and FSHR and LHR cDNAs were amplified using specific primers in a touchdown PCR. The, FSHR sense primer was (5') AAG AGC CTG GGG AAT CTG TGG AAG and the antisense primer was (3') CAG TAG GAT TGC CAT TTA GTC CAT G, the LHR sense primer was (5') T CCT TTG TTA ACT

CTA GAA AGA ATG CAA AG and the antisense primer was (3') CGT CCT CTG AAG CAG GTA CAA TTC. The cDNAs were cloned into pcDNA3.1/V5-His-TOPO-TA vector and were transformed into JM109 competent cells and amplified. The cDNA clones were sequenced in an automatic sequencer PE Applied Biosystems 373A (Perkin Elmer, Foster City, CA).

Transient Co-transfections of FSHR or LHR and RGS3 or RGS10

The human embryonic kidney-derived cell line HEK-293 was maintained in DMEM/10% FCS/20 µg/mL gentamicin (DFG), in a 5% CO_2 atmosphere at 37°C; 10^5 cells/0.5 mL DFG were plated in a 24-well plate. Twenty-four hours later, the cells were transfected with 50 ng of FSHR or LHR cDNA and 50 ng of RGS3, RGS10, or control pcDNA3.1 vector cDNA, per well, using 2 µL of lipofectamine in 0.25 mL of OPTI-MEM, after 5 h 0.25 mL of DMEM/20% FCS was added. Proteins were allowed to express for 72 h after transfection *(26)*.

Scatchard Binding Assay

Scatchard binding was assessed in a range of concentrations of [^{125}I]FSH or LH, from 62,500 to 1,000,000 cpm. Fifty-one hours after transfection the cells were washed with DMEM/0.1% BSA containing 20 µg/mL gentamicin (DBG), and plain DMEM was added. Seventy-two hours after transfection, the cells were washed twice with 0.5 mL cold DMEM/BSA/10 m*M* HEPES. The radioactivity was added to each well in the same medium and the cells were allowed to incubate for 3 h at 4°C. The cells were washed twice with 1 mL of cold PBS, and 0.5 mL of 0.2 *M* NaOH/0.1% SDS was added to each well. The cell lysate was aspirated and radioactivity was determined using a Packard gamma counter (Downers Grove, IL). Scatchard transformation of the binding data was employed to determine the number of membrane-expressed receptors and to calculate receptor–ligand affinity. The *x*-intercept was taken as maximal binding; this number was used to convert the number of molecules bound to average number of receptors expressed on each in the cell population. To determine the effect of RGS proteins on the affinity of the FSHR and the LHR for ligand, the slope of the curve was calculated using the *x* and *y* intercepts *(27)*.

Quantification of cAMP Release

Forty-eight hours after transfection, cells were washed twice with DBG and then stimulated with 0, 12.5, 25, or 50 ng of FSH or LH per 0.5 mL DBG containing 0.2 m*M* methylisobutylxanthine (to prevent cAMP degradation) for 24 h at 37°C. After stimulation, the medium from each well was collected into tubes containing 50 µL of 10 m*M* theophylline (also to prevent cAMP degradation). The samples were heated at 95°C for 5 min to disrupt enzyme activity and the amount of medium-released cAMP was determined by radioimmunoassay (RIA) as previously described *(26)*.

Quantification of IP Accumulation

Fifty-four hours after transfection, cells were washed twice with DBG, and incubated in 0.5 mL/well DMEM (inositol-free) containing 4 µCi/mL of [^{3}H]inositol for 18 h at 37°C. The cells were washed twice with 0.5 mL DMEM (inositol-free) containing 5 m*M* LiCl and stimulated with 0, 12.5, 25, or 50 ng of FSH or LH per 0.5 mL of the same DMEM/LiCl (inositol-free) for 2 h at 37°C. The treatment solutions were removed, and 1 mL of 0.1 *M* formic acid was added to each well. The cells were frozen and thawed to disrupt cell membranes, and the intracellular IP accumulation was determined by Dowex anion exchange chromatography and liquid scintillation spectroscopy *(28)*.

Data Analysis

Data are presented as the means ± SEM of triplicate assay wells in each experiment. The data were analyzed using one-, two-, or three-way ANOVA followed by the Tukey's HSD test, and by the Student's *t* test; $p < 0.05$ was considered significant.

Acknowledgments

This study was supported by grant IMSS-2003/018 from the Coordinación de Investigación en Salud, Instituto Mexicano del Seguro Social, México (to J.P.M.). The authors thank Dr. P. Michael Conn, from the Oregon National Primate Research Center, Oregon Health and Science University, Beaverton, OR, who generously provided the RGS3 and RGS10 cDNAs and the RGS3 polyclonal antibody, and for critical reading of the manuscript and advice. We thank Dr. Ana Pasapera, from the Research Unit in Reproductive Medicine, Instituto Mexicano del Seguro Social, México, D.F., México, who kindly provided the HEK-293 cell line.

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