Activation of the Luteinizing Hormone/Choriogonadotropin Hormone Receptor Promotes ADP Ribosylation Factor 6 Activation in Porcine Ovarian Follicular Membranes*

Received for publication, February 16, 2001, and in revised form, June 4, 2001
Published, JBC Papers in Press, July 11, 2001, DOI 10.1074/jbc.M101498200

Lisa M. Salvador‡, Sutapa Mukherjee‡, Richard A. Kahn§, Marilyn L. G. Lamm¶, Asgerally T. Fazleabas, Evelyn T. Maizels‡, Marie-France Bader**, Heidi Hamm§§, Mark M. Rascenick††, James E. Casanova‡‡, and Mary Hunzicker-Dunn‡‡‡

From the Departments of §Cell and Molecular Biology and ¶¶Molecular Pharmacology and Biological Chemistry and the Neuroscience Institute, Northwestern University Medical School, Chicago, Illinois 60611, the §Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322, **INSERM, U-338 Biologie de la Communication Cellulaire, 5 rue Blaise Pascal, Strasbourg 67084 Cedex, France, the ¶¶Departments of Physiology & Biophysics and Psychiatry and the ¶¶Department of Obstetrics and Gynecology, University of Illinois College of Medicine, Chicago, Illinois 60612, and the ‡‡Department of Cell Biology, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

Previously we demonstrated in a cell-free ovarian follicular plasma membrane model that agonist-dependent desensitization of the luteinizing hormone/choriogonadotropin receptor (LH/CG R) is GTP-dependent, mimicked by the addition of ADP-ribosylation factor (ARF) nucleotide binding site opener, which acts as a guanine nucleotide exchange factor for ARFs 1 and 6, and selectively inhibited by synthetic N-terminal ARF6 peptides. We therefore sought direct evidence that activation of the LH/CG R promotes activation of ARF1 and/or ARF6. Using a classic ARF activation assay, the cholera toxin-catalyzed ADP-ribosylation of Gαq, results show that LH/CG R activation stimulates an ARF protein by a brefeldin A-independent mechanism. Synthetic N-terminal inhibitory ARF6 but not ARF1 peptide blocks LH/CG R-stimulated ARF activity. LH/CG R activation also promotes the binding of a photoaffinity GTP analog to a protein that migrates on one- and two-dimensional polyacrylamide gel electrophoresis with ARF6. These results suggest that ARF6 is the predominant ARF activated by the LH/CG R. To activate ARF6, the LH/CG R does not appear to signal through the C-terminal regions of Gαi or Gαq or through the second or third intracellular loops or the N terminus of the cytoplasmic tail of the LH/CG R. Although exogenous recombinant ARNO promotes only a small increase in ARF6 activation in the presence of activated LH/CG R, hCG-stimulated ARF6 activation is reduced to basal levels by catalytically inactive ARF nucleotide binding-site opener. These results provide direct evidence that LH/CG R activation leads to the activation of membrane-delimited ARF6.

We have recently shown in a cell-free plasma membrane model that binding of endogenous βarrestin1 (Arrestin 2) to the third intracellular (3i) loop of the active luteinizing hormone/choriogonadotropin (LH/CG) receptor promotes receptor desensitization by reducing the ability of the receptor to activate the stimulatory guanine nucleotide binding protein (Gαs) and resulting adenylyl cyclase (AC) (1, 2). The binding of βarrestin1 to the active LH/CG receptor is obligatory for LH/CG receptor desensitization, and there is sufficient βarrestin1 present in the membranes to promote ~80% LH/CG receptor desensitization (1–3). The pool of membrane-delimited βarrestin1 is made available to the activated LH/CG receptor by one or more steps that occur in response to LH/CG receptor activation and are dependent upon GTP (3). We therefore sought to elucidate the basis for the GTP dependence of βarrestin1-dependent LH/CG receptor desensitization. To this end, we have shown that LH/CG receptor desensitization appears to be independent of heterotrimeric Gαi, Gαq, and Gαs proteins, and of the Ras, Rap, and Rac families of small G proteins, based on the inability of C-terminal peptides or antisera directed toward the C termini of the Gα proteins, sequestration of Gβγ (4), or clostridial toxins (3) to disrupt LH/CG receptor desensitization. Rather, LH/CG receptor desensitization appears to be dependent on activation of the small G protein ADP-ribosylation factor 6 (ARF6) (3). This conclusion is based on results showing that both βarrestin1 release from its membrane docking site and subsequent LH/CG receptor desensitization are inhibited by preincubation of membranes with the inhibitory N-terminal ARF6 peptide but not with the analogous ARF1 peptide. As all G protein activation is dependent on a guanine nucleotide exchange factor (GEF), we sought to identify a GEF that might be involved in βarrestin1-dependent LH/CG receptor desensitization. LH/CG receptor desensitization is insensitive to brefeldin A (3), a fungal metabolite, which inhibits the guanine

* This work was funded by National Institutes of Health Grants R01 HD/KD 38060 (to M. H. D.), R01 AI 32991 (to J. E. C.), R01 MH59585 and AG15482 (to M. M. R., a Lalar Foundation Fellowship (to S. M.), and U.S. Army Breast USAMRMC Grant DAMD17-00-1-0386 (to L. M. S.). Preliminary results were presented at the 13th Ovarian Workshop, 2000, in Madison, Wisconsin. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Current address: Dept. of Urology, Northwestern University Medical School, Chicago, Illinois 60611.

§ 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@northwestern.edu.

¶¶ The abbreviations used are: LH/CG, luteinizing hormone/choriogonadotropin; ARF6, ADP-ribosylation factor 6; ARNO, ARF nucleotide binding-site opener; PI3K, phosphatidylinositol 3-kinase; GEF, guanine nucleotide exchange factor; i, intracellular; G protein, guanine nucleotide-binding protein; CTX, cholera toxin; FSII, follicle-stimulating hormone; BTP, bis-Tris propane; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; AC, adenylyl cyclase; PLD, phospholipase D; GPCR, G protein-coupled receptor; IEF, isoelectric focusing, dg, deglycosylated; BSA, bovine serum albumin; [32P]AAGTP, P5(4'-azidoanilido)-P5-P5-GTP, AMP-PNP, adenosine 5'-β,y-imino-triphosphate.
nucleotide exchange activity of most GEFs that activate ARFs 1–5, including Gea1p, Gea2p, GNOM, Sec7p, and BIG1 and 2, but not that of the GEFs comprising the ARNO/cytohesin-1/GRP1, EF-A6, or ARF-GEP_105 subfamilies, which activate ARF 1 and 6 (5, 6). Moreover, β-arrestin1 release from its membrane docking site and LH/CG receptor desensitization are stimulated by the addition of recombimt ARNO, a GEF for ARFs 1 and 6 (3), and blocked by a catalytically inactive recombinant ARNO (7). These results suggest that endogenous ARNO, or an ARNO-like GEF, activates ARF1 and/or ARF6 to promote β-arrestin1 release and consequent LH/CG receptor desensitization. It was therefore important to ascertain directly whether LH/CG receptor activation indeed promotes activation of an ARF and if the activated ARF corresponds to ARF1 and/or ARF6.

The classic method used to demonstrate ARF activation is the ability of cholera toxin (CTX) to catalyze the ADP-ribosylation of Gαi (8). ARF functions in the reaction as a cofactor by lowering the K_m for both the ADP-riboside donor NAD and Gαi (9), stimulating the reaction 50-fold (10). We have previously reported that preincubation of ovarian follicular membranes with hCG but not with BSA promotes CTX-catalyzed ADP-ribosylation of especially the long form but also the short form of Gαi, both of which are immunoprecipitated with anti-Gαi antisera (11, 12). CTX-catalyzed ADP-ribosylation of Gαi was dependent on the concentration of hCG and increased with time of incubation in the presence but not in the absence of hCG (11). These results are consistent with our hypothesis that LH/CG receptor activation promotes activation of an ARF in follicular membranes.

In the following studies we therefore sought to determine directly whether the agonist-activated LH/CG receptor promotes activation of ARF1 and/or ARF6 in follicular membranes. Using three different ARF activation assays, results show that ARF6 is the predominant ARF activated by the LH/CG receptor.

**EXPERIMENTAL PROCEDURES**

Purified hCG (CR-127) and FSH (oFSH-19) were kindly provided by Dr. A. F. Parlow of the National Hormone and Pituitary Program Harbor-UCLA Medical Center (Torrance, CA). Purified deglycosylated (dg) hCG was kindly provided by Dr. Patrick Roche, Mayo Clinic, Rochester MN. The ovarian follicular membrane fraction, which was purified by sucrose gradient centrifugation and enriched in AC activity, was incubated with recombinant ARF6 and if the activated ARF corresponds to ARF1 and/or ARF6. These results suggest that endogenous ARNO (7). These results suggest that endogenous ARNO, or an ARNO-like GEF, activates ARF1 and/or ARF6 to promote β-arrestin1 release and consequent LH/CG receptor desensitization. It was therefore important to ascertain directly whether LH/CG receptor activation indeed promotes activation of an ARF and if the activated ARF corresponds to ARF1 and/or ARF6.
hCG Stimulates the Binding of the GTP Photoaffinity Analog ([32P]AAGTP to ARF6.—We next sought to determine which plasma membrane-localized ARF(s) is activated as a consequence of LH/CG receptor activation. One technique to assess the activation of any G protein is its agonist-dependent binding of GTP. Release of GDP from G proteins is rate-limiting and stimulated by GEFs like the G protein–coupled receptors (GPCRs) for heterotrimeric G proteins or specific GEFs for the many small G proteins (33, 34). Hormone-dependent binding of GTP or its photoaffinity analog ([32P]AAGTP provides a method to detect G protein activation. Initial experiments showed that hCG stimulates binding of the photoaffinity GTP analog ([32P]AAGTP to one or more proteins of ~21 kDa in porcine follicular membranes (Fig. 2A). However, because the porcine follicular membrane fraction likely contains many small G proteins such as Ras (11) and the ARFs, we first sought to obtain a membrane fraction enriched in ARF6 to ascertain whether hCG promotes activation of ARF6. We determined that upon extraction of membrane proteins with 1% Triton X-100, ARF6 as well as the Triton-insoluble marker protein caveolin (35) remain in the Triton-insoluble fraction whereas Goα (12) and the LH/CG receptor are localized to a Triton-insoluble fraction (Fig. 2B). Neither ARF1 (Fig. 2C) nor ARFs 3, 4, or 5 (not shown) is detectable in the Triton-insoluble fraction. Consistent with this result, 2D-PAGE (with 70% pH 8–10, 30% pH 3–10 ampholines) of the Triton-insoluble fraction revealed that the immunoreactive ARF proteins in this fraction are basic, with pl values of ~8.0 consistent with the pl of ARF6 (28, 30) and not with that of ARF1. The more acidic ARFs 2–5 (31), which should migrate to the acidic end of the isoelectric focusing (IEF) gel (marked by the “<”), were not detected. These results indicate that, among the ARFs, only ARF6 segregates into the Triton-insoluble membrane fraction. We next determined whether hCG promotes binding of the photoaffinity GTP analog ([32P]AAGTP to ARF6. Follicular membranes were incubated for 10 min with [32P]AAGTP in the presence of BSA or hCG, UV-irradiated to covalently bind the GTP analog to protein, then extracted with 1% Triton X-100. Results (Fig. 2E) show that hCG increased binding of [32P]AAGTP to a protein retained in the Triton-insoluble membrane fraction ~21 kDa, which corresponds to the molecular weight of ARF6. We next sought to determine whether the 21-kDa protein, which binds [32P]AAGTP in the total membrane fractions, corresponds to ARF6. 2D-PAGE of the total membrane fraction incubated with hCG and [32P]AAGTP shows that only a single protein of ~21 kDa with a pl of ~8.0 covalently binds [32P]AAGTP (Fig. 2F, upper panel) and that this protein comigrates with immunoreactive ARF6 (lower panel). No labeling of the more acidic ARFs, which should migrate toward the acidic end of the IEF gel (marked by the “>”), was detected. These results therefore suggest that ARF6 is indeed activated to bind [32P]AAGTP in response to LH/CG receptor activation.

hCG Stimulates ARF6 Activity.—The ability of a receptor agonist to stimulate CTX-catalyzed ADP-ribosylation of Goα proteins has been used as evidence that an agonist-activated receptor signals to one or more G proteins (36–38). This technique is based on the observation that Goα subunits serve as optimal substrates for CTX-catalyzed ADP-ribosylation when the guanine nucleotide-binding pocket of the Go subunit of the G protein heterotrimer becomes devoid of nucleotide through release of bound GDP (36, 38). This obligatory GDP release normally occurs in response to agonist-dependent receptor activation. Alternatively, in the absence of receptor activation, GDP release from the GoαGTP heterotrimer can be stimulated by addition of exogenous GTP, resulting in the generation of a

**Fig. 1. Immunoreactive ARF6 is detected in porcine follicular membranes.** In A, proteins in porcine follicular membranes (75 µg of membrane protein for lanes 1 and 2, 100 µg for lane 3) purified by sucrose density gradient centrifugation were separated by SDS-PAGE, blotted to Hybond, and then probed with pan-ARF antibody 1D9 (lane 1), ARF6-specific antibody (lane 2), or ARF1-specific antibody (lane 3). Immunoreactive bands below 21 kDa on the ARF6-specific blot (lane 2) are believed to represent proteolytic breakdown products of ARF6. In B, proteins in follicular membranes (75 µg of membrane protein) were separated by 2D-PAGE, as described under “Experimental Procedures.” Amorpholines consisted of 100% pH 3–10. An aliquot of total membrane fraction not subjected to IEF (Total) was loaded onto SDS-PAGE gel, as indicated. The symbol “<” marks the edges of the tube IEF gel. Following SDS-PAGE, proteins were transferred to Immobilon membrane and blotted to Hybond, and then probed with pan-ARF antibody 1D9. As described under “Experimental Procedures,” immunoreactive bands below 21 kDa on the ARF6-specific blot were separated by 2D-PAGE, as described under “Experimental Procedures.” Amorpholines consisted of 100% pH 3–10. In C, the amount of ARF6 in porcine follicular membranes was determined whether hCG promotes binding of the photoaffinity GTP analog ([32P]AAGTP to ARF6. Follicular membranes were incubated for 10 min with [32P]AAGTP in the presence of BSA or hCG, UV-irradiated to covalently bind the GTP analog to protein, then extracted with 1% Triton X-100. Results (Fig. 2E) show that [32P]AAGTP binds to a protein retained in the Triton-insoluble membrane fraction ~21 kDa, which corresponds to the molecular weight of ARF6. We next sought to determine whether the 21-kDa protein, which binds [32P]AAGTP in the total membrane fractions, corresponds to ARF6. 2D-PAGE of the total membrane fraction incubated with hCG and [32P]AAGTP shows that only a single protein of ~21 kDa with a pl of ~8.0 covalently binds [32P]AAGTP (Fig. 2F, upper panel) and that this protein comigrates with immunoreactive ARF6 (lower panel). No labeling of the more acidic ARFs, which should migrate toward the acidic end of the IEF gel (marked by the “>”), was detected. These results therefore suggest that ARF6 is indeed activated to bind [32P]AAGTP in response to LH/CG receptor activation.

**Fig. 2. Immunoreactive ARF6 is detected in porcine follicular membranes.** A. Immunoreactive ARF6 was detected in porcine follicular membranes (75 µg of membrane protein for lanes 1 and 2, 100 µg for lane 3) purified by sucrose density gradient centrifugation were separated by SDS-PAGE, blot to Hybond, and then probed with pan-ARF antibody 1D9 (lane 1), ARF6-specific antibody (lane 2), or ARF1-specific antibody (lane 3). Immunoreactive bands below 21 kDa on the ARF6-specific blot (lane 2) are believed to represent proteolytic breakdown products of ARF6. In B, proteins in follicular membranes (75 µg of membrane protein) were separated by 2D-PAGE, as described under “Experimental Procedures.” Amorpholines consisted of 100% pH 3–10. An aliquot of total membrane fraction not subjected to IEF (Total) was loaded onto SDS-PAGE gel, as indicated. The symbol “<” marks the edges of the tube IEF gel. Following SDS-PAGE, proteins were transferred to Immobilon membrane and blotted to Hybond, and then probed with pan-ARF antibody 1D9. In C, the amount of ARF6 in porcine follicular membranes was determined whether hCG promotes binding of the photoaffinity GTP analog ([32P]AAGTP to ARF6. Follicular membranes were incubated for 10 min with [32P]AAGTP in the presence of BSA or hCG, UV-irradiated to covalently bind the GTP analog to protein, then extracted with 1% Triton X-100. Results (Fig. 2E) show that [32P]AAGTP binds to a protein retained in the Triton-insoluble membrane fraction ~21 kDa, which corresponds to the molecular weight of ARF6. We next sought to determine whether the 21-kDa protein, which binds [32P]AAGTP in the total membrane fractions, corresponds to ARF6. 2D-PAGE of the total membrane fraction incubated with hCG and [32P]AAGTP shows that only a single protein of ~21 kDa with a pl of ~8.0 covalently binds [32P]AAGTP (Fig. 2F, upper panel) and that this protein comigrates with immunoreactive ARF6 (lower panel). No labeling of the more acidic ARFs, which should migrate toward the acidic end of the IEF gel (marked by the “>”), was detected. These results therefore suggest that ARF6 is indeed activated to bind [32P]AAGTP in response to LH/CG receptor activation.
ARF6 fractionates into the Triton X-100 insoluble membrane fraction and binds \(^{32}P\)AAGTP in response to LH/CG receptor activation. In A, follicular membranes (30\(\mu\)g of membrane protein) were incubated in the presence of 1\(\mu\)M AMP-PNP, 3\(\mu\)M MgCl\(_2\), 0.5 mM EDTA, 1 mM EGTA, and 25 mM bis-Tris propane (BTP), pH 7.2, 10\(\mu\)M \(^{32}P\)AAGTP, and 10\(\mu\)g/ml BSA or hCG at 30 °C for 10 min; membranes were washed, resuspended in incubation medium containing 100 \(\mu\)M GDP, and UV-irradiated 3 min at 4°C. Corresponding Coomassie blue-stained membrane proteins are shown below the autoradiogram. Results are representative of three experiments. In B, follicular membranes (300\(\mu\)g of membrane protein) were incubated with 10\(\mu\)g/ml BSA or hCG in an incubation medium (IM) consisting of 1 mM ATP, 5 mM MgCl\(_2\), 0.4 mM EDTA, 1 mM EGTA, 10\(\mu\)M GTP, and 25 mM BTP, pH 7.2, for 40 min at 30 °C. Membrane proteins were then pelleted, resuspended in buffer containing 1% Triton X-100, and stirred at 4°C for 1 h (12), then separated into a Triton-soluble and -insoluble fractions by centrifugation at 105,000 \(\times\) g for 60 min. Pellet and supernatant fractions were then heat-denatured. Blots were probed with ARF6-specific, caveolin, and LH/CG receptor antibodies, as indicated. 100% of the Triton-insoluble and 33% of the Triton-soluble fraction was loaded onto the gel for SDS-PAGE for ARF6 and caveolin blots (12); 100% of both fractions was loaded for the LH/CG receptor blot shown.

Results for each antibody are from separate experiments, and each are representative of three experiments. In C, follicular membranes (150 \(\mu\)g) were extracted with 1% Triton X-100 as in B, and 100% of the Triton-soluble and -insoluble fractions was loaded onto the gel for SDS-PAGE. The blot was probed with ARF1-specific antibody. In D, proteins in the Triton X-100-insoluble fractions were separated by 2D-PAGE, as described under “Experimental Procedures.” Amphilines consisted of 70% pH 6–10, and 30% pH 3–10. An aliquot of total membrane fraction not IEF was loaded onto the 2D-PAGE gel, as indicated. The symbol “\(" marks the edges of the IEF tube gel. Following SDS-PAGE, proteins were transferred to Immobilon membrane and probed with pan-ARF antibody ID9. In E, membranes (80 \(\mu\)g of membrane protein) were incubated in an incubation medium consisting of 1 mM ATP, 5 mM MgCl\(_2\), 0.4 mM EDTA, 1 mM EGTA, 10 \(\mu\)M GDP, and 25 mM BTP, pH 7.2, 25 mM creatine phosphate, and 0.2 mg/ml bovine serum albumin. Membrane proteins were then solubilized in 1% Triton X-100 (12). Triton-insoluble proteins were heat-denatured and subjected to SDS-PAGE. Results are representative of two separate experiments. In F, membranes were incubated as in E but only in the presence of hCG, and following UV irradiation total membrane fraction was pelleted, heat-denatured, and subjected to 2D-PAGE with 70% pH 8–10 and 30% pH 3–10 amphilones; proteins transferred to Immobilon membranes were then subjected first to autoradiography (upper panel) then to Western blotting using pan-ARF antibody ID9 (lower panel). Results are representative of two experiments.
the addition of inhibitory synthetic myristoylated (Mryr)- and non-Myr-(2–13)ARF6 peptides (3). We therefore sought to determine whether the ARF6 N-terminal peptide inhibited ARF6-dependent CTX-stimulated AC activity. When membranes were incubated with 100 μM GTP, CTX raised AC activities over levels seen without CTX (H2O), as a consequence of the ARF-dependent ADP-ribosylation of Goβi and resulting inhibition of the GTPase activity of Goβi (Fig. 4). Addition of Myr-ARF6 N-terminal peptide promoted a concentration-dependent reduction of CTX-stimulated AC activities whereas Myr-ARF1 N-terminal peptides were ineffective (Fig. 4).

We also previously showed that, by preventing βarrestin1 release from its membrane docking site, ARF6 N-terminal peptides inhibit LH/CG receptor desensitization (3). We therefore sought to determine whether the ARF6 N-terminal peptide also inhibited hCG-stimulated CTX-catalyzed ADP-ribosylation of Goβi. Myr-ARF6 N-terminal peptide reduced hCG-stimulated CTX-catalyzed ADP-ribosylation of Goβi in a concentration-dependent manner whereas the corresponding Myr-ARF1 peptide was ineffective (Fig. 5). Taken together, the results of both ARF activation assays suggest that ARF6 and not ARF1 is the predominant cofactor in the ADP-ribosylation of Goβi in porcine follicular membranes and that LH/CG receptor activation leads to the activation primarily of ARF6.

**LH/CG Receptor Stimulation of the ADP-ribosylation of Goβi: Potential Role for ARNO, a Guanine Nucleotide Exchange Factor for ARF6—**Brefeldin A fails to inhibit the GTP exchange activity of ARNO, cytohesin-1, GRP-1, ARF-GEF100, and EFA6 GEFs for ARF1 and/or ARF6 but does inhibit the guanine nucleotide exchange activity of most other GEFs that activate ARFs 1–5 (5, 6). We therefore determined whether hCG-stimulated ARF6 activation was inhibited by brefeldin A. Results in Fig. 6 show that neither the basal nor hCG-stimulated CTX-catalyzed ADP-ribosylation of the short or long forms of Goβi is inhibited by brefeldin A. Thus, the LH/CG receptor activates ARF6 through a brefeldin A-insensitive ARF GEF present in follicular membranes to trigger CTX-catalyzed ADP-ribosylation of Goβi.

Because our follicular membrane model contains relatively high levels of endogenous ARNO (~1.5 μg/mg of membrane protein (7)), we sought to determine whether catallytically inactive ARNO, containing a mutation at E156, could function in a dominant negative manner to inhibit the ability of the LH/CG receptor to promote the ADP-ribosylation of Goβi. As shown in Fig. 7, E156K ARNO significantly reduced the ability of hCG to activate ARF6 to promote the ADP-ribosylation of Goβi. These results suggest that the guanine nucleotide exchange activity of endogenous ARNO or an ARNO-like GEF is obligatory for the LH/CG receptor to activate ARF6.

We next sought to determine whether addition of exogenous ARNO to the already high levels of ARNO present in follicular membranes further enhances the ADP-ribosylation of Goβi. Addition of exogenous recombinant ARNO did not increase the CTX-catalyzed ADP-ribosylation of Goβi in the absence of receptor agonist (Fig. 8A, lanes 1, 3, 5, and 7; Fig. 8B, lanes 1 and 5). However, in the presence of hCG, exogenous recombinant ARNO promoted a small increase in ARF activation (Fig. 8A, lane 4 versus 2). Addition of 4 mM MgCl2 to the reaction mix was found to diminish the ability of hCG to stimulate the ADP-ribosylation of Goβi (Fig. 8A, lanes 5 and 6). The addition of exogenous recombinant ARNO to a reaction mix containing 4 mM MgCl2 restored hCG-stimulated CTX-catalyzed ADP-ribosylation of both the long and short forms of Goβi (Fig. 8A, lanes 5 and 6 versus 7 and 8; note reduced protein load in lanes 7 and 8). Activation of the FSH receptor also stimulated the ADP-ribosylation of Goβi (Fig. 8B, lanes 1 and 3), and this response is also slightly enhanced by the addition of exogenous ARNO (lanes 3 and 4). Aluminum fluoride (AlF3) promoted only a very modest activation of the ADP-ribosylation of Goβi (Fig. 8C), and this response was unaffected by the addition of exogenous ARNO. The inability of AlF3 to promote robust CTX-catalyzed ADP-ribosylation of Goβi is consistent with an earlier report (47) and likely results from dissociation of Gαi GDP/AlF3 from βγ subunits based on the ability of AlF3 to mimic the terminal phosphate on GTP to promote dissociation of GDP-bound heterotrimeric G proteins (33). These results show that in the presence of already high levels of endogenous ARNO, exogenous recombinant ARNO promotes only a small further increase in hCG-stimulated CTX-catalyzed ADP-ribosylation of Goβi but under experimental conditions where endogenous ARNO is either inactive or unavailable, then exogenous ARNO promotes a robust increase in hCG-stimulated CTX-catalyzed ADP-ribosylation of Goβi probably by enhancing ARF6 activity. The inability of exogenous ARNO to promote CTX-catalyzed ADP-ribosylation of Goβi in the absence of LH/CG receptor activation is most likely attributable to the absence of substrate, i.e. the presence of Gαi GDP/βγ conformation. Additionally, these results might suggest that the active conformation of the LH/CG receptor is required to activate ARNO or that receptor activation releases ARF6, potentially from the receptor, to be activated by available ARNO.
Effect of Heterotrimeric G Protein C-terminal Peptides and Antisera and of Synthetic Peptides Corresponding to Selected Regions of the LH/CG Receptor on the Ability of LH/CG Receptor Activation to Activate ARF6—Finally, we sought to determine whether the ability of the LH/CG receptor to activate the membrane-delimited ARF6 requires selected regions of the LH/CG receptor or the C-terminal regions of Gαi, Gαs, or Gαq. Synthetic C-terminal Gα peptides have been shown to compete specifically with Gα proteins for binding to a receptor and therefore to inhibit downstream events (48–52). Gα C-terminal peptide-directed antisera have also been shown to inhibit receptor coupling to specific Gα proteins (53–58). We have previously shown that Gαs (354–372) inhibits (∼50%) the ability of the LH/CG receptor to activate Gs and AC (4) based on the ability of this peptide to reduce Gαs signaling to AC (50), and that LH/CG receptor 3i and 3TM6 peptides selectively ablate LH/CG receptor desensitization based on their ability to compete with endogenous receptor for βarrestin1 (1). We therefore determined whether we could block the ability of the LH/CG receptor to activate ARF6 to stimulate the ADP-ribosylation of Gαs by preincubating membranes with antibodies directed to the C-terminal domains of Gαs, Gαo, or Gαq, or with synthetic peptides directed to the C termini of Gαs, Gαo, or Gαq, or with synthetic peptides directed toward selected intracellular loops of the LH/CG receptor. As seen in Fig. 9, A–C, none of these reagents blocks the ability of hCG to activate ARF6 to stimulate the ADP-ribosylation of Gαs. However, the LH/CG receptor antagonist dghCG does not stimulate the ADP-ribosylation of Gαs (Fig. 9C, lane 3). These results suggest that the ability of the LH/CG receptor to activate ARF6 is dependent on LH/CG receptor activation but independent of the C-terminal regions of Gαo and Gαo and of the 2i and 3i loops and the N terminus of the cytoplasmic tail (4i) of the LH/CG receptor or that it involves regions on the effector that are not sensitive to these reagents. The availability of substrate for ADP-ribosylation (i.e. Gαo, βγ) even in the presence of Gαs C-terminal peptide, which reduces signaling to Gs/AC by ∼50% (4), likely shows that Gs is not limiting in our membranes. Results (Fig. 9C) also show that ARF6 activation by the LH/CG receptor is not inhibited by wortmannin, a phosphatidylinositol 3-kinase (PI3K) inhibitor.

**DISCUSSION**

We have obtained direct evidence that agonist-dependent activation of the LH/CG receptor promotes the activation of a membrane-delimited ARF. The predominant ARF in follicular membranes that is activated upon engagement of the LH/CG receptor is ARF6, but we cannot rigorously exclude actions of other ARF isoforms. Under experimental conditions where receptor activation leads to the binding of a photoaffinity GTP analog to activated G proteins (12), we detect binding of GTP to a ∼21-kDa protein, which exhibits a basic pI consistent with the pI of ARF6 and not with that of the other ARFs (31, 32). hCG-stimulated GTP binding to the 21-kDa protein is still detected when we segregate ARF6 from the other ARFs following hCG-stimulated ARF activation by removing proteins soluble in Triton X-100. ARF6 is abundant in purified follicular membranes, present at a concentration of ∼8 μg/mg of membrane protein, and based on 2D-PAGE followed by Western blotting with the pan-ARF antibody, predominaates over ARF1 in this membrane model. Moreover, ARF activation stimulated by hCG or high concentrations of GTP (100 μM) is blocked by the inhibitory synthetic N-terminal Myr-ARF6 peptide and not by the corresponding Myr-ARF1 peptide. Our conclusion that agonist-dependent LH/CG receptor activation leads to activation of ARF6 is consistent with our earlier report (3), which showed that an inhibitory N-terminal ARF6 but not ARF1 peptide also prevents GTP-stimulated βarrestin1 release from its membrane docking site and LH/CG receptor desensitization.
We have shown that ARF6 is localized to the Triton X-100-insoluble fraction in membranes treated either with BSA or with hCG to promote receptor activation. In contrast to ARF6, the majority of Gaα (12) and LH/CG receptors is restricted to the Triton X-100-soluble membrane fraction. The basis for the localization of ARF6 to the Triton X-100-insoluble fraction is not known and requires additional studies. However, based on evidence of an association with actin or other cytoskeletal proteins. Our results also indicate that, as in many (19, 27, 28) but certainly not all cell models (27, 29, 59, 62–64), ARF6 in ovarian follicular cells appears to be constitutively associated with the plasma membrane both in its inactive and active conformation. Studies in rat ovarian granulosa cells also show that the majority of ARF6 is detected by Western blotting in the membrane/pellet fraction of cells and is not redistributed in response to LH/CG receptor activation (3), even in a magnesium-containing buffer that can dislodge ARF6 from the pellet fraction in other cells (29).

We designed a series of experiments to begin to determine how the LH/CG receptor promotes ARF6 activation. We first determined whether the active conformation of the LH/CG receptor signals through ARNO to activate ARF6. ARNO has been shown to catalyze the exchange of GDP for GTP on ARFs (12) and LH/CG receptors is restricted to the Triton X-100-insoluble membrane fraction. The basis for the localization of ARF6 to the Triton X-100-insoluble fraction is not known and requires additional studies. However, based on evidence of an association between ARF6 and cortical actin (59–62), the Triton-insolubility of ARF6 might reflect its association with actin or other cytoskeletal proteins. Our results also indicate that, as in many (19, 27, 28) but certainly not all cell models (27, 29, 59, 62–64), ARF6 in ovarian follicular cells appears to be constitutively associated with the plasma membrane both in its inactive and active conformation. Studies in rat ovarian granulosa cells also show that the majority of ARF6 is detected by Western blotting in the membrane/pellet fraction of cells and is not redistributed in response to LH/CG receptor activation (3), even in a magnesium-containing buffer that can dislodge ARF6 from the pellet fraction in other cells (29).

We designed a series of experiments to begin to determine how the LH/CG receptor promotes ARF6 activation. We first determined whether the active conformation of the LH/CG receptor signals through ARNO to activate ARF6. ARNO has been shown to catalyze the exchange of GDP for GTP on ARFs (12) and LH/CG receptors is restricted to the Triton X-100-insoluble membrane fraction. The basis for the localization of ARF6 to the Triton X-100-insoluble fraction is not known and requires additional studies. However, based on evidence of an association between ARF6 and cortical actin (59–62), the Triton-insolubility of ARF6 might reflect its association with actin or other cytoskeletal proteins. Our results also indicate that, as in many (19, 27, 28) but certainly not all cell models (27, 29, 59, 62–64), ARF6 in ovarian follicular cells appears to be constitutively associated with the plasma membrane both in its inactive and active conformation. Studies in rat ovarian granulosa cells also show that the majority of ARF6 is detected by Western blotting in the membrane/pellet fraction of cells and is not redistributed in response to LH/CG receptor activation (3), even in a magnesium-containing buffer that can dislodge ARF6 from the pellet fraction in other cells (29).

We designed a series of experiments to begin to determine how the LH/CG receptor promotes ARF6 activation. We first determined whether the active conformation of the LH/CG receptor signals through ARNO to activate ARF6. ARNO has been shown to catalyze the exchange of GDP for GTP on ARFs 1 and 6 (22, 23). Based on our evidence that follicular membranes contain ~1.5 μg of ARNO/mg of membrane protein (7), we determined whether the approximate tripling of the endogenous level of ARNO, by adding ~0.25 μg of recombinant ARNO to 100 μg of membrane protein, promoted a further increase in ARF6 activation, assessed as CTX-stimulated ADP-ribosylation of Gaα. Our results showed that the addition of exogenous ARNO to follicular membranes in the presence of the active LH/CG receptor promoted only a minimal increase in ARF6 activation, consistent with the notion that levels of endogenous ARNO are sufficient to support ARF6 activation. Under experimental conditions where endogenous ARNO was
either inactive or unavailable and hCG did not activate ARF6 to stimulate CTX-catalyzed ADP-ribosylation of Goα, addition of recombinant ARNO restored ARF6 activation in the presence of hCG to levels seen in the absence of added MgCl₂. Our results showing that the addition of ~8-fold molar excess of recombinantly catalytically inactive ARNO abolished hCG-stimulated ARF6 activation to stimulate CTX-catalyzed ADP-ribosylation of Goα support our conclusion that endogenous ARNO is obligatory for ARF6 activation. However, we cannot rule out the possibility that catalytically inactive ARNO is acting to sequester ARF6 and thus indirectly inhibiting ARF6 activation or that another brefeldin A-insensitive GEF promotes ARF6 activation in response to LH/CG receptor activation. Either ARNO or another ARNO-like GEF is activated in response to LH/CG receptor activation and consequently activates available ARF6, or ARF6 might be potentially bound to the inactive receptor and, upon engagement of the receptor, is freed to be actuated by available ARNO, or aspects of both scenarios might apply, such that receptor activation leads to both ARF release from the receptor and ARNO activation. None of these alternatives can be excluded by the present results. However, our earlier result showing that addition of exogenous recombinant ARNO in the absence of LH/CG receptor activation promotes LH/CG receptor desensitization (7) suggests that LH/CG receptor activation increases the availability of ARNO rather than promotes activation of ARNO. In the absence of hCG, exogenous ARNO was ineffective in activating ARF6 to stimulate CTX-catalyzed ADP-ribosylation of Goα, likely because of a lack of substrate for ADP-ribosylation.

We also determined whether the activated LH/CG receptor directs ARF6 activation via its second or third intracellular loops or the N terminus of its cytoplasmic tail, or via the C-terminal regions of Goα or Goβ. However, reagents established to test each of these regions of the LH/CG receptor and Go proteins yielded negative results. Although these results suggest that these regions of the Go proteins and LH/CG receptor do not participate in ARF6 activation, participation of other regions of the LH/CG receptor cannot be excluded and indeed are expected.

It is interesting that FSH also activates an ARF in porcine follicular membranes. We have linked ARF6 activation by the activated LH/CG receptor to LH/CG receptor desensitization (3). We do not yet know if this ARF6-dependent pathway, which promotes release of the βarrestin1 obligatory for LH/CG receptor desensitization, is unique to the LH/CG receptor or applies more universally to other G protein-coupled receptors (GPCRs). The ability of FSH to activate an ARF, however, is consistent with the possibility that GPCRs other than the LH/CG receptor can promote receptor desensitization via an ARF. The β-adrenergic receptor upon agonist but not antagonist binding has also been shown to stimulate CTX-catalyzed ADP-ribosylation of Goα, i.e. to activate an ARF. Moreover, there is recent evidence that overexpression of an ARF6 GTPase activating protein GIT1 inhibits β-adrenergic receptor internalization, consistent with the notion that the β-adrenergic receptor activates an ARF, such as ARF6 (65–67). ARF activation also occurs in response to activation of other GPCRs, including the m3 muscarinic acetylcholine (68), fMet-Leu-Phe (69), GnRH, H1 histamine, and B2 bradykinin (70) receptors and in response to activation of receptor tyrosine kinases like the insulin (25) and epidermal growth factor (71) receptors. At least in some of these cell models, receptor-stimulated ARF activation leads to activation of PLD² (70, 72, 73). ARF activation in a number of these models involves the recruitment of the ARF and/or its GEF via phosphatidylinositol 3,4,5-trisphosphate produced by activation of PI3K and is thus inhibited by the PI3K inhibitor wortmannin (71, 73, 74). We have shown that wortmannin does not inhibit LH/CG receptor-stimulated ARF activation or LH/CG receptor desensitization in our plasma membrane model (7), consistent with our evidence that neither ARNO nor ARF6 needs to be recruited. Additional studies are required to elucidate how the active LH/CG receptor promotes the activation of ARF6 in ovarian follicular membranes.

Acknowledgment—We thank Dr. Subhendu Mukhopadhy for preparing E156K ARNO.

REFERENCES

1. Mukherjee, S., Palczewski, K., Gurevich, V. V., and Hunzicker-Dunn, M. (1999) J. Biol. Chem. 274, 12884–12889
2. Mukherjee, S., Palczewski, K., Benovic, J. L., Gurevich, V. V., and Hunzicker-Dunn, M. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 493–498
3. Mukherjee, S., Gurevich, V. V., Jones, J. C. R., Casanova, J. E., Frank, S. R., Maizels, E. T., Baer, M. F., Kahn, R. A., Palczewski, K., Aktories, K., and Hunzicker-Dunn, M. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 5901–5906
4. Rajagopalan-Gupta, R. M., Mukherjee, S., Zhu, X., Ho, Y. K., Hamm, H., Birnbaumer, M., Birnbaumer, L., and Hunzicker-Dunn, M. (1999) Endocrinology 140, 1612–1621
5. Jackson, C. L., and Casanova, J. E. (2000) Trends Cell Biol. 10, 60–67
6. Someya, A., Sata, M., Takeda, K., Paccheco-Rodriguez, G., Ferrans, V. J., Moss, J., and Vaughan, M. (2000) Proc. Natl. Acad. Sci. U.S.A. 98, 2413–2418
7. Mukherjee, S., Casanova, J. E., and Hunzicker-Dunn, M. (2001) J. Biol. Chem. 6524–6528
8. Kahn, R. A., and Gilman, A. G. (1986) J. Biol. Chem. 261, 7906–7911
9. Noda, M., Tasi, S. C., Adamik, R., Moss, J., and Vaughan, M. (1996) Biochim. Biophys. Acta 1034, 195–199
10. Randazzo, P. A., Terui, T., Sturch, S., and Kahn, R. A. (1994) J. Biol. Chem. 269, 29490–29494
11. Rajagopalan-Gupta, R. M., Rasenick, M. M., and Hunzicker-Dunn, M. (1997) Mol. Endocrinol. 11, 538–549
12. Rajagopalan-Gupta, R. M., Lamm, M. L., Mukherjee, S., Rasenick, M. M. and Hunzicker-Dunn, M. (1998) Endocrinology 138, 4547–4555
13. Ekstrom, R. C., and Hunzicker-Dunn, M. (1989) Endocrinology 124, 956–963
14. Noda, M., Ning, Y., Venable, S. F., Pereira-Smith, O. M., and Smith, J. R. (1994) Exp. Cell Res. 218, 164–168
15. Bockaert, J., Hunzicker-Dunn, M., and Birnbaumer, L. (1976) J. Biol. Chem. 251, 2653–2663
16. Lamm, M. L. G., Rajagopalan-Gupta, R. M., and Hunzicker-Dunn, M. (1999) Endocrinology 140, 29–36
17. Rasenick, M. M., Talluri, M., and Dunn, W. J., III (1994) Methods Enzymol. 237, 110–110
18. Hunzicker-Dunn, M., Cutler, R. E., Jr., Maizels, E. T., DeManno, D. A., Lamm, M. L. G., Erlichman, J., Sanwal, B. D., and LaBarbera, A. R. (1991) J. Biol. Chem. 266, 7166–7175
19. Cavenagh, M. M., Whitney, J. A., Carroll, K. Zhang, C., Boman, A. L., Rosenwald, A. G., Meallan, I., and Kahn, R. A. (1996) J. Biol. Chem. 271, 21767–21774
20. Bender, F. E., Douglass, L. W., and Kramer, A. (1982) Statistical Methods for Food and Agriculture, AVI Publishing Co., Inc., Westport, CT
21. Caumont, A.-S., Vitale, N., Gense, M., Galas, M.-C., Casanova, J. E., and Bader, M.-P. (2000) J. Biol. Chem. 275, 15637–15644
22. Chardin, P., Paris, S., Antonny, B., Robineau, S., Berrada-Dufour, S., Jackson, C. L., and Chabre, M. (1996) Nature 384, 481–484
23. Frank, S., Upender, S., Hansen, S. H., and Casanova, J. E. (1998) J. Biol. Chem. 273, 23–27
24. Paris, S., Berrada-Dufour, S., Robineau, S., Begay, J., Antonny, B., Chabre, M., and Chardin, P. (1997) J. Biol. Chem. 272, 22221–22226
25. Shone, K., Vasudevan, C., and Romero, G. (1997) Curr. Biol. 7, 387–396
26. Palczewski, A., Whatmore, J., Morgan, C., Jones, D., and Cockcroft, S. (2000) J. Biol. Chem. 275, 13157–13164
27. Peters, P. J., Hsu, W. Y., Ooi, C. E., Finazzi, D., Teal, S. B., Oorschot, V., Donaldson, J. G., and Rossouw, S. D. (1995) J. Biol. Chem. 270, 1003–1017
28. Yang, C. Z., Heimberg, H., D’Souza-Schorey, C., Mueckler, M. M., and Stahl, P. D. (1998) J. Biol. Chem. 273, 4006–4011

2 PLD does not appear to be activated in porcine follicular membranes by GTP plus recombinant ARF1 (I. Lopez, unpublished observation).
Activation of the Luteinizing Hormone/Choriogonadotropin Hormone Receptor Promotes ADP Ribosylation Factor 6 Activation in Porcine Ovarian Follicular Membranes

Lisa M. Salvador, Sutapa Mukherjee, Richard A. Kahn, Marilyn L. G. Lamm, Asgerally T. Fazleabas, Evelyn T. Maizels, Marie-France Bader, Heidi Hamm, Mark M. Rasenick, James E. Casanova and Mary Hunzicker-Dunn

J. Biol. Chem. 2001, 276:33773-33781.
doi: 10.1074/jbc.M101498200 originally published online July 11, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101498200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 72 references, 47 of which can be accessed free at http://www.jbc.org/content/276/36/33773.full.html#ref-list-1