Desensitization of the Luteinizing Hormone/Choriogonadotropin Receptor in Ovarian Follicular Membranes Is Inhibited by Catalytically Inactive ARNO

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We have investigated the participation of endogenous ADP-ribosylation factor (ARF) nucleotide-binding site opener (ARNO) in desensitization of the luteinizing hormone/choriogonadotropin (LH/CG) receptor, independent of receptor internalization, using a cell-free plasma membrane model. We recently showed that the addition of recombinant ARNO promotes binding of β-arrestin1 to the third intracellular (3i) loop of the active LH/CG receptor, thereby reducing the ability of the receptor to activate the stimulatory G protein and signal to adenylyl cyclase. In the present report we determined whether ARNO is detectable in follicular membranes and whether the catalytically inactive E156K ARNO mutant, containing a mutation in the Sec7 domain, can act in a dominant negative manner to block LH/CG receptor desensitization. Results show that ARNO is readily detected in follicular membranes and that levels of membrane-associated ARNO increase with follicular maturation. The addition of catalytically inactive E156K ARNO blocks both the release of β-arrestin1 from its membrane docking site, based on Western blot analysis, and development of LH/CG receptor desensitization. We also investigated whether a point mutation in the pleckstrin homology (PH) domain of ARNO (R280D), which blocks binding of phosphoinositides like phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 4,5-bisphosphate (PIP2) but not catalytic activity, disrupts LH/CG receptor desensitization. R280D ARNO neither promotes nor inhibits LH/CG receptor desensitization, consistent with a requirement of the PH domain of ARNO for its association with the plasma membrane. LH/CG receptor activation of ARNO is not mediated by activation of phosphatidylinositol 3-kinase (PI 3-kinase) or by G protein βγ subunits. Taken together, these results suggest that LH/CG receptor promotes β-arrestin1 release from its membrane docking site to bind to the 3i loop of the LH/CG receptor via activation of membrane delimited endogenous ARNO. As ARNO activation is independent of PI 3-kinase and Gβγ, our results are consistent with a role for PIP2 in receptor-stimulated ARNO activation.

The binding of saturating concentrations of agonist to guanine nucleotide-binding (G) protein-coupled receptors initially results in productive coupling to an effector, resulting in increased effector activity. Thereafter, effector activity often declines or becomes desensitized as a result of the uncoupling of the agonist-bound receptor from its cognate G protein (1). We have used a cell-free plasma membrane model to investigate the cellular mechanism of homologous luteinizing hormone/choriogonadotropin (LH/CG) receptor desensitization independent of LH/CG receptor internalization (2, 3).

We have recently shown that desensitization of the LH/CG receptor requires the binding of membrane-delimited β-arrestin1 (Arrestin 2) to the third intracellular loop (3i) of the LH/CG receptor, resulting in reduced cAMP production (4, 5). This conclusion is based on evidence that preincubation of membranes with neutralizing anti-arrestin antibodies prevents development of LH/CG receptor desensitization (5), that β-arrestin1 binds directly and selectively to a synthetic peptide corresponding to the 3i loop of the LH/CG receptor (4), and that preincubation of membranes with a synthetic peptide corresponding to the 3i loop of the LH/CG receptor completely blocks development of receptor desensitization (4). We have also recently shown that LH/CG receptor activation not only exposes a binding site for β-arrestin1 at the 3i loop on the receptor but also leads to the apparent activation of the small G protein ADP-ribosylation factor 6 (ARF6), resulting in the release of a pool of β-arrestin1 from its membrane docking site (6). These conclusions are based on the following results. (a) ARNO (25 nM), a guanine nucleotide exchange factor (GEF) for ARFs 1 and 6 (7, 8), promotes desensitization of the LH/CG receptor in the presence but not the absence of GTP concomitant with the release of β-arrestin1 from its membrane docking site (6). (b) Catalytically dead E156K ARNO mutant (at 25 or 50 nM) does not cause LH/CG receptor desensitization (6). (c) Preincubation of membranes with synthetic N-terminal ARF6 peptide, but not with the corresponding ARF1 peptide, prevents the release of β-arrestin1 from its membrane docking site and thereby prevents development of LH/CG receptor desensitization (6). LH/CG receptor-dependent activation of the small G protein ARF6 is consistent with earlier studies showing that LH/CG receptor desensitization exhibits an obligatory requirement for GTP (3, 9–11).

The abbreviations used are: LH/CG, luteinizing hormone/choriogonadotropin; hCG, human choriogonadotropin; ARF, ADP ribosylation factor; ARNO, ARF nucleotide binding site opener; BSA, bovine serum albumin; 3i, third intracellular loop; Gs, stimulatory guanine nucleotide-binding protein; GTPγS, guanine nucleotide exchange factor; AC, adenylyl cyclase; PI, phosphatidylinositol; PIP2, phosphatidylinositol 4,5-bisphosphate; PH, pleckstrin homology.

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Catalytically Inactive ARNO Inhibits LH/CGR Receptor Desensitization

Based on evidence that ARNO is readily detected in membranes of preovulatory-size follicles, we next sought to determine whether catalytically inactive ARNO could compete with endogenous ARNO to block agonist-dependent LH/CGR receptor desensitization. The E156K mutation in the Sec7 domain of ARNO blocks the ability of ARNO to promote GTP exchange at ARF6 but does not disrupt its ability to bind phosphoinositides via its pleckstrin homology (PH) domain (14). Although preincubation of membranes with 50 (not shown) or 100 nM (Fig. 2A) E156K ARNO did not affect the extent of LH/CGR receptor desensitization (hCG/hCG conditions), preincubation of membranes with 200 nM E156K ARNO significantly reduced (p < 0.05) the extent of LH/CGR receptor desensitization. Thus, when membranes were preincubated with 200 (Fig. 2A), 300, or 400 (not shown) nM E156K ARNO, hCG-stimulated AC activity measured with hCG in stage 1 (i.e., desensitization conditions) was no longer significantly different from values measured with BSA in stage 1. The capability of E156K ARNO to block LH/CGR receptor desensitization was lost when E156K ARNO was boiled (Fig. 2B). The addition of E156K ARNO directly to the 5-min AC assay did not affect basal or hCG-, forskolin-, or aluminum fluoride-stimulated AC activities (Fig. 2C). This result shows that E156K ARNO does not have a nonspecific effect on AC activity or on the ability of the LH/CGR receptor or Gs to activate AC. To ascertain whether E156K ARNO indeed blocks development of LH/CGR receptor desensitization by preventing ARF6 activation, we determined whether E156K ARNO blocks the release of β-arrestin1 from its membrane docking site. In the absence of LH/CGR receptor activation, β-arrestin1 was readily detectable in follicular membranes (Fig. 2D, lane 4), presumably bound at a docking site distinct from the LH/CGR receptor (6). Incubation of membranes with hCG resulted in the retention of a fraction of β-arrestin1 at the membrane (Fig. 2D, lane 3). This fraction of β-arrestin1 was competed away with a synthetic peptide corresponding to the θ loop of the LH/CGR receptor (lane 2), consistent with the premise that LH/CGR receptor activation results in the binding of a fraction of β-arrestin1 to the θ loop of the activated LH/CGR receptor (4, 6). However, when E156K ARNO was included in the membrane incubation mix with hCG and θ peptide, this fraction of β-arrestin1 was retained in the membrane (lane 1), presumably at its membrane docking site as it was no longer competed away by the θ peptide. These results suggested that exogenous catalytically dead E156K ARNO effectively competes with endogenous ARNO to block the ability of the activated LH/CGR receptor to promote β-arrestin1 release and consequent LH/CGR receptor desensitization. Based on these results we can therefore conclude that endogenous ARNO is activated to promote GTP exchange at ARF6 in response to LH/CGR receptor activation.

ARNO contains a C-terminal PH domain that can bind phosphatidylinositol 3,4,5-trisphosphate (Pi(3,4,5)P₃), the product of PtdIns(3,4,5)P₃ (inositol 3,4,5-trisphosphate) and PI(4,5)P₂ (inositol 1,4,5-trisphosphate), with high selectivity and affinity (Kᵦ ≈ 85 nM (16)). A point mutation at R280D in the PH domain of ARNO results in a mutant protein that retains its catalytic activity to promote GTP exchange on ARF6 but cannot bind phosphoinositides (14). We have shown that, unlike catalytically active wild-type ARNO (at 25 nM), which promotes LH/CGR receptor desensitization, R280D ARNO at 50 nM does not promote LH/CGR receptor desensitization (6). This result suggests that the binding of phosphoinositides to the PH domain of ARNO is obligatory for LH/CGR receptor to promote activation of ARNO. We therefore sought to determine whether R280D ARNO could function like E156K ARNO in a dominant negative manner to block development of LH/CGR receptor desensitization in response to receptor activation. Preincubation

**EXPERIMENTAL PROCEDURES**

All chemicals were from previously described sources (4, 5). Recombinant ARNO and E156K ARNO were expressed and purified as described previously (7). A sucrose gradient-purified membrane fraction enriched in adenylyl cyclase (AC) activity was isolated from preovulatory-size porcine ovarian follicles and stored at −70 °C (6). The two-stage desensitization reaction (4) is summarized in the Fig. 2 legend. Supernatant and pellet fractions of porcine ovarian follicles were obtained by homogenizing tissue in 10 mM TRIS-HCl, pH 7.0, 1 mM EDTA with a glass-clasp Dounce homogenizer (10 strokes) followed by centrifugation at 1,000 × g for 5 min and then at 10,000 × g for 30 min. The final pellet was resuspended in 1 volume of the original homogenate, and an aliquot of the final supernatant and pellet fractions was obtained for protein determination. SDS-STOP was then added, and samples were boiled for 10 min and stored at 70 °C. Western blotting of anti-ARNO antibody (7) was performed as described previously (6, 7). The results were analyzed using Student’s t test (p < 0.05) (12).

**RESULTS**

We first determined whether ARNO is detectable in follicular membranes enriched in AC activity. Results (Fig. 1A, lane 4) show that a band reactive with affinity-purified anti-ARNO antibody (7) is readily detectable in porcine ovarian follicular membranes and migrates on SDS-polyacrylamide gel electrophoresis somewhat faster than His6-tagged recombinant human ARNO (lanes 5–8). Quantitation of the amount of ARNO in partially purified ovarian follicular membranes by Western blotting, using the signal generated by recombinant ARNO as the standard, yielded a concentration of ~1.5 μg of ARNO protein (or 32 nmol/mg of membrane protein. The basis for the ARNO doublet (see Fig. 1A, lanes 1–3) is not known but might correspond to phosphorylated and unphosphorylated ARNO (13, 14). We additionally determined whether the amount of ARNO present in unpurified 10,000 × g membrane and supernatant fractions is regulated by follicular maturation. Results (Fig. 1B) show that whereas ARNO levels are relatively low and unregulated in the supernatant fractions of small (1–2 mm) compared with large (8–10 mm) follicles, the levels of ARNO in the membrane fraction are higher (~6-fold) in large preovulatory follicles enriched in LH/CGR receptors compared with levels in small immature follicles that do not contain LH/CGR receptors (15). These results show that the expression of ARNO is increased with development of porcine follicles from an immature to a preovulatory phenotype and that the majority of ARNO is localized to the pellet fraction.
of follicular membranes with 200 (Fig. 3A) or 300 nm (not shown) R280D ARNO does not affect the ability of receptor activation to promote LH/CG receptor desensitization. Consistent with this result, preincubation of membranes with the PI 3-kinase inhibitor wortmannin at 100 nm does not reduce the extent of LH/CG receptor desensitization (Fig. 3B). As some PH domains also bind the βγ subunits of activated G proteins (17), we tested whether a βγ inhibitor peptide (18), corresponding to residues 956–982 of AC2, could block development of LH/CG receptor desensitization. The results (Fig. 3C) show that preincubation of follicular membranes with 10 μM βγ-inhibitor peptide QEHA (QEHAQEPEROYMCIGHTMVEFAAYALVGK), which blocks βγ-dependent stimulation of AC2, β-adrenergic receptor kinase, and phospholipase C (18), does not affect LH/CG receptor desensitization. Higher concentrations of the QEHA peptide inhibited basal and hCG- and forskolin-stimulated AC activities (Fig. 3C and not shown). Taken together, these results indicate that PIP3 or βγ signaling through the PH domain of ARNO does not appear to be obligatory for the activated LH/CG receptor to promote GTP exchange at ARF.

**DISCUSSION**

ARNO is detected in a purified membrane fraction enriched in AC activity obtained from preovulatory-size porcine ovarian follicles at a concentration of ~32 nmol/mg of membrane protein. Moreover, the expression of ARNO appears to be regulated with follicular maturation. Higher levels of ARNO are detected in membranes of mature preovulatory (8–10 mm) porcine follicles, which express LH/CG receptors, compared with small (1–2 mm) follicles, which do not express LH/CG receptors (15). That increased expression of ARNO correlates with the induction of LH/CG receptors is very interesting and suggests that the same stimulus that induces LH/CG receptors, namely follicle-stimulating hormone (19), might also increase expression of ARNO. These results further suggest that ARNO, in an apparently inactive conformation, is constitutively associated with the plasma membrane of preovulatory ovarian follicles. The conclusion that ARNO is present in an inactive conformation and is activated upon engagement of the LH/CG receptor is based not only on results presented in Fig. 2, showing that LH/CG receptor desensitization is blocked by E156K ARNO, but is also consistent with our previous experiments showing that catalytically inactive ARNO blocks LH/CG receptor desensitization (20). The results shown support the idea that the catalytically inactive conformation of ARNO plays a role in the regulation of LH/CG receptor desensitization, possibly by blocking the activation of β-arrestin and preventing desensitization of the LH/CG receptor.
ARNO, but also on evidence that ARF activation, measured as cholera toxin-catalyzed ADP-ribosylation of the long form of Gαs, is negligible in the absence of LH/CG receptor activation (20). The basis for the apparently constitutive membrane association of ARNO is not clear. However, in other cellular models there is also evidence that a large portion of total cellular ARNO can be associated constitutively with the plasma membrane in the absence of directed membrane-receptor activation both on overexpression in various cell lines (7) and in chromaffin cells isolated from bovine adrenal glands (21).

We have shown with the porcine follicular membrane model that catalytically inactive E156K ARNO acts in a dominant negative manner to selectively block the development of LH/CG receptor desensitization in response to LH/CG receptor activation. Catalytically inactive E156K ARNO blocks the obligatory release of β-arrestin1 from its membrane docking site. As a result, the LH/CG receptor remains active to signal to Gs and AC despite the continued presence of saturating concentrations of receptor agonist. This result suggests that LH/CG receptor desensitization requires the activation of endogenous ARNO. However, based on the recent identification of the exchange factor for ARF6 (EFA6) (22), in which the guanine nucleotide exchange activity is also insensitive to brefeldin A, we cannot rule out the possibility that the apparent dominant negative effect of E156K ARNO is attributable to its potential ability to sequester available ARF6, thus indirectly blocking LH/CG receptor desensitization potentially mediated by EFA6.

ARNO, along with GRP1 and cytohesin-1, comprise a subfamily of guanine nucleotide exchange factors for the ARFs in which the guanine nucleotide exchange activity is not inhibited by the fungal metabolite brefeldin A (7, 8, 23–25). Each of these factors contains an N-terminal coiled-coil domain, a central Sec7 domain, that is sufficient for guanine nucleotide exchange activity on ARF (26) and is highly homologous with the Sec7 gene in yeast which encodes a GEF required for protein secretion (reviewed in Ref. 27), a C-terminal PH domain, and an adjacent cluster of basic residues (c domain) (8).

The PH domain of the proteins in this subfamily appears to be necessary both for their plasma membrane association and for subsequent guanine nucleotide exchange activity toward membrane-localized ARFs (14, 23, 24, 28, 29). These guanine nucleotide exchange proteins are often localized to the cytosolic fraction of unstimulated cells and have been shown to translocate to the plasma membrane in response to receptor activation by insulin or epidermal growth factor in 3T3 L1 adipocytes and Chinese hamster ovary cells (16, 30). Consistent with these results, transfection of cells with ARNO or cytohesin-1 containing a mutation in the PH domain disrupts the plasma membrane association of these proteins and consequent biological responses, namely cytohesin-1-dependent β2 integrin adhesion to adhesion molecule 1 in Jurkat E6 leukemia cells (23) and ARNO-dependent actin reorganization in HeLa cells (14).

The predominant ligands that have been shown to bind to the PH domains of these GEFs are phosphatidylinositol 4,5-bisphosphate (PIP2) and PIP3 (31). Although there have been seemingly contradictory results on the affinities of the PH domains of these proteins for PIP2 or PIP3, recent evidence from the Czech laboratory (32) has resolved this apparent controversy by showing that ARNO, GRP1, and cytohesin-1 can seemingly contradictory results on the affinities of the PH domains of these GEFs for PIP3 and PIP2 depending on whether the PH domain contains a diglycine or triglycine motif, respectively. Moreover, isoforms of each of the proteins in this subfamily appear to exist that express either the diglycine or triglycine motif even in the same tissue (7, 8, 33, 34). Consistent with this result, recruitment and activation of these GEFs can depend on either PIP2 or PIP3, contingent upon whether the diglycine or trigly-
Although ARNO activation by the LH/CG receptor is independent of the PH domain and receptor desensitization based on the ability of ARNO to promote LH/CG receptor desensitization, since R280D ARNO retains its GTP exchange activity (14). Yet, R280D ARNO does not promote LH/CG receptor desensitization (Ref. 6 and present report). The inability of R280D ARNO to promote LH/CG receptor desensitization might be attributable to its inability to appropriately associate with the plasma membrane because of the mutation in its PH domain. A similar argument can be made for the inability of R280D to block desensitization in a dominant negative manner. Our results showing that R280D ARNO neither stimulates nor inhibits LH/CG receptor desensitization indirectly suggest that the PH domain of ARNO is indeed necessary at least for its membrane association. However, the ineffectiveness of the PI 3-kinase inhibitor wortmannin to block desensitization indicates that ARNO activation is not dependent on PI3 generated by PI 3-kinase activation. As PI3 does not appear to participate in the activation of ARNO leading to LH/CG receptor desensitization, it is likely that the predominant ARNO present in follicle membranes contains the triglycine motif in its PH domain, which does not selectively bind PI3 but rather binds PI2 with equivalent affinity (32, 36). The recombinant E156K ARNO and ARNO proteins that we have used contain the triglycine motif and are regulated by PIP2 rather than PIP3. ARNO and ARNO proteins that we have used contain the triglycine motif and are regulated by PIP2 rather than PIP3. ARNO and ARNO proteins that we have used contain the triglycine motif and are regulated by PIP2 rather than PIP3.

In conclusion, these studies show that endogenous membrane-delimited ARNO appears to be obligatory for the LH/CG receptor to promote β-arrestin1 release from its membrane docking site and receptor desensitization based on the ability of catalytically inactive ARNO to block both of these responses. Although ARNO activation by the LH/CG receptor is independent of PI3 and Gβγ, ARNO activation could well be dependent on PI2. The PH domain of ARNO also appears to be obligatory for the LH/CG receptor to promote membrane binding and is likely to be the same one that leads to its activation.
