Minireview

ARF6: a newly appreciated player in G protein-coupled receptor desensitization

Mary Hunzicker-Dunn[a,*], Vsevolod V. Gurevich[b], James E. Casanova[c], Sutapa Mukherjee[a]

[a] *Department of Cell and Molecular Biology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611, USA*
[b] *Department of Pharmacology, Vanderbilt University School of Medicine, 454 Robinson Research Building, Nashville, TN 37232, USA*
[c] *Department of Cell Biology, University of Virginia Health Sciences Center, Charlottesville, VA 22908, USA*

Received 16 April 2002; revised 2 May 2002; accepted 7 May 2002

First published online 21 May 2002

Edited by Veli-Pekka Lehto

Abstract The luteinizing hormone/choriogonadotropin hormone receptor (LH/CG R) signals to regulate ovulation, corpus luteum formation, and fetal survival during pregnancy. Agonist binding to the LH/CG R is poorly reversible, emphasizing the importance of a cellular mechanism to temper signaling by a potentially persistently active receptor. Like other G protein-coupled receptors (GPCRs), signaling by this receptor is modulated by its binding of an arrestin. We have identified ADP ribosylation factor 6 (ARF6) as a protein whose activation state is regulated by the LH/CG R and which functions to regulate the availability of plasma membrane-docked arrestin 2 to this receptor. We hypothesize that ARF6 might also serve GPCRs other than the LH/CG R to regulate the availability of arrestin 2 for receptor desensitization. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: ADP ribosylation factor 6; ADP ribosylation factor nucleotide binding site opener; Arrestin; G protein-coupled receptor

1. Introduction

ADP ribosylation factor 6 (ARF6) is an abundant plasma membrane-localized protein [1] poised to modulate signaling of the large family of guanine nucleotide-binding (G) protein-coupled receptors (GPCRs). ARF6 is a monomeric G protein which cycles between a GDP-bound inactive conformation and a GTP-bound active conformation [2]. Rate-limiting GDP release is catalyzed by a class of guanine nucleotide exchange factors (GEFs), which include the ARF nucleotide binding site opener (ARNO)/cytohesin, exchange factor for ARF6 (EFA6), and ARF-guanine nucleotide exchange protein 100 (ARF-GEP100) subclasses [3,4]. The GTPase activity of ARF6 [2] is stimulated by a family of GTPase activating proteins (GAPs) [5–7]. Functions attributed to ARF6 include regulation of membrane ruffling and cell motility, aspects of endocytosis, exocytosis, and membrane recycling, as well as reorganization of the cortical actin cytoskeleton and activation of phospholipase D [6,8,9]. Although it has been recognized for some time that activation of an ARF occurs upon agonist activation of a number of different GPCRs, including the β_2-adrenergic, m3 muscarinic acetylcholine, fMet-Leu-Phe, H1 histamine, gonadotropin releasing hormone, and B2 bradykinin receptors [10–13], the function of this ARF is poorly understood. We showed that an ARF is also activated following agonist binding to the luteinizing hormone/choriogonadotropin hormone receptor (LH/CG R) [14], and identified that ARF as ARF6 [15].

We recently described an unappreciated role for the activation of ARF6 in desensitization of the LH/CG R. We have established that the activation state of ARF6 in ovarian follicular membranes regulates the availability to the LH/CG R of a pool of arrestin 2, as summed in the model presented in Fig. 1. In the inactive $ARF6^{GDP}$ conformation, arrestin 2 is bound to ARF6 (directly or indirectly) and is not accessible to the receptor (Fig. 1A). With ARF6 activation, arrestin 2 is undocked from ARF6 (Fig. 1B) and now available to bind to active LH/CG R to promote desensitization (Fig. 1C). Recent evidence shows that desensitization of the LH/CG R heterologously expressed in human embryonic kidney 293 (HEK293) cells also requires ARF6 activation [16]. This result supports the hypothesis that the availability of arrestin 2 for desensitization of GPCRs other than the LH/CG R might also be regulated by the activation state of ARF6. Data which led to identification of a role for ARF6 in regulating the availability of arrestin 2 to the LH/CG R are reviewed in this report.

2. GPCR desensitization and internalization

GPCR desensitization describes the uncoupling of a GPCR from its cognate G protein and results in reduced signaling of the receptor to the G protein-linked effector. Based initially on rhodopsin [17,18] and subsequently on β_2-adrenergic receptor (β_2-AR) [19,20], desensitization of GPCRs is generally believed to be triggered by the recruitment of and consequent phosphorylation of the GPCR by a G protein-regulated kinase (GRK). An arrestin is then recruited from the cytosol [21] and upon interacting with the phospho-receptor, binds to receptor with high affinity [22]. The mammalian arrestins include the visual arrestins (arrestin 1 and arrestin 4) and the ubiquitous arrestin 2 (or βarrestin 1) and arrestin 3 (or βarrestin 2) [23]. Arrestin mediates receptor desensitization by sterically hindering the ability of the receptor to activate its cognate G protein [24]. For receptors other than rhodopsin, arrestin also serves as a scaffold to target the receptor for endocytosis, by binding clathrin and the β subunit of the clathrin adapter protein AP-2 [25,26], and as a docking site for enzymes associated with other signaling pathways, including those for the mitogen-activated protein kinase/extracellular regulated kinases [19,27]. Therefore, uncoupling of GPCRs from their associated G proteins can lead

*Corresponding author.
E-mail address: mhd@northwestern.edu (M. Hunzicker-Dunn).

0014-5793/02/$22.00
PII: S0014-5793(02)02822-3

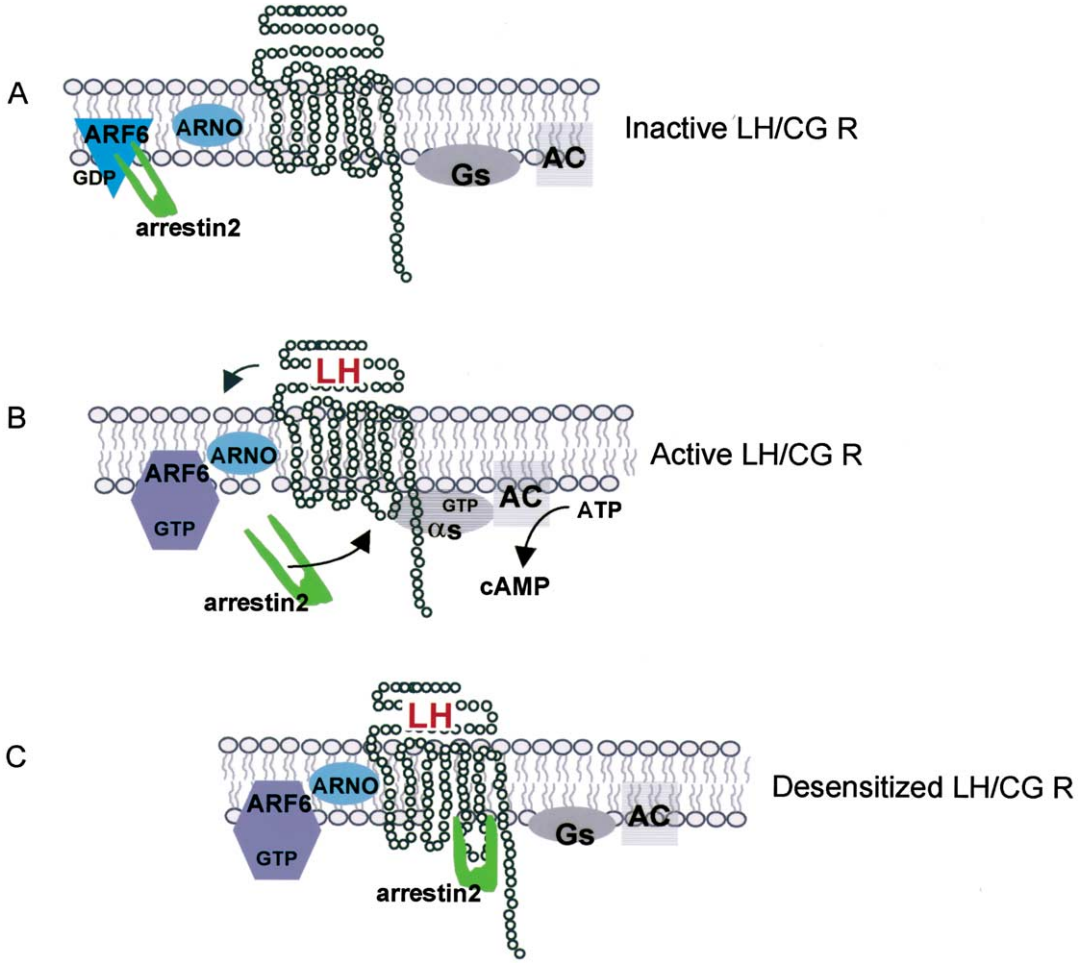

Fig. 1. Model of LH/CG R desensitization. Shown in A is the docking of arrestin 2 at a membrane location distinct from the inactive LH/CG R and in association with inactive $ARF6^{GDP}$. Activation of the LH/CG R upon binding agonist, as shown in B, not only promotes activation of the stimulatory heterotrimeric G protein Gs and consequent adenylyl cyclase (AC) activation by αs^{GTP}, but also promotes activation of ARF6 and liberation of arrestin 2. ARNO or a similar ARF6 GEF promotes GDP release from ARF6 in response to LH/CG R activation. Arrestin 2 binding to the 3i loop of the active LH/CG R, shown in C, mediates desensitization by interfering with the ability of the agonist-activated receptor to activate Gs.

to the coupling of GPCRs to other signaling pathways. The majority of results leading to these conclusions are based on the heterologous expression of the GPCR, GRK, and the arrestin. Integrated into the literature of receptor desensitization is an extensive literature on receptor internalization that is based on evidence that for many GPCRs, receptor internalization results from GRK-dependent receptor phosphorylation and subsequent binding of an arrestin [20]. However, it is not clear for most GPCRs outside the visual system whether the binding of arrestin to a particular site mediates both receptor desensitization and internalization or whether there are two separate binding sites possibly for distinct non-visual arrestins. Recent evidence for the chemokine R CXCR4 [28], the β_2-AR [29,30], and the LH/CG R [16,31,32] suggests that desensitization and internalization of GPCRs may be mediated by distinct pools of non-visual arrestins.

However, for a few receptors such as the LH/CG R, even though this receptor exhibits GRK- and agonist-dependent phosphorylation in a heterologous expression model leading to receptor desensitization and internalization [33,34], receptor phosphorylation is not obligatory for desensitization [35–39]. Moreover, LH/CG R desensitization in a cell-free membrane model exhibits a critical requirement for GTP [37,38,40,41]. Similar reports on the obligatory role of GTP in GPCR desensitization exist for the endogenous β_2-AR [42] and vasopressin R [43] in kidney cell membranes. Desensitization of the LH/CG R can also be reversed by the non-hydrolyzable GDP analog GDPβS, generating an active receptor [44]. These results suggest either that desensitization of the LH/CG R is mediated by a unique mechanism or that the cell-free membrane model afforded identification of elements of desensitization not readily discernible in an intact cellular model.

3. ARF6/arrestin 2-dependent LH/CG R desensitization

3.1. LH/CG R desensitization is mediated by membrane-delimited arrestin 2

Desensitization of the native LH/CG R can be readily demonstrated in a cell-free membrane model prepared from ovarian follicles [45] (which endogenously express ~10 000 receptors/cell [46]). All of the proteins required to mediate desensitization must therefore be present in this membrane preparation. We initially determined whether uncoupling of the LH/CG R from Gs and adenylyl cyclase (AC) was dependent on the binding of a membrane-associated arrestin to the receptor. The addition of neutralizing anti-arrestin antibodies that block arrestin's interaction with all GPCRs prevented agonist from promoting desensitization [47]. Consistent with this result, arrestin 2 but not arrestin 3 was readily detected by Western blotting in an AC-enriched membrane preparation

[47]. These results show that desensitization of the LH/CG R requires a pool of membrane-delimited endogenous arrestin 2 and that this obligatory arrestin 2 need not be recruited from another cellular compartment. However, arrestin 2 coimmunoprecipitated only with active but not with inactive native receptor [16,48]. This result indicates that arrestin 2 must be 'docked' in the membrane at a site that is distinct from the inactive LH/CG R (see Fig. 1A).

Arrestin 2 binds specifically to the third intracellular (3i) loop of the active LH/CG R. This conclusion is based on evidence that a synthetic peptide corresponding to the 3i loop of the receptor selectively competed with receptor for arrestin 2 binding and blocked agonist-dependent receptor desensitization [49]. Neither a scrambled 3i loop peptide nor a 2i loop peptide blocked receptor desensitization [49]. Moreover, surface plasmon resonance studies showed that arrestin 2 bound with pM affinity to the 3i loop peptide and exogenous arrestin 2 promoted desensitization of the active intact LH/CG R in follicular membranes with an ED_{50} of ~10 pM [16]. Surprisingly, however, while visual arrestin 1 also bound with pM affinity to the 3i loop peptide of the LH/CG R and promoted desensitization of intact receptor in follicular membranes with an ED_{50} of ~10 pM, arrestin 3 bound with only mM affinity to the 3i loop peptide and did not promote receptor desensitization [16]. Thus, desensitization of the LH/CG R is selectively mediated by arrestin 2 binding to the 3i loop of the active receptor. Consistent with the absence of detectable levels of arrestin 3 in follicular membranes, arrestin 3 did not coimmunoprecipitate with active (or inactive) LH/CG R [16]. Thus, arrestin 3 does not participate in LH/CG R desensitization.

An Asp residue in the 3i loop of the LH/CG R is crucial for arrestin 2 binding [16]. Mutation of the Asp to a Gly yielded a receptor that did not bind arrestin 2 or become desensitized [16] and which exhibited mild constitutive activity [50–52]. Mutation of the Asp to the similarly charged Glu, to Asn which is uncharged but has similar hydrogen bonding capability, or to non-polar Val yielded receptor that did not bind arrestin 2 [16]. This Asp residue in the 3i loop is therefore crucial for arrestin 2 binding to the LH/CG R. However, this Asp residue does not simply substitute for the negatively charged phosphate group of other GPCRs since a D564G mutant LH/CG R expressed in HEK293 cells was not desensitized by arrestin 2 mutants that are 'constitutively active' and do not require phospho-receptor to achieve high affinity binding to receptor [16]. The homologous Asp residue is conserved in the glycoprotein hormone and cannabinoid receptors but is generally replaced with a similarly charged Glu residue in most other GPCRs [53]. Perhaps this conserved Asp may also allow the other glycoprotein hormone and cannabinoid receptors to bind arrestin 2 in a phosphorylation-independent manner to promote desensitization.

3.2. The GTP-dependent step of LH/CG R desensitization is the undocking of arrestin 2

It can be readily demonstrated in a cell-free membrane model that agonist-dependent LH/CG R desensitization critically requires GTP [37,38,40,41]. However, arrestin 2 binding to the active LH/CG R does not constitute the GTP-dependent step of LH/CG R desensitization since addition of exogenous arrestin 2 promotes desensitization in the absence of GTP [48]. This result indicates that the GTP-dependent step is upstream of arrestin 2 binding to the LH/CG R. To determine whether the GTP-dependent step of LH/CG R desensitization was the release of arrestin 2 from its membrane docking site, membranes were incubated (30 min at 30°C) without agonist but in the presence or absence of 100 μM GTP. Membranes were then washed, and pellets assayed for arrestin 2 content by Western blotting and for LH/CG R desensitization by AC assays. GTP promoted the release of the majority of membrane-delimited arrestin 2 from its docking site (Fig. 2A, compare lanes 1 and 2). Moreover, these membranes were no longer capable of agonist-dependent LH/CG R desensitization [48], suggesting that the obligatory arrestin 2 had been washed away. These results establish that the arrestin 2 required for LH/CG R desensitization is not simply trapped in the membranes. Rather, GTP is required to free arrestin 2 so that it is accessible to bind to active LH/CG R.

3.3. Activation of ARF6 triggers the undocking of arrestin 2

That desensitization requires GTP in the presence of agonist suggests the probable involvement of a G protein. As our earlier studies suggested that desensitization appeared to be independent of heterotrimeric G proteins [54], we directed our attention to the large families of monomeric small G proteins. Preincubation of follicular membranes with active toxins which selectively inhibited Rho, Ras, Rap and Rac GTPases did not affect desensitization [48]. Desensitization was also unaffected by preincubation with brefeldin A [48], a fungal metabolite that inhibits the guanine nucleotide exchange activity of many GEFs that activate ARFs 1–5 but not those like ARNO that activate ARF6 [4,55]. ARF6 was thus a

Fig. 2. The release of arrestin 2 from its membrane docking site in the absence of receptor agonist requires either high concentrations of GTP or exogenous ARNO and low GTP concentrations. A: Ovarian follicular membranes were incubated (30 min at 30°C, in 25 mM bis-Tris-propane buffer, pH 7.2, 5 mM $MgCl_2$, 1 mM EGTA, 0.4 mM EDTA) in the absence of receptor agonist but in the presence of water, 100 μM GTP, or 100 μM GTP plus 25 μM Non-Myr ARF6 peptide, as indicated, diluted ~25-fold with 10 mM Tris–HCl, and pelleted. Pelleted membranes were subjected to SDS–PAGE and Western blot analysis using anti-arrestin 2 antibody. B: Membranes were incubated as in A in the presence of indicated additions, then diluted and subjected to SDS–PAGE and Western blot analysis. From Mukherjee et al. [48].

candidate for the G protein required for LH/CG R desensitization. Addition of nM concentrations of ARNO, a GEF which activates both ARF1 and ARF6, in the absence of agonist promoted desensitization of active LH/CG R [48]. ARNO was effective only in the presence and not in the absence of GTP [48]. ARNO containing a mutated PH domain (ARNO[R280D]) or a mutation in the catalytic site (ARNO[E156K]) did not promote desensitization [48]. Most importantly, the effect of ARNO was completely reversed by neutralizing anti-arrestin antibodies that prevent interactions of all arrestins with GPCRs [48]. Overall, these results suggest that ARNO promoted the apparent release of arrestin 2 from its membrane docking site (see Fig. 1A). Critical tests yielded results that support this model. When receptor was not activated, the addition of exogenous ARNO plus 1 μM GTP promoted the release of arrestin 2 from its membrane docking site which was washed away when membranes were repelleted (Fig. 2B, compare lanes 1 and 2) [48]. When receptor was activated, catalytically inactive ARNO blocked agonist-dependent LH/CG R desensitization by preventing arrestin 2 release from its docking site, evidenced by the retention of membrane-associated arrestin 2 which was not competed away by synthetic 3i loop peptide [56]. Taken together these results indicate that a pool of arrestin 2 is sequestered at the plasma membrane and that this pool can be discharged by a GTP-dependent pathway regulated by LH/CG R activation and ARNO.

As ARNO activates both ARF1 and ARF6, we sought to determine whether the activation of ARF1 or ARF6 was mediating LH/CG R-stimulated desensitization by releasing sequestered arrestin 2. Upon preincubation of follicular membranes with synthetic N-terminal peptides of ARF1 and ARF6 which inhibit activation of ARF1 and ARF6 [57,58], respectively, only the ARF6 inhibitory peptide blocked arrestin 2 release (Fig. 2A, lane 3). The ARF6 inhibitory peptide, but not the ARF1 inhibitory peptide, also inhibited the ability of LH/CG R to activate ARF6 activity in follicular membranes [15] and abrogated agonist-dependent desensitization [48]. These results indicate that LH/CG R activation indeed stimulates the activation of ARF6 and consequent release of a pool of arrestin 2. Conversion of ARF6 from its GDP-bound to its active GTP-bound state thus appears to comprise the GTP-dependent step of LH/CG R desensitization.

Our model for the roles of ARNO and ARF6 in LH/CG R desensitization is depicted in Fig. 1. ARNO, ARF6, and arrestin 2 are readily detected by western blotting in the AC-enriched follicular membrane preparation [15,47,48,56]. Upon receptor activation and consequent ARF6 activation (panel B), a pool of arrestin 2 is released from its membrane docking site and binds with high affinity to the 3i loop of the active but unphosphorylated receptor (panel C). We have shown that ARNO causes LH/CG R desensitization [48], that ARNO is present in follicular membranes at a relatively high concentration (1.5 μg or 32 nmol/mg membrane protein), and that catalytically inactive ARNO blocks agonist-stimulated LH/CG R desensitization [56]. We have not proven, however, whether ARNO is the GEF responsible for ARF6 activation downstream of LH/CG R activation by agonist or if another GEF is responsible. Arrestin 2 binding to the receptor uncouples the R from Gs, leading to a reduced rate of cAMP production. ARF6 activation thus serves as a trigger to release arrestin 2.

4. Does ARF6 play a restricted role in LH/CG R desensitization or a more universal role in GPCR desensitization and internalization?

We have recently shown that the murine LH/CG R stably expressed in HEK293 cells also exhibits agonist-dependent cell-free receptor desensitization [16]. This desensitization is dependent on membrane-bound arrestin 2 and inhibited by neutralizing anti-arrestin antibodies [16]. LH/CG R desensitization in HEK293 cells is also specifically inhibited by the inhibitory N-terminal ARF6 peptide [16]. The presence of the agonist-dependent ARF6-regulated release of arrestin 2 from a docking site in HEK293 cell membranes (which do not normally express LH/CG Rs) suggests that this pathway may be a more widespread mechanism to regulate the accessibility of arrestin 2 to bind to active GPCRs. Support for this hypothesis was provided by evidence that ARF activation occurs in response to the activation of a number of GPCRs, including the FSH R [15], β_2-AR [10], m3 muscarinic acetylcholine R [11], fMet-Leu-Phe R [12], H2 histamine R and B2 bradykinin R [13]. While some of these examples of GPCR-stimulated ARF activation are believed to lead to activation of phospholipase D [13,59,60], it is entirely possible that they also stimulate arrestin 2 release. Support for this hypothesis was also provided by evidence that over-expression of the ARF GAP GIT1 inhibits β_2-AR internalization [61], and by recent evidence that over-expressed ARNO can stimulate while GTP binding-deficient ARF6 retards the rate of β_2-AR internalization in HEK293 cells [62]. The relationship between GPCR desensitization and internalization is complex, probably receptor-specific, and has not been extensively evaluated. For some GPCRs, the same arrestin binding to a single site on the receptor probably leads to both desensitization and internalization [63,64]. For other GPCRs, there is recent evidence that arrestins (same or distinct?) bind to two different regions on the receptor, one of which drives desensitization and the other drives internalization [28]. For the latter group of GPCRs, whether the availability of the arrestin which drives receptor internalization is regulated by ARF6 or more likely is recruited from the cytosol [21] has not been clarified.

5. Concluding remarks

It is our hypothesis that the GPCR-stimulated ARF6 activation which liberates a pool of arrestin 2 to bind to active receptor may be a relatively universal mechanism to trigger arrestin release for desensitization. Future studies are needed to test this hypothesis. For some GPCRs, an additional restraint is placed on the receptor by the requirement that the receptor be phosphorylated by a GRK in order to achieve high affinity arrestin binding. For the LH/CG R, this impediment to arrestin 2 binding is not imposed, possibly to insure that uncoupling of the active receptor from Gs and cAMP formation occurs and/or to insure the initiation of additional signaling pathways as a consequence of arrestin 2-LH/CG R interactions. It is not surprising, based on the importance of the LH/CG R to ovulation, corpus luteum formation, and fetal survival, that the activity of the LH/CG R to signal is precisely regulated.

Acknowledgements: We gratefully acknowledge members of the Hun-

zicker-Dunn lab who contributed to these results, including Evelyn T. Maizels, Regina D. Horvat, and Lisa M. Salvador. We also gratefully acknowledge the gifts of extremely valuable reagents from the following collaborators: Richard A. Kahn, Mariel and Lutz Birnbaumer, Heidi Hamm and Anita Preninger, Krzysztof Palczewski, Larry Donoso, and Klaus Aktories.

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