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MINIREVIEWS

Arrestin-mediated signaling: Is there a controversy?

Vsevolod V Gurevich, Eugenia V Gurevich

ORCID number: Vsevolod V Gurevich (0000-0002-3950-5351); Eugenia V Gurevich (0000-0002-0563-8295).

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Corresponding author to: Vsevolod V Gurevich, PhD, Professor, Department of Pharmacology, Vanderbilt University, 2200 Pierce Ave, PRB Rm 417D, Nashville, TN 37232, United States. vsevolod.gurevich@vanderbilt.edu

Vsevolod V Gurevich, Eugenia V Gurevich, Department of Pharmacology, Vanderbilt University, Nashville, TN 37232, United States

Abstract

The activation of the mitogen-activated protein (MAP) kinases extracellular signal-regulated kinase (ERK)1/2 was traditionally used as a readout of signaling of G protein-coupled receptors (GPCRs) *via* arrestins, as opposed to conventional GPCR signaling *via* G proteins. Several recent studies using HEK293 cells where all G proteins were genetically ablated or inactivated, or both non-visual arrestins were knocked out, demonstrated that ERK1/2 phosphorylation requires G protein activity, but does not necessarily require the presence of non-visual arrestins. This appears to contradict the prevailing paradigm. Here we discuss these results along with the recent data on gene edited cells and arrestin-mediated signaling. We suggest that there is no real controversy. G proteins might be involved in the activation of the upstream-most MAP3Ks, although *in vivo* most MAP3K activation is independent of heterotrimeric G proteins, being initiated by receptor tyrosine kinases and/or integrins. As far as MAP kinases are concerned, the best-established role of arrestins is scaffolding of the three-tiered cascades (MAP3K-MAP2K-MAPK). Thus, it seems likely that arrestins, GPCR-bound and free, facilitate the propagation of signals in these cascades, whereas signal initiation *via* MAP3K activation may be independent of arrestins. Different MAP3Ks are activated by various inputs, some of which are mediated by G proteins, particularly in cell culture, where we artificially prevent signaling by receptor tyrosine kinases and integrins, thereby favoring GPCR-induced signaling. Thus, there is no reason to change the paradigm: Arrestins and G proteins play distinct non-overlapping roles in cell signaling.

Key words: G protein-coupled receptors; Arrestin; G protein; Signaling; Extracellular signal-regulated kinase 1/2; c-Jun N-terminal kinase 3

Core tip: Both arrestins and G proteins play important roles in G protein-coupled receptor (GPCR) signaling, including GPCR-initiated activation of mitogen-activated protein (MAP) kinases extracellular signal-regulated kinase (ERK)1/2 and c-Jun N-terminal kinase 3 (JNK3). Their roles do not overlap. G proteins participate in signal initiation, by activating MAP3Ks. Arrestins, free and GPCR-bound, function as scaffolds of the three-tiered MAP kinase cascades, facilitating signal transduction. Cells express other scaffolds, so that no MAPK cascade relies solely on arrestins. Different experimental paradigms highlight the role of G proteins or arrestins in this process, and

Telephone: +1-615-3227070
Fax: +1-615-3436532

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neither can be discounted based on available evidence.

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INTRODUCTION

G-protein-coupled receptors (GPCRs) respond to hormones, neurotransmitters, light, odorants, taste molecules, extracellular calcium, extracellular protease activity, cell adhesion, and a variety of other stimuli[1]. All members of the GPCR super-family (which includes hundreds of receptors encoded by different genes in animals) share a common transmembrane domain consisting of seven α-helices, which are connected by intra- and extra-cellular loops of variable lengths[2]. Their extracellular N-termini and intracellular C-termini also differ widely in size and structure[2]. Upon activation by an appropriate input most GPCRs serve as guanyl nucleotide exchange factors of heterotrimeric G proteins, facilitating the release of guanosine diphosphate (GDP) bound to their inactive α-subunits and its exchange for guanosine triphosphate (GTP), which is a lot more abundant in cells. Activated G proteins then dissociate from the receptors, their α- and βγ-subunits separate and activate or inhibit various effectors. Active GPCRs can sequentially activate several molecules of G proteins, providing signal amplification at this level. Active GPCRs are also specifically phosphorylated by G protein-coupled receptor kinases (GRKs)[3], of which most mammals have seven. Nocturnal rodents only have six, as they are missing GRK7, specialized GRK expressed in cone photoreceptors, which function in relatively bright light.

ARRESTIN-MEDIATED GPCR DESENSITIZATION

The first arrestin family member (current systematic name arrestin-1) was discovered in the visual system as the protein that specifically binds active phosphorylated rhodopsin and suppresses its signaling[4]. Thus, desensitization, *i.e.*, the suppression of G protein-dependent signal transduction, was the first arrestin function discovered. Subsequently the first[5] and then the second non-visual arrestin[6-8] were cloned. The demonstration that the first non-visual arrestin preferentially desensitized phosphorylated β2-adrenergic receptors (β2AR) (which gave it the original name, β-arrestin; systematic name arrestin-2), whereas visual arrestin-1 preferentially desensitized phosphorylated rhodopsin[9], suggested the idea that all arrestins desensitize cognate GPCRs *via* specific binding to their active phosphorylated state[10]. Thus, the field came to believe that the model of two-step desensitization, phosphorylation of active GPCRs by specific GRKs, reviewed in[3], followed by arrestin binding to the active phosphorylated receptor, applies to all GPCRs[10-12]. In this paradigm, the role of arrestins is to stop GPCR signaling *via* G proteins. This remains the best characterized biological function of all arrestin proteins[11]. Subsequent findings that receptor-associated non-visual arrestins directly bind clathrin[13] and clathrin adaptor, adaptor protein 2 (AP2)[14], the key components of the coated pit, and that the binding to both is enhanced by arrestin-receptor interactions[15], suggested that arrestins participate in the next step of desensitization, *i.e.* receptor removal from the plasma membrane *via* internalization.

GPCR-DEPENDENT ARRESTIN SIGNALING

The arrestin-mediated cellular signaling was first discovered upon GPCR stimulation, and therefore was assumed to be strictly receptor-dependent. The binding of non-visual arrestins to their cognate receptors was shown to facilitate the activation of protein kinases proto-oncogene tyrosine-protein kinase Src (c-Src)[16], c-Jun N-terminal kinase 3 (JNK3)[17], then extracellular signal-regulated kinase (ERK)1/2[18]. As JNKs and ERKs are mitogen-activated protein kinases (MAPKs) activated *via* the three-tiered kinase cascade (in general terms, MAP3K, MAP2K, and MAPK[19,20]), the latter two cases suggested that receptor-bound arrestins scaffold the three-kinase modules,

thereby facilitating signal transduction in them. Initial studies detected direct arrestin binding to both MAP3Ks, proto-oncogene serine/threonine-protein kinase (cRaf) (a.k.a. Raf1) and apoptosis signal-regulating kinase 1 (ASK1), and corresponding MAPKs, ERK1/2 and JNK3, but not to the MAP2Ks of these cascades, MEK1 or MKK4/7[17,18]. However, subsequently arrestin interactions with MEK1[21], as well as with MKK4 and MKK7[22,23] were documented. Thus, the idea of scaffolding of MAP kinase cascades by arrestin bound to a GPCR received further experimental support. The binding of arrestins to ERK1/2 is barely detectable in the absence of activated GPCR[24], and both arrestin binding to ERK1/2 and arrestin-dependent ERK1/2 activation are greatly facilitated by GPCR stimulation[18]. Therefore, arrestin-dependent ERK1/2 activation following GPCR stimulation in the experimental conditions excluding other inputs (see below) became a readout of choice for arrestin-mediated signaling. It has been shown that GPCRs that form stable complexes with arrestins tend to increase ERK1/2 activity in the cytosol, presumably *via* retaining ERK1/2 activated by the GPCR-bound arrestin scaffold in that compartment, whereas GPCRs that form transient complexes with arrestins induce mitogenic response due to the translocation of active ERK1/2 to the nucleus, where it acts on its nuclear substrates[25]. Moreover, using siRNA knockdown ERK1/2 activation by angiotensin II type 1A receptor *via* G proteins (likely $G_{q/11}$) was found to be transient, peaking at 2 min and then rapidly declining, whereas arrestin-mediated activation of ERK1/2 was shown to peak later and last much longer[26]. Even though ERK1/2 can be activated *via* a variety of pathways in the cell[27], it became widely accepted that the late phase (10-30 min after the stimulus) of ERK activation reflects GPCR signaling *via* arrestins[28,29]. However, it has been shown that G protein-mediated ERK1/2 activation can also have a late phase (see as the first report of this phenomenon[30], reviewed in[31]). As the late phase of ERK1/2 activation was subsequently shown to be mediated by G proteins in several other studies involving different GPCRs, the time course of ERK1/2 activation cannot be regarded as an indication of it being G protein- or arrestin-dependent.

The molecular mechanism of arrestin-mediated connection between GPCRs and proteins containing Src homology 3 (SH3) domains was recently extensively investigated using biophysical methods[32]. The data suggest that arrestin "unwinds" the auto-inhibited conformation of c-Src, thereby directly activating this kinase. Free arrestin-2 was found to have detectable effect, but an increase in Src activity upon binding to the arrestin-2 engaged by an appropriately phosphorylated GPCR, or, to a lesser extent, receptor-derived phosphopeptide, was much greater[32]. The authors detected notable differences between conformational changes in arrestin-2 induced by differentially phosphorylated peptides[32], which is consistent with the barcode hypothesis positing that GPCRs phosphorylated at different sites by different GRKs have differential effects on arrestin conformation, which is translated into the activation of distinct branches of arrestin-mediated signaling[33,34].

GPCR-INDEPENDENT ARRESTIN SIGNALING

Interestingly, whereas both non-visual arrestin-2 and -3 (a.k.a. β-arrestin1 and 2) appeared to facilitate the activation of c-Src and ERK1/2, only one subtype, arrestin-3, facilitated the activation of JNK3[24,35,36], as well as at least some isoforms of ubiquitously expressed JNK1 and JNK2[37]. Early studies revealed that arrestin-3 can facilitate JNK3 activation even in receptor-independent manner, when the upstream-most kinase, MAP3K ASK1, is overexpressed[24,35]. This finding was confirmed by documenting that arrestin-3 mutant incapable of GPCR binding (which has a deletion in the inter-domain hinge region, precluding domain movement[38,39] necessary for the binding to GPCRs[40-42]) promotes JNK3 activation as effectively as wild type (WT) arrestin-3[43]. It was also shown that replacement of certain residues in the arrestin-3 with their homologues from closely related arrestin-2[6,7] impedes its ability to activate JNK3, rendering it arrestin-2-like[36]. Systematic comparison of the effects of β2AR ligands acting *via* endogenous receptor on the activation of ERK1/2 and JNK3 in the same cells expressing various forms of arrestin-3 proved beyond reasonable doubt receptor-independence of this arrestin-3 function: While the levels of active phosphorylated ERK1/2 reflected the functional state of the receptor, the levels of active phospho-JNK3 did not depend on it, reflecting only the nature of arrestin-3 mutant expressed[43]. Arrestin-3-mediated scaffolding of the two modules of the JNK3-activating cascade, MKK4-JNK3 and MKK7-JNK3, was demonstrated using purified proteins *in vitro* in the absence of any GPCRs, confirming yet again that receptors are not necessary for this arrestin-3 function[22,23]. Recent structure of the arrestin-3 trimer crystallized in the presence of a fairly abundant intracellular small molecule, inositol-

hexakisphosphate (IP6)[44] revealed that all three protomers in the trimer are in the "active" (receptor-bound-like) conformation. It was similar to the conformation of arrestin-1 in complex with rhodopsin[41,42], as well as the conformations of constitutively active arrestin-1 splice variant p44[45] and C-terminally truncated arrestin-2 in complex with the phosphopeptide derived from the angiotensin receptor C-terminus[40], thereby suggesting a molecular mechanism of receptor-independent activation of arrestin-3. These data suggest that at least one of non-visual arrestins, arrestin-3, can assume "active" (GPCR bound-like) conformation without the help of GPCRs[44]. Curiously, the molecular mechanism of arrestin-3 activation in this case appears to resemble the mechanism of activation of all arrestins by GPCRs: The phosphates of IP6 engage the same positively charged side chains in arrestin as receptor-attached phosphates[46]. It is also noteworthy that GPCRs might activate arrestins catalytically, *i.e.*, that arrestins can maintain active conformation after dissociation from GPCRs[47]. Thus, multiple mechanisms can generate "active" arrestins in the cytoplasm that are not bound to GPCRs.

The propensity of arrestin-3 mutants to form trimers in the presence of IP6 appeared to correlate with their ability to facilitate JNK3 activation in cells[44]. However, no IP6 was used in the experiments where MKK4-JNK3 and MKK7-JNK3 modules were reconstituted *in vitro* from purified proteins with arrestin-3[22,23], suggesting that this subtype can assume active (at least in terms of the ability to facilitate signaling in the JNK3 activation cascade) conformation spontaneously, without the help of IP6. Indeed, structural data[48] and molecular dynamics simulations[49] indicate that arrestin-3 is more flexible than other arrestin subtypes. A short arrestin-3-derived peptide comprising the first 25 residues was found to facilitate JNK3 activation both *in vitro* and in cells[50]. This peptide is unlikely to trimerize, as it does not contain most of the inter-protomer interfaces observed in the crystal trimer[44]. It was expressed as a fusion with well-folded proteins (MBP in *E. coli* and YFP in mammalian cells), which suggests that it simply needs to have loose conformation to function as a scaffold[50]. Interestingly, the arrestin-3 N-terminus, containing this peptide, does not appear to be particularly loose in the crystal trimer[44], so that the detailed molecular mechanism of arrestin-3-mediated scaffolding of the ASK1-MKK4/7-JNK3 cascade still remains to be elucidated.

The facilitation of JNK3 phosphorylation by arrestin-3 is not the only receptor-independent function of arrestin proteins documented. It was recently shown that arrestin-2-(1-380) fragment generated by caspase cleavage in the absence of receptor stimulation translocates to the mitochondria, where it assists caspase-cleaved tBid in releasing cytochrome c, thereby promoting apoptotic cell death[51]. Both non-visual arrestins and their receptor binding-deficient mutants affect cell spreading and motility *via* disassembly of focal adhesions and regulation of small GTPases[52,53]. Thus, several signaling functions of arrestins do not appear to be dependent on GPCRs, and, by extension, on G proteins.

ROLE OF G PROTEINS

As discussed above, some signaling functions could be performed by free arrestins independently of their interaction with GPCRs and, consequently, of G proteins activated by these receptors. However, arrestin-dependent signaling has long been considered to require arrestin binding to GPCR but at the same time to be G protein-independent serving as an alternative pathway of the GPCR signaling (*e.g.*, see[54]; recently reviewed in[55,56]). Indeed, free arrestins have minimal effect on certain signaling pathways, such as ERK1/2 activation, whereas arrestins bound to agonist-activated phosphorylated GPCRs are able to facilitate signaling in these pathways. Recently the notion of G protein independence of the GPCR-initiated arrestin signaling function has been called into question.

The use of CRISPR-Cas9 gene editing enabled the creation of cells lacking individual G proteins or several G proteins at the same time[57]. The only class of G proteins that could not be eliminated by CRISPR-Cas9 gene editing was $G_{i/o}$ subtypes, but these G proteins can be inactivated by pertussis toxin[57,58]. Thus, the combination of inactivation of $G_{i/o}$ proteins by pertussis toxin in cells where all other G protein subtypes were knocked out by CRISPR-Cas9 made possible the construction of cells lacking all G protein-mediated signaling (termed "zero functional G" cells). A comprehensive study was performed in these cells, with numerous GPCRs, including β2AR and angiotensin1 receptor often used to demonstrate arrestin-dependent ERK1/2 activation[58]. The results showed that neither arrestin recruitment to GPCRs nor receptor internalization requires G protein signaling. However, the authors did not detect any arrestin-mediated ERK1/2 activation in "zero functional G" cells using

a variety of methods, including label-free dynamic mass redistribution and ERK1/2 phosphorylation in response to receptor stimulation[58]. Interestingly, the authors documented the role of arrestins in ERK1/2 activation by comparing "zero arrestin" cells with parental line, but only when at least some G protein-mediated signaling remained (illustrated by the Supplementary Figure 4 in Grundmann *et al*[58]). The main take-home message of that study was that while GPCR-induced arrestin-mediated signaling exists, it requires G protein action. The results suggested that without G proteins arrestins do not regulate ERK1/2 activation. Thus, the field has to decide whether these data call for yet another paradigm change.

MAPKS ARE ACTIVATED BY VARIOUS INPUTS

It is important to note that *in vivo* the main activators of MAPK cascades are not GPCRs. In most cases upstream MAP3Ks are activated by growth factor receptors[19,59], death receptors[60], integrins[61], or various stressors[62]. We should keep in mind that experimental paradigms used to study arrestin-mediated signaling actually exclude non-GPCR inputs. Cultured cells are usually plated on supports that do not activate integrins. In addition, cells where GPCR-induced MAPK activation is studied are routinely serum-starved, *i.e.*, maintained in growth factor-deficient conditions, which prevents MAPK activation *via* growth factor and/or death receptors, likely the prevalent mechanisms *in vivo*. Mammals have 20 different MAP3Ks that integrate signaling inputs[20]. The mechanisms of MAP3Ks activation are usually complex. For example, one of the MAP3Ks of the ERK1/2 cascade, cRaf (a.k.a. Raf1) is activated by active (GTP-liganded) small G proteins of Ras family, which recruit it to the membrane and promote its dimerization. cRaf dimerizes with other members of RAF family and kinase suppressor of Ras (KSR). An element adjacent to the Ras-binding domain, cysteine-rich domain stabilized by zinc, binds phosphatidylserine, facilitating membrane anchoring[63]. Several additional events contribute to cRaf activation[63]: Ras binding facilitates dephosphorylation of the site upstream of the kinase domain that in the inactive state of Raf1 binds 14-3-3 protein. Dimerized cRaf molecules apparently phosphorylate the activation segment, which stabilizes the active form of the kinase. For full activity, the negatively charged N-terminal region and the C-terminal 14-3-3 binding site also need to be phosphorylated. Another example of complex activation mechanism is ASK1, one of the MAP3Ks of JNK1/2/3 cascades. It is activated by oxidative stress, endoplasmic reticulum (ER) stress, calcium influx, or mechanical stress, and inhibited by the interactions with reduced thioredoxin and 14-3-3 protein[62]. Its phosphorylation on three different serines in the N- and C-terminal elements is inhibitory, whereas the phosphorylation of the three threonines in the kinase domain is stimulatory[62]. Thus, in addition to being phosphorylated on threonines, for full activation the three serines in ASK1 must be dephosphorylated, and both thioredoxin and 14-3-3 protein must dissociate[62]. To the best of our knowledge, none of these events is regulated by heterotrimeric G proteins. It is entirely possible that when MAP3Ks are activated *via* GPCR-independent mechanisms by integrins, death or growth factor receptors, or stressors, G proteins are not involved, whereas arrestins might still act as scaffolds bringing the three kinases of MAPK cascades together.

MAPK ACTIVATION IN DIFFERENT SUBCELLULAR COMPARTMENTS

Another important aspect of MAP kinase signaling is related to cell compartmentalization. Most MAPKs phosphorylate transcription factors in the nucleus, although practically every MAP kinase has cytoplasmic or even plasma membrane-localized substrates[64]. Naturally, the biological impact of MAPK activity towards nuclear and non-nuclear proteins has very different biological meaning. Free arrestins are soluble cytoplasmic proteins, whereas GPCR-bound arrestins localize even more restrictively, to the plasma membrane and endosomes. Localization of scaffolds determines where active MAPKs are generated, thus directing their signaling towards substrates in a particular cellular compartment. The original studies suggested that ERK1/2 activated *via* arrestin scaffold remains in the cytoplasm[18], where it phosphorylates its non-nuclear substrates, whereas ERK1/2 activated *via* G protein- and growth factor receptor-mediated mechanisms translocates to the nucleus[18]. Indeed, in some cases arrestin-mediated activation of ERK1/2 was shown not to affect transcription[65]. However, a recent study showed that arrestin-2 in adrenocortical zona glomerulosa facilitates aldosterone production by ERK1/2

activation[66], apparently *via* transcription regulation. Similarly, ERK1/2 activation by angiotensin 1A receptor in vascular smooth muscle *via* both G_q and arrestin was shown to involve transactivation of EGF receptor[67]. Thus, direct biological consequences of ERK1/2 activation also cannot be used to distinguish between arrestin-dependent and -independent mechanisms of its activation.

BIASED GPCR SIGNALING

Recently GPCR ligands that bias the signaling towards G proteins or arrestins have attracted a lot of attention as tools that might help achieving desired therapeutic outcome while minimizing unwanted side effects[55,56]. Ligand-activated GPCRs[68], as well as light-activated prototypical GPCR rhodopsin[69], exist in an equilibrium of multiple conformational states (reviewed in[70]). Thus, distinct subsets of active GPCR conformations might preferentially bind particular signal transducers, such as different G proteins and/or arrestins. The data suggesting that G protein action is required for arrestin-mediated signaling appears to be inconsistent with the concept of arrestin-biased signaling. Indeed, if we envision a ligand with the 100% bias towards arrestin, then it might have to rely on alternative signaling inputs to provide an initial "push", at least, for some signaling pathways such as the ERK activation, before arrestins could step in. However, these findings do not contradict the idea that GPCR ligands that promote arrestin recruitment to a greater extent than G protein activation can yield signaling outcomes quite different from those generated by unbiased ligands promoting the activation of both G proteins and arrestins.

Indeed, it is likely that a relatively low level of G protein activity is sufficient to provide the initial activation of the MAPK pathways where arrestins play the role of scaffolds or signaling enhancers. Furthermore, practically all G proteins have measurable rate of spontaneous exchange of GDP for GTP[71], *i.e.*, activation. In addition, non-GPCR activators, such as AGS proteins[72], or other proteins containing G protein regulatory (GoLoco) motif[71], were shown to catalyze nucleotide exchange, leading to G protein activation. Thus, a fraction of the G protein pool in the cell is always active. In practical terms, considering that GPCRs and their endogenous ligands were designed by evolution to signal in both directions, it is highly unlikely that 100% effective bias can be achieved by manipulation of ligand structure. For example, it was recently shown that carvedilol, which was traditionally considered to be a "clean" arrestin-biased ligand of β-adrenergic receptors, actually promoted β1-adrenoreceptor coupling to Gi proteins, and this unconventional Gi activation by the receptor that was believed to be strictly Gs-specific is required for observed "arrestin-biased" signaling[73]. In the same vein, recent comprehensive analysis of 65 different ligands of β2AR identified many G protein-biased ones, but none specifically biased towards arrestin recruitment[74], reinforcing the notion that the evolution "designed" GPCRs primarily to activate G proteins. Thus, it appears likely that any synthetic arrestin-biased ligand will have sufficient ability to produce necessary G protein activation, so that the proposed model suggesting the involvement of G proteins in arrestin-mediated signaling does not negate the possibility of exploiting biased signaling for therapeutic purposes. For example, even weak partial agonism towards G proteins combined with a stronger agonism towards arrestins might generate sufficient "push" to activate MAP3Ks and enable arrestin-mediated scaffolding, but not enough G protein signaling to yield the biological effect of a full agonist. Conversely, a strong GPCR agonist biased towards G proteins might produce an effect without activating the arrestin brunch, thereby avoiding arrestin-mediated signaling.

Alternatively, when MAP3Ks are activated *via* G protein-independent mechanisms (which is the most likely scenario *in vivo*), GPCR-bound arrestins might function as signal-enhancing scaffolds, facilitating MAPK activation and other pathways independently of G proteins. Arrestin-mediated scaffolding would restrict the localization of generated active MAPKs to the vicinity of GPCRs, *i.e.*, to plasma membrane and endosomes, thereby directing them to substrates in these locales. Thus, arrestin-biased GPCR agonists would affect cell signaling in a different manner than unbiased ones. These ideas must be explored experimentally, preferably in cells that are meant to be targeted under the conditions where the cell receives all inputs, including stimulation *via* growth factor receptors and integrins.

COOPERATION OF ARRESTINS AND G PROTEINS

Conceivably, there might be situations *in vivo* where the bulk of MAP kinase

activation depends on GPCRs, similarly to the experimental conditions used to study arrestin-mediated signaling to MAPKs. In these cases, it might appear counter-intuitive that the signaling of arrestins, which suppress G protein coupling to GPCRs, might require G protein activity. However, it is very likely that any GPCR agonist, including those with arrestin bias, also activates G proteins to a certain extent, as arrestin binding to any GPCR is inevitably delayed by the need of receptor phosphorylation by GRKs to increase arrestin affinity[10,11]. There are known examples where particular biological outcomes, such as Rho A activation and stress fiber formation, require simultaneous input from active G proteins and arrestins[75]. Another known mechanism where G proteins and arrestins might cooperatively participate in signaling that requires both types of transducers involves complexes between certain GPCRs and growth factor receptors that create distinct signaling platforms (reviewed in[76]).

PUTTING PIECES TOGETHER

Here the focus of the discussion is GPCR-dependent activation of ERK1/2 in the experimental conditions used to study arrestin-mediated signaling, which exclude non-GPCR inputs. The data obtained with gene knockout appear to be less ambiguous than those obtained with siRNA knockdown often used earlier[28,29,54]: The knockdown is never complete and one can never be sure that only the targeted proteins were knocked down. A good example demonstrating problems with knockdown specificity are the two siRNA studies[77,78], where opposite conclusions regarding the role of arrestins and arrestin domain-containing proteins in β2AR trafficking were made based on the data. Thus, strictly speaking, without the demonstration of rescue by the expression of knockdown-resistant exogenous protein substituting for that targeted by siRNA, the results of knockdown cannot be unambiguously interpreted[79,80]. The same applies to knockout: Only rescue with knocked out protein proves that the phenotype observed emerged due to the elimination of an intended target. In addition, complete knockout of an important signaling protein has other caveats: The cells might be inadvertently selected for their ability to survive without eliminated protein due to changes in signaling pathways. For example, simultaneous knockout of both non-visual arrestins is embryonic lethal in mice[81], whereas mouse embryonic fibroblasts[81] and HEK293 cells[57,58,82,83] lacking arrestins are viable and can be transfected to generate cells exclusively expressing individual arrestins or particular mutants[52,53,84,85]. Thus, some cells can live without arrestins, whereas others cannot. It is possible (and very hard to check) that "zero functional G" cells also have unanticipated and uncontrolled changes in their signaling pathways. A recent study using three independently generated lines of "zero arrestin" HEK293 cells suggested that these lines are quite different, particularly in terms of signaling: The elimination of arrestins resulted in enhanced, reduced, or unchanged ERK1/2 phosphorylation in response to GPCR activation, as compared to parental cell lines[83]. These data clearly showed that non-visual arrestins do play a role in signaling, at least in GPCR-dependent ERK1/2 activation[83].

So, does arrestin-mediated signaling *via* GPCRs that is G protein-independent, as previously claimed[54], exist? While unambiguous answers require further experimentation, one plausible explanation for the apparent controversy between a large body of data describing arrestin-dependent signaling (reviewed in[55,56]) and recent findings in "zero functional G" and "zero arrestin" cells[57,58,82] can be proposed. An important point that was consistently overlooked in studies of GPCR-dependent arrestin-mediated signaling *via* MAP kinases is the issue of signal initiation. MAP kinase cascades are highly conserved in eukaryotes, from yeast to mammals, and always contain three protein kinases (MAP3K-MAP2K-MAPK) that sequentially activate each other by phosphorylation[86]. The signaling in these cascades is initiated by the activation of the upstream-most MAP3Ks[19]. Yet is was never taken into account that MAP3Ks of the ERK1/2 and JNK3 cascades (cRaf and ASK1, respectively), have to be activated to initiate signaling that eventually leads to the observed phosphorylation of ERK1/2 or JNK3, which usually depends on various protein scaffolds bringing the three kinases of each cascade together. Non-visual arrestins were found to serve as scaffolds but were never shown to facilitate MAP3K activation (reviewed in[12,87-89]). Thus, it is entirely possible that in "real life" the first "push" leading to the activation of MAP3Ks is provided by GPCRs *via* G proteins, or, more likely, by numerous non-GPCR signaling mechanisms, whereas signal propagation is facilitated by scaffolds, including receptor-bound or free arrestins. In case of MAP3K activation by growth factor receptors or integrins arrestin-dependent ERK1/2 activation might appear GPCR-dependent but G protein-independent. In

contrast, under experimental conditions so far used to study arrestin-mediated signaling G proteins activated in response to GPCR stimulation might be the only remaining source of MAP3K activation, which would translate into G protein dependence of arrestin signaling to the ERK pathway, as described recently[58]. The need, or lack thereof, of active G proteins for apparently receptor-independent JNK3 activation by arrestin-3 and arrestin-3-derived peptide has never been tested experimentally, although ASK1 activation *via* G protein-independent mechanisms is more likely in this case.

The existing evidence of the role of non-visual arrestins in cell signaling[83] does not actually contradict the idea that G protein activity might be necessary for the arrestin-mediated signaling under conditions where the inputs from growth factor receptors, integrins, and stressors are excluded. Arguably, the situation where GPCRs assume the leading role in the MAPK activation can be encountered only in rather artificial experimental conditions, although we cannot exclude that this situation sometimes exists *in vivo*. The data obtained in "zero functional G" cells do not contradict the notion that arrestin-mediated signaling exists and plays a role in cell biology. Regardless of the potential role of G proteins, signal propagation to MAPKs would still depend on scaffolds, possibly including non-visual arrestins. Experiments where the activity of MAP3Ks and MAP2Ks in each cascade, rather than only the phosphorylation state of downstream MAPKs, such as ERK1/2 and JNK3, is determined in cells expressing non-visual arrestins with or without functional G proteins are necessary to test this hypothesis. It would be instructive to test whether the activation of growth factor receptors, which are the main known activators of MAP3Ks[19], or the activation of integrins (*e.g.*, by plating cultured cells on fibronectin) bypasses the requirement for the G protein activity. If non-visual arrestin scaffolds contribute to MAPK activation under any of these conditions, their function is likely to be G protein-independent.

CONCLUSION

Available evidence strongly indicates that non-visual arrestins scaffold three-tiered MAP kinase cascades, facilitating signal propagation. Other signaling functions of arrestins are also well documented. However, arrestins were never implicated in the activation of upstream-most MAP3Ks. Cells have numerous MAP3Ks that are activated by various inputs, including, but not limited to, G protein-mediated GPCR signaling. Thus, arrestins and heterotrimeric G proteins have distinct non-overlapping functions in cell signaling. In MAPK cascades, under experimental conditions that exclude non-GPCR inputs, G proteins might play a role in MAP3K activation, whereas arrestins act as scaffolds facilitating signal transduction.

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