Arrestin-mediated signaling: Is there a controversy?

Vsevolod V Gurevich, Eugenia V Gurevich

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

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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. 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
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\(^6\) 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\(^10\). 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\(^12\) 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\(^19\)), the latter two cases suggested that receptor-bound arrestins scaffold the three-kinase modules,
crystallized in the presence of a fairly abundant intracellular small molecule, inositol—
not necessary for this arrestin-3 function. Recent structure of the arrestin-3 trimer [22,23]
in the absence of any GPCRs, confirming yet again that receptors are proteins in vitro activating cascade, MKK4-JNK3 and MKK7-JNK3, was demonstrated using purified mutant expressed [43]. Arrestin-3-mediated scaffolding of the two modules of the JNK3-active phospho-JNK3 did not depend on it, reflecting only the nature of arrestin-3 receptor-independence of this arrestin-3 function: While the levels of active same cells expressing various forms of arrestin-3 proved beyond reasonable doubt endogenous receptor on the activation of ERK1/2 and JNK3 in the via [36]. Systematic comparison of the effects of β2AR [6,7] with their homologues from closely related arrestin-2 [43] arrestin-3 [40-42]) promotes JNK3 activation as effectively as wild type (WT) binding to GPCRs in the inter-domain hinge region, precluding domain movement [38,39] necessary for the
hypothesis positing that GPCRs phosphorylated at different sites by different GRKs differentially phosphorylated peptides, which is consistent with the barcode detected notable differences between conformational changes in arrestin-2 induced by GRKs [32]. The authors investigated using biophysical methods proteins containing Src homology 3 (SH3) domains was recently extensively
facilitated the activation of JNK3, as well as at least some isoforms of 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 [32,33].

**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 [24,35,39], as well as at least some isoforms of ubiquitously expressed JNK1 and JNK2 [97]. Early studies revealed that arrestin-3 can facilitate JNK3 activation even in receptor-independent manner, when the upstream-most kinase, MAP3K ASK1, is overexpressed [24,39]. 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 [35,39] necessary for the binding to GPCRs [31,32]) 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 [26-28] impedes its ability to activate JNK3, rendering it arrestin-2-like [41]. 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) 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, as well as the conformations of constitutively active arrestin-1 splice variant p44 and C-terminally truncated arrestin-2 in complex with the phosphopeptide derived from the angiotensin receptor C-terminus, thereby suggesting a molecular mechanism of receptor-independent activation of arrestin-3. These data suggest that at least one of non-functional arrestins, arrestin-3, can assume “active” (GPCR-bound-like) conformation without the help of GPCRs. 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. It is also noteworthy that GPCRs might activate arrestins catalytically, i.e., that arrestins can maintain active conformation after dissociation from GPCRs. 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. 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, 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 and molecular dynamics simulations 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. This peptide is unlikely to trimerize, as it does not contain most of the inter-protomer interfaces observed in the crystal trimer. 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. Interestingly, the arrestin-3 N-terminus, containing this peptide, does not appear to be particularly loose in the crystal trimer, 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 Bid in releasing cytochrome c, thereby promoting apoptotic cell death. 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. 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 recently reviewed in). 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. The only class of G proteins that could not be eliminated by CRISPR-Cas9 gene editing was Gαi subtypes, but these G proteins can be inactivated by pertussis toxin. Thus, the combination of inactivation of Gαi 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. 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\(^6\). 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\(^{53,59}\), 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 two different MAPK pathways that have distinct signaling inputs\(^6\). 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-ligated) 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\(^6\). Several additional events contribute to cRaf activation\(^6\): 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\(^6\). 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\(^6\). 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\(^6\). 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\(^6\). 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\(^6\), where it phosphorylates its non-nuclear substrates, whereas ERK1/2 activated via G protein- and growth factor receptor-mediated mechanisms translocates to the nucleus\(^6\). Indeed, in some cases arrestin-mediated activation of ERK1/2 was shown not to affect transcription\(^6\). However, a recent study showed that arrestin-2 in adrenocortical zona glomerulosa facilitates aldosterone production by ERK1/2...
activation[69], apparently via transcription regulation. Similarly, ERK1/2 activation by angiotensin 1A receptor in vascular smooth muscle via both Gs 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[66-68]. Ligand-activated GPCRs[69], 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[73], 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[74]. 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[66], 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 arrests, 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[105]. 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[74]. 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[83]).

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[52,54]. 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[72,78], where opposite conclusions regarding the role of arrests and arrestin-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[96,99]. 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 arrests is embryonic lethal in mice[19], whereas mouse embryonic fibroblasts[85] and HEK293 cells[86,87] lacking arrests are viable and can be transfected to generate cells exclusively expressing individual arrests or particular mutants[52,53,54,92]. Thus, some cells can live without arrests, 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 arrests 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 arrests 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[114], 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[53,54]) and recent findings in “zero functional G” and “zero arrestin” cells[52,53,54] 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[84]. The signaling in these cascades is initiated by the activation of the upstream-most MAP3Ks[84]. 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 arrests were found to serve as scaffolds but were never shown to facilitate MAP3K activation (reviewed in[111,114]). 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 arrests. 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\(^{[83]}\). 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 \textit{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 MAP3Ks 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\(^{[83]}\), 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.

REFERENCES

1. Bockaert J, Pin JP. Molecular tinkering of G-protein-coupled receptors: an evolutionary success. EMBO J 1999; 18: 1723-1729 [PMID: 10202136 DOI: 10.1093/emboj/18.7.1723]
2. Fredriksson R, Lagerström MC, Lundin LG, Schölth HB. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol 2003; 63: 1256-1272 [PMID: 12761335 DOI: 10.1124/mol.63.6.1256]
3. Gurevich EV, Tesmer JJ, Mushhegan A, Gurevich VV. G protein-coupled receptor kinases: more than just kinases and not only for GPCRs. Pharmacol Ther 2012; 133: 40-69 [PMID: 21903131 DOI: 10.1016/j.pharmthera.2011.08.001]
4. Wilden U, Hall SW, Kühn H. Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. Proc Natl Acad Sci USA 1986; 83: 1174-1178 [PMID: 3006038 DOI: 10.1073/pnas.83.5.1174]
5. Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ. beta-Arrestin: a protein that regulates beta-adrenergic receptor function. Science 1990; 248: 1547-1550 [PMID: 21631101 DOI: 10.1126/science.2163110]
6. Attramadal H, Arriza JL, Aoki C, Dawson TM, Codina J, Kwatra MM, Snyder SH, Caron MG, Lefkowitz RJ. Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family. J Biol Chem 1999; 267: 17882-17890 [PMID: 1517224]
7. Sterne-Marr R, Gurevich VV, Goldsmith P, Bodine RC, Sanders C, Donoso LA, Benovic JL. Polypeptide variants of beta-arrestin and arrestin3. J Biol Chem 1993; 268: 15640-15648 [PMID: 8340386]
8. Rapoport B, Kaufman KD, Chazenbalk GD. Cloning of a member of the arrestin family from a human thyroid cDNA library. Mol Cell Endocrinol 1992; 84: R39-43 [PMID: 1587386 DOI: 10.1016/0303-7207(92)90038-8]
9. Lohse MJ, Andexinger S, Pitcher J, Trukawinski S, Codina J, Faure JP, Caron MG, Lefkowitz RJ. Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of beta-arrestin and arrestin in the beta 2-adrenergic receptor and rhodopsin systems. J Biol Chem 1992; 267: 8558-8564 [PMID: 1349018]
10. Gurevich VV, Gurevich EV. The molecular acrobatics of arrestin activation. Trends Pharmacol Sci
cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following Tohgo A, Pierce KL, Choy EW, Lefkowitz RJ, Luttrell LM. beta-Arrestin scaffolding of the ERK surface to the nucleus. 

Cell Signal 6 Guan KL. The mitogen activated protein kinase signal transduction pathway: from the cell to the nucleus. 

2015; 16, Therrien M. Regulation of RAF protein kinases in ERK signalling. Nat Rev Mol Cell Biol Lavoie H: 34-43 [PMID: 29133163 DOI: 10.1016/j.cellsig.2017.11.003] 2016; 26, 2014; 26, 2007; 26, 2014; 9, 2013; 288 Isoforms in cells via scaffolding. J Biol Chem 2013; 288: 37332-37342 [PMID: 24257757 DOI: 10.1074/jbc.M113.510412]

38 Vishnivetskiy SA, Hirsch JA, Velez MG, Gurevich VV, Gurevich VV. Transition of arrestin into the active receptor-binding state requires an extended interdomain hinge. J Biol Chem 2002; 277: 43961-43967 [PMID: 12213448 DOI: 10.1074/jbc.M206951200]

39 Hansson SM, Cleghorn WM, Francis DJ, Vishnivetskiy SA, Raman D, Song X, Nair KS, Slepak VZ, Klug CS, Gurevich VV. Arrestin mobilizes signaling proteins to the cytoskeleton and redirects their activity. J Biol Chem 2007; 282: 575-387 [PMID: 17359998 DOI: 10.1016/j.jbc.2007.02.053]

34 Shukla AK, Manglik A, Kruse AC, Xiao K, Reis RL, Tseng WC, Staup DS, Hilger D, Uysal S, Huang LY. Structure of active beta-arrestin-1 bound to a G-protein-coupled receptor phosphophosphate. Nature 2013; 497: 137-141 [PMID: 23604255 DOI: 10.1038/nature12120]

40 Kang Y, Zhou XE, Gao X, He Y, Liu W, Ishchenko A, Barty A, White TA, Yefanov O, Han GW. Crystal structure of rhodopsin-bound to arrestin by femtosecond X-ray laser. Nature 2013; 523: 561-567 [PMID: 26200343 DOI: 10.1038/nature14656]

41 Zhou XE, He Y, de Waal PW, Gao X, Kang Y, Van Eps N, Yin Y, Pal K, Goswami D, White TA. Identification of Phosphorylation Codes for Arrestin Recruitment by G Protein-Coupled Receptors. Cell 2017; 170: 457-469.e13 [PMID: 28753425 DOI: 10.1016/j.cell.2017.07.002]

42 Breitman M, Kook S, Gimenez LE, Lizada BN, Palazzo MC, Gurevich EV, Gurevich VV. Silent scaffolds: inhibition of c-Jun N-terminal kinase 3 activity in cell by dominant-negative arrestin-3 mutant. J Biol Chem 2012; 287: 19653-19664 [PMID: 22525077 DOI: 10.1074/jbc.M112.358912]
angiotensin AT1a receptor stimulation. J Biol Chem 2002; 277: 9429-9436 [PMID: 11777902 DOI: 10.1074/jbc.M106457200]

66 Lympenosopoulos A, Rengo G, Zicarelli C, Kim J, Soltsy-S, Koch WJ. An adrenal beta-arrestin-1-mediated signaling pathway underlies angiotensin II-induced aldosterone production in vitro and in vivo. Proc Natl Acad Sci USA 2009; 106: 5825-5830 [PMID: 19289825 DOI: 10.1073/pnas.0817061106]

67 Kim J, Ahn S, Rajagopal K, Lefkowitz RJ. Independent beta-arrestin2 and Gq/ protein kinase Ceta pathways for ERK stimulated by angiotensin type 1A receptors in vascular smooth muscle cells converge on transactivation of the epidermal growth factor receptor. J Biol Chem 2009; 284: 11953-11962 [PMID: 19254952 DOI: 10.1074/jbc.M808176200]

68 Manglik A, Kim TH, Masureel M, Altenbach C, Yang Z, Hilger D, Lerch MT, Kobilska TS, Thian FS, Hubbell WL. Structural Insights into the Dynamic Process of β2-Adrenergic Receptor Signaling. Cell 2017; 161: 1101-1111 [PMID: 25981663 DOI: 10.1016/j.cell.2015.04.043]

69 Van Eps N, Caro LN, Morizumi T, Kusnetzow AK, Szczepek M, Hofmann KP, Bayburt TH, Sligar SG, Ernst OP, Hubbell WL. Conformational equilibria of light-activated rhodopsin in nanodiscs. Proc Natl Acad Sci USA 2017; 114: E3268-E3275 [PMID: 28373559 DOI: 10.1073/pnas.1620405114]

70 Wei W, Kobilska BK. The Molecular Basis of G Protein-Coupled Receptor Activation. Annu Rev Biochem 2018; 87: 897-919 [PMID: 29092558 DOI: 10.1146/annurev-biochem-060614-033910]

71 Natochin M, Gasimov KG, Artemyev NO. Inhibition of GDP/GTP exchange on G alpha subunits by proteins containing G-protein regulatory motifs. Biochemistry 2001; 40: 5322-5328 [PMID: 11318607 DOI: 10.1021/bi010505w]

72 Gismowski MJ, Lanier SM. Activation of heterotrimeric G-proteins independent of a G-protein coupled receptor and the implications for signaling process. Rev Physiol Biochem Pharmacol 2005; 155: 57-80 [DOI: 10.1007/3-540-28217-3_3]

73 Wang J, Hanada K, Staus DP, Makara MA, Dahal GR, Chen Q, Atles A, Engelhardt S, Rockman HA. Gaq is required for carvedilol-induced β2 adrenergic signal β-arrestin biased signaling. Nat Commun 2017; 8: 4736 [PMID: 29167435 DOI: 10.1038/s41467-017-01148-0]

74 Littmann T, Götte M, Reintartz MT, Kälble S, Wainer IW, Ozawa T, Seifert R. Recruitment of β-arrestin 1 and 2 to the β2-adrenoceptor: analysis of 65 ligands. J Pharmacol Exp Ther 2015; 355: 183-190 [PMID: 26306761 DOI: 10.1124/jpet.115.227759]

75 Barnes WG, Reiter E, Violin JD, Ren XR, Milligan G, Lefkowitz RJ. beta-Arrestin 1 and Galphai/11 coordinate activate RhoA and stress fiber formation following receptor stimulation. J Biol Chem 2005; 280: 8041-8050 [PMID: 15611106 DOI: 10.1074/jbc.M412924230]

76 Pyne NJ, Pyne S. Receptor tyrosine kinase-G-protein-coupled receptor signalling platforms: out of the shadow? Trends Pharmacol Sci 2011; 32: 443-450 [PMID: 21612832 DOI: 10.1016/j.tips.2011.04.002]

77 Nabhani JF, Pan H, Lu Q. Arrestin-domain-containing protein 3 recruits the NEDD4 E3 ligase to mediate ubiquitination of the beta2-adrenergic receptor. EMBO Rep 2010; 11: 605-611 [PMID: 20559325 DOI: 10.1038/embojr.2010.80]

78 Han SO, Kommandi RP, Shenoy SK. Distinct roles for β-arrestin2 and arrestin-domain-containing proteins in β2 adrenergic receptor trafficking. EMBO Rep 2013; 14: 164-171 [PMID: 23208550 DOI: 10.1002/embr.2012.187]

79 Gurevich VV, Gurevich EV. Differential manipulation of arrestin-3 binding to basal and agonist-activated G protein-coupled receptors. Cell Signal 2017; 29: 1706 [PMID: 28461104 DOI: 10.1016/j.cellsig.2017.04.021]

80 Coffa S, Breitman M, Spiller BW, Gurevich VV. A single mutation in arrestin-2 prevents ERK1/2 activation by reducing c-Raf binding. Biochemistry 2011, 50: 6951-6958 [PMID: 21732673 DOI: 10.1021/bi200745k]

81 Prokop S, Perry NA, Vishnivetskii SA, Toth AD, Inoue A, Milligan G, Iversen TM, Hunyady L, Gurevich VV. Differential manipulation of arrestin-3 binding to basal and agonist-activated G protein-coupled receptors. Cell Signal 2017; 29: 98-107 [PMID: 29846104 DOI: 10.1016/j.cellsig.2017.04.021]

82 Tian T, Harding A. How MAP kinase modules function as robust, yet adaptable, circuits. Cell Cycle 2014; 13: 2579-2590 [PMID: 25483189 DOI: 10.4161/cc.29349]

83 Gurevich EV, Gurevich VV. Arrestin: ubiquitous regulators of cellular signaling pathways. Genome Biol 2006; 7: 236 [PMID: 17020596 DOI: 10.1186/gb-2006-7-9-236]

84 Peterson YK, Luttrell LM. The Diverse Roles of Arrestin Scaffolds in G Protein-Coupled Receptor Signaling. Pharmacol Rev 2017; 69: 256-297 [PMID: 28626043 DOI: 10.1124/pr.116.13367]

85 Gurevich VV, Gurevich EV. Arrestins and G proteins in cellular signaling: The coin has two sides. Sci Signal 2018; 11: eaav1646 [PMID: 30254054 DOI: 10.1126/scisignal.aav1646]

P- Reviewer: Alcántara-Hernández R, Lympenosopoulos A, Rajagopal S
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