Arrestins as Signaling Molecules Involved in Apoptotic Pathways: A Real Eye Opener

William E. Miller and Robert J. Lefkowitz

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The seven membrane-spanning or G protein-coupled receptor (GPCRs) superfamily comprises molecules (≥1000) that regulate a remarkably diverse array of physiological functions in organisms ranging in complexity from yeast to man. Studies carried out over the past 25 years with prototypic model systems, such as rhodopsin for the visual system and the β2-adrenergic receptor (β2-AR) for the catecholamine system, have established the basic paradigms that seem to explain many of the "conventional" signaling events emanating from this type of receptor. These paradigms include activation of heterotrimeric ($\alpha\beta\gamma$) G proteins and dissociation of the G protein into its α and βγ subunits. Activation and dissociation of G proteins in turn lead to stimulation of effector enzymes, alterations in the concentration of cellular second messenger levels, or opening of ion channels. Receptor activation is countered by phosphorylation of the activated receptors by G protein-coupled receptor kinases, followed by the binding of an arrestin molecule, which dampens or desensitizes receptor signaling. Increasingly, these simple paradigms appear to be inadequate to explain a growing list of receptor-mediated effects in which the G protein-coupled receptor kinases (GRKs) and β-arrestins seem to function as adaptors or intermediates in the internalization and signaling of the receptors, rather than simply as desensitizing elements. Two papers provide a dramatic new example of this emerging theme: Alloway *et al*. (*1*) and Kiselev *et al*. (*2*) demonstrate that *Drosophila* arrestin2 plays an important role in the activation of an apoptotic signaling pathway leading to retinal degeneration.

In the *Drosophila* compound eye, light-induced conversion of rhodopsin to metarhodopsin results in the stimulation of a G_q-mediated signaling pathway that activates an eye-specific phospholipase C (norpA). After conversion to metarhodopsin and phosphorylation by rhodopsin kinase, arrestin2 interacts with the activated receptor and quenches G protein signaling. Dephosphorylation of rhodospin by a receptor phosphatase (rdgC), combined with phosphorylation of arrestin2 by calcium/calmodulin-dependent kinase II (CamKII), results in the disassembly of rhodopsin/arrestin2 complexes and ultimately leads to regeneration of active rhodopsin. This visual signaling pathway differs somewhat from the perhaps more familiar mammalian system in which rhodopsin activates the G protein transducin, leading to activation of a cyclic 3′, 5′ guanosine monophosphate (cGMP) phosphodiesterase. Moreover, the *Drosophila* arrestin2 is actually more similar to mammalian β-arrestin2 than to mammalian visual arrestin. Mammalian visual arrestin is also referred to as arrestin1, whereas the two peripheral isoforms are variably termed β-arrestin1 (arrestin2) and β-arrestin2 (arrestin3).

Taking advantage of mutations in the phosphatase rdgC or norpA (*Drosophila* phospholipase C, which is essential for activation of rdgC and CamKII), these recent studies demonstrate that exposure to light leads to the formation of a stable internalized complex of rhodopsin and arrestin2 that signals apoptosis (Fig. 1). These mutations lead either to hyperphosphorylated rhodopsin (rdgC and norpA) or hypophosphorylated arrestin2 (norpA) in response to light, both of which promote the stable assembly and internalization of the receptor/arrestin2 complex. Whereas chronic stimulation of rhodopsin signaling in photoreceptor cells leads to retinal degeneration through a G protein-mediated pathway (*3*, *4*), Alloway *et al.* and Kiselev *et al.* now indicate that the formation of stable rhodopsin/arrestin2 complexes in *Drosophila* leads to an additional form of retinal degeneration that is largely independent of G protein-mediated signaling (*1*, *2*). Alloway *et al*. indicate that the stable rhodopsin/arrestin2 complexes induce retinal degeneration specifically through an apoptotic pathway. Thus, overexpression of the p35 caspase inhibitor in flies blocks retinal degeneration in light-stimulated photoreceptor cells that contain stable complexes of rhodopsin and arrestin2, but not retinal degeneration induced through classical G protein signaling pathways. This important finding suggests that the stable formation of rhodopsin/arrestin2 complexes in the intracellular region of *Drosophila* photoreceptor cells leads to the activation of a signaling pathway (as yet unknown) resulting in apoptosis. The requirement for arrestin2 in the apoptosis-inducing complex is further supported by the observation that conversion of metarhodopsin back to rhodopsin, which disassembles the arrestin complex, blocks the induction of apoptosis. Although it is clear that arrestin must remain bound to rhodopsin in these intracellular complexes in order for apoptosis to occur, it is not entirely clear whether the hyperphosphorylated rhodopsin merely stabilizes the receptor/arrestin2 interaction or if it mediates an additional signal important for the induction of apoptosis.

Prevention of rhodopsin internalization with a temperature-sensitive mutant of shibire [the *Drosophila* homolog of dynamin, a guanosine triphosphatase (GTPase) required for clathrin-mediated internalization] also blocks the induction of apoptosis, indicating that internalization of rhodopsin/arrestin2 complexes is also necessary for apoptosis. Interestingly, although these reports document that *Drosophila* rhodospin undergoes internalization, its mammalian counterparts in the visual system have not thus far been reported to internalize. Kiselev *et al*. (*2*) provide a potential explanation for this difference in that the *Drosophila* arrestin2 protein interacts with clathrin to promote internalization, whereas mammalian visual arrestins do not interact with clathrin. In contrast, the mammalian β-arrestins promote internalization of many different GPCRs through interaction with clathrin. This represents another respect in which the consequences of the *Drosophila* rhodopsin/arrestin2 interaction appear more similar to the GPCR/β-arrestin situation than to the classical rhodopsin/arrestin system in mammalian cells. Clathrin is the major component of coated vesicles, and interaction of mammalian β-arrestins with clathrin and the clathrin adapter AP-2 is an important factor in

The authors are at the Howard Hughes Medical Institute, Departments of Medicine and Biochemistry, Duke University Medical Center, Box 3821, Durham, NC 27710, USA. E-mail: millerw@receptor-biol.duke.edu and lefko001@receptor-biol.duke.edu

promoting internalization of many GPCR family members. However, internalization of GPCRs into clathrin-coated vesicles is a complex process involving many proteins in addition to clathrin and AP-2 (*5*), such as *N*-ethylmaleimide-sensitive fusion protein (NSF) (*6*) and GRK interactor 1 (GIT1) (*7*).

Recent work from a number of laboratories focusing on mammalian GPCR/β-arrestin signaling pathways may provide further insight into the mechanisms by which the formation of stable intracellular rhodopsin/arrestin2 complexes in flies leads to the induction of apoptosis. β-arrestin serves as an adaptor that interacts directly with a number of proteins that are components of signaling pathways involved in both cell proliferation and apoptosis.

The first β-arrestin-interacting signaling protein to be discovered was the nonreceptor tyrosine kinase c-Src (*8*). Interaction of c-Src with β-arrestin mediates recruitment of c-Src to agonist-activated β2-ARs. Recruitment of c-Src to activated GPCRs is involved in numerous cellular processes, including receptor internalization, as well as activation of mitogen-activated protein kinase (MAPK) pathways. β-arrestin can also recruit c-Src and other Src family tyrosine kinases to other GPCRs, including the chemokine receptor for interleukin-8 (CXCR1) and the neurokinin receptor (NK1) (*9*, *10*).

The mammalian β-arrestins also interact with several members of the MAPK family. These interactions have revealed that the β-arrestins function as receptor-controlled scaffolds for the activation of several different MAPK modules. The β-arrestin molecule promotes assembly of upstream kinases [MAPK kinase kinase (MAPKKK) and MAPK kinase (MAPKK)] to facilitate the phosphorylation and activation of the MAPK. Under the control of the agonist-receptor complex, not only the activity but also the subcellular distribution of the β-arrestin-scaffolded complex are regulated. This has been demonstrated for the MAP kinases ERK1 and ERK2 and Jun NH_2-terminal kinase 3 (JNK3), establishing the β-arrestins as potentially important intermediates in both anti- and proapoptotic pathways, respectively.

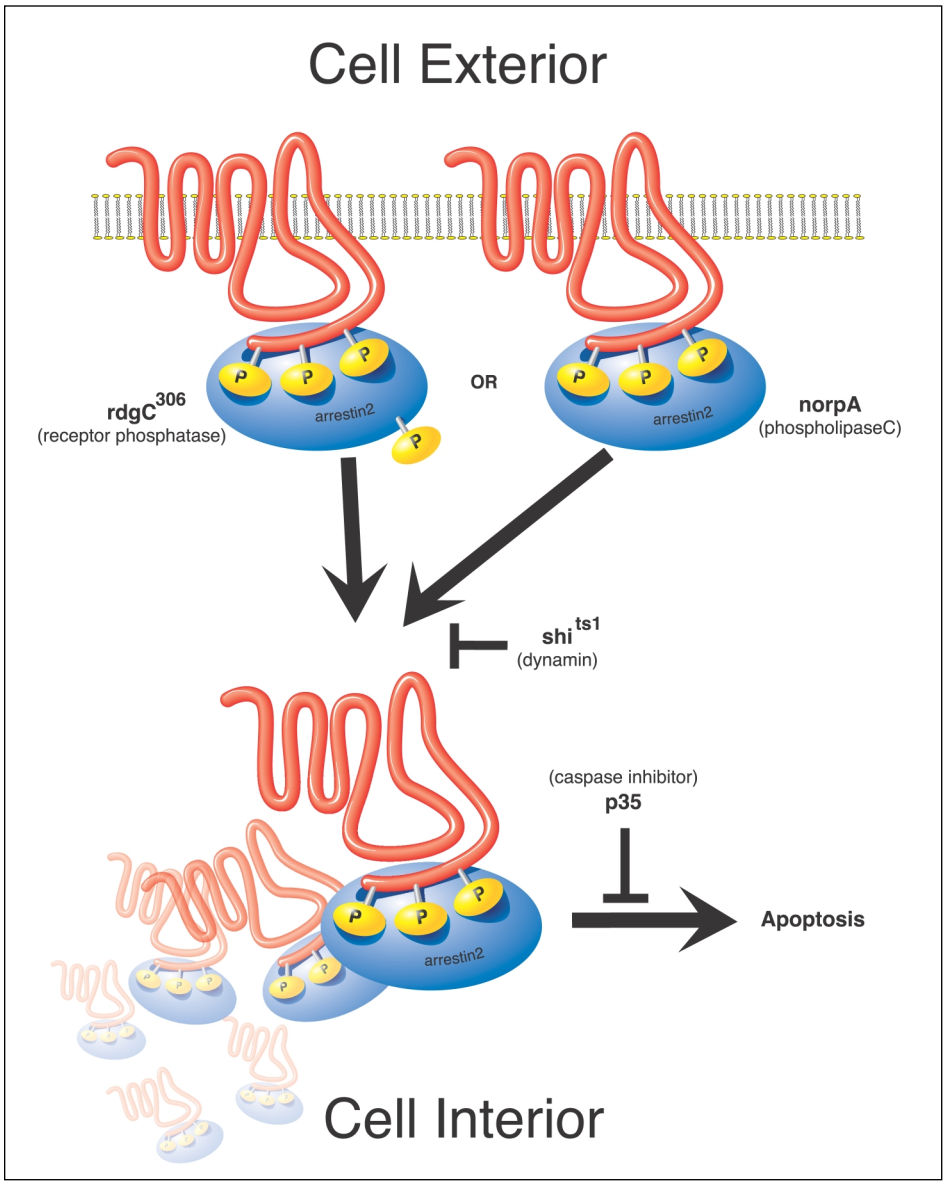

Fig. 1. *Drosophila* arrestin2 engages a signaling pathway leading to the induction of apoptosis. Stimulation of rhodopsin receptors leads to phosphorylation of the receptor and binding of arrestin2. Dephosphorylation of receptor by rdgC coupled with phosphorylation of arrestin2 by CamKII (stimulated by the *Drosophila* phospholipase C, norpA) normally leads to disassembly of the receptor/arrestin complexes. Mutations in either rdgC or norpA in *Drosophila* cause the stable formation of intracellular rhodopsin/arrestin2 complexes that induces death of photoreceptor cells. The $rdgC^{306}$ allele results in the formation of hyperphosphorylated rhodopsin and stable rhodopsin/arrestin2 complexes. norpA is upstream of a pathway involved in inositol trisphosphate (IP_3) accumulation and Ca^{2+} release and is required for the activation of both rdgC and CamKII. Therefore, in norpA mutant flies, not only will rhodopsin will be hyperphosphorylated, but arrestin2 will be hypophosphorylated, resulting in the formation of stable rhodopsin/arrestin2 complexes. The induction of cell death is blocked by the caspase inhibitor p35. This finding formally designates the cell death pathway as apoptotic. Furthermore, the use of *shibire* mutants (*Drosophila* dynamin) indicates that the rhodopsin/arrestin2 complex must be internalized to induce the apoptotic effect.

Assembly of β-arrestin-scaffolded ERK1 and ERK2 complexes has been described for the NK1, protease-activated receptor 2 (PAR2), and angiotensin II 1A (AT1A) receptors (*10*-*12*). These β-arrestin-scaffolded complexes also contain Raf, the

MAPKKK in the ERK pathway. Furthermore, the subcellular localization of these complexes appears to be important for the outcome of signal generation, because binding of proteins such as ERK to β-arrestin in the cytoplasm can block nuclear localization of the active kinase and prevent the induction of gene expression. In the case of the agonist-activated NK1 receptor, the complex is formed at or near the membrane and is followed by nuclear accumulation of active ERK1 and ERK2 (*11*). In contrast, the agonist-activated PAR2 and AT1A receptor-associated complexes appear to be sequestered in the cytoplasm (*10*, *12*). Activation of NK1 receptors and the subsequent formation of a receptor/β-arrestin complex stimulate ERK activity, which presumably mediates the proliferative and antiapoptotic effects of NK1 receptor signaling. In contrast, the complex formed in response to activation of PAR2 receptors appears to localize to the cytoplasm of cells, where it sequesters ERK and promotes the nonproliferative effects of PAR2 agonists.

JNK proteins are involved in regulating responses to cell stress that can ultimately lead to apoptosis. Accordingly, JNKs are also known as stress-activated protein kinases (SAPKs). Stimulation of numerous GPCRs, including the AT1A receptor, leads to stimulation of JNK activity. With the use of yeast two-hybrid screening, the JNK3 isoform was identified as a binding partner for mammalian β-arrestin2 (*13*). β-Arrestin2 interacts not only with JNK3 in cells, but also with an upstream activator of JNK3, the MAPKKK ASK1. Thus, β-arrestin2 acts as a scaffold to increase the local concentration of members of the JNK signaling cascade, resulting in the phosphorylation and activation of JNK. Activation of AT1A receptors leads to the formation of stable internalized complexes containing receptor, β-arrestin, and JNK3. The formation of stable internalized GPCR complexes containing β-arrestin, Src, and MAPK (such as JNK and ERK) may be analogous to the light-induced rhodopsin/arrestin2 complex formed in *Drosophila* photoreceptor cells.

One can envision scenarios in which the balance of proliferative and apoptotic signaling cascades is dependent on the nature of the MAPK modules engaged by the receptor/arrestin complexes. JNKs have been clearly implicated in apoptotic signaling. Although ERKs have generally been viewed as antiapoptotic, recently their involvement in apoptotic signaling has also been reported (*14*). Alternatively, proliferative signaling mediated by ERKs, which requires nuclear translocation of the active enzyme, might be opposed by arrestin-mediated cytosolic retention of activated ERK. It is presently unknown if the *Drosophila* rhodopsin/arrestin2 complexes contain homologs of the JNK, ERK, or Src proteins. It is, however, interesting to speculate that stimulation of rhodopsin in the *Drosophila* mutants leads to the activation of similar signaling proteins and pathways.

The accepted paradigm of GPCR signaling involving the activation of signaling pathways downstream of G proteins continues to be an actively studied and validated area of research. However, the combined efforts of groups studying rhodopsin signaling in *Drosophila* photoreceptor cells and of groups studying the involvement of β-arrestins in mammalian GPCR signaling seem to suggest that arrestins play previously unsuspected roles in GPCR signaling. There is mounting evidence that internalized activated receptors signal in an arrestin-dependent manner that may be largely independent of G proteins. Several obvious questions arise from the interesting work of Alloway *et al*. (*1*) and Kiselev *et al.* (*2*). For example, in a normal nonpathological setting (wild-type rdgC and norpA), do transient complexes of internalized rhodopsin and arrestin2 complexes assemble? The transient formation of such complexes could signal in a similar way but not lead to apoptosis because of the rapid disassembly of the complexes. Alternatively, these complexes may only form under pathological conditions, which would certainly have ramifications for retinal degeneration in both fly and human systems. Stimulation of mammalian cells with several agonists as described above leads to the stable formation of receptor/β-arrestin complexes containing ERK1, ERK2, or JNK3 that localize to endocytic vesicles. Thus, there is precedent for the formation of stable receptor and arrestin complexes under nonpathological settings in mammalian cells.

Perhaps the most important question concerns the nature of the stabilized rhodopsin/arrestin2 complex. Do these complexes contain other proteins like c-Src, Raf, ERK, ASK1, and JNK3 that are intimately involved in the generation of the apoptotic signal? The readily available genetic approaches in *Drosophila* provide a unique system for studying arrestin signaling in vivo and should soon provide additional details to the emerging picture of arrestins as signaling molecules. These studies with the *Drosophila* photoreceptor system have the potential to illuminate novel arrestin-dependent signaling mechanisms used by a wide variety of mammalian and invertebrate GPCRs.

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