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PERSPECTIVE

Arrestin-Independent Internalization of G Protein-Coupled Receptors

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A feature common to most G protein-coupled receptors (GPCRs) is the cyclic process of signaling, desensitization, internalization, resensitization, and recycling to the plasma membrane. These coordinated events prevent cells from undergoing excessive receptor stimulation or periods of prolonged inactivity. In the "canonical" pathway of GPCR regulation, agonist-activated GPCR induces receptor phosphorylation mediated by GPCR kinases (GRKs), which phosphorylate serine and threonine residues in the third intracellular loop and/or the carboxyl terminus of the receptor (Ferguson, 2001) (Fig. 1). Receptor-G protein interaction, however, is not inhibited by GRK-mediated phosphorylation. For this, the phosphorylated receptor recruits another molecule, β-arrestin. β-Arrestin sterically hinders further coupling of the receptor with the heterotrimeric G proteins, and this interruption of signaling generates receptor desensitization. In the case of G_s-coupled β_2-adrenergic receptors, β-arrestins may also recruit cAMP phosphodiesterases of the PDE4D family into a complex with the activated receptor, where they are positioned to degrade cAMP at enhanced rate (Perry et al., 2002).

Upon receptor phosphorylation and β-arrestin binding, most GPCRs internalize into clathrin-coated vesicles. β-Arrestins also play a central role in this process. By serving as an adaptor molecule, β-arrestin links GPCRs to several proteins of the endocytotic machinery, including clathrin (Goodman et al., 1996) and the clathrin adaptor complex AP-2 (Laporte et al., 1999). In the endosomes, most GPCRs are dephosphorylated and return to the plasma membrane as resensitized receptors to undergo another round of signal transduction. Receptor resensitization requires dissociation of β-arrestin before the receptors can be dephosphorylated. Internalized GPCRs may also be down-regulated via lysosomal degradation or may direct activation of additional intracellular signaling pathways. In these processes, β-arrestins play regulatory roles as well. GPCRs that display strong and persistent β-arrestin binding and cointernalize with β-arrestins recycle relatively slowly or are more likely to be transported to the lysosomes for receptor degradation. In contrast, GPCRs that bind β-arrestin transiently and dissociate from β-arrestin shortly after movement of the receptor into the clathrin-coated vesicles recycle rapidly (Oakley et al., 2000; Shenoy and Lefkowitz, 2003). β-Arrestins may also function as scaffolds for GPCR-mediated activation of Src family tyrosine kinases, extracellular signal-regulated kinase 1/2, c-Jun NH_2-terminal kinase 3, and p38 mitogen-activated protein kinases (Lefkowitz and Whalen, 2004). Thus, β-arrestins are important multifunctional adaptor molecules regulating desensitization, internalization, intracellular signaling, and recycling of a large number of GPCRs.

It has become increasingly clear that these molecular mechanisms are by no means universal for all GPCRs. Several GPCRs do not require β-arrestins for internalization, at least in certain cell types. These include the metabotropic glutamate receptor 1 (Dhami et al., 2004), secretin receptor (Walker et al., 1999), serotonin 5-HT_{2A} receptor (Gray et al., 2003), protease-activated receptor-1 (Paing et al., 2002), prostacyclin receptor (Smyth et al., 2000), formyl peptide receptor (Bennett et al., 2000; Vines et al., 2003), and M_2 muscarinic cholinergic receptor (van Koppen and Kaiser, 2003).

In this issue of *Molecular Pharmacology*, Chen et al. (2004) elegantly show another example of β-arrestin-independent internalization of a GPCR: the high-affinity leukotriene B_4 receptor BLT1. Signaling by this GPCR involves the activation of phospholipase C via pertussis toxin-sensitive (G_i/G_o) and -insensitive (G_{16}/G_{14}) G proteins (Gaudreau et al., 2002). Chen et al. show that BLT1 readily internalizes after recep-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; AP-2, adaptor protein complex-2.

tor activation in cells that express sufficient levels of GRK2. Internalization is blocked by coexpressing dominant-negative GRK2 but not by dominant-negative β-arrestins. In addition, β-arrestin-GFP does not redistribute upon BLT1 activation, and β-arrestin does not associate with BLT1, whereas GRK2 does. Moreover, a C-terminal mutant BLT1, which does not associate with GRK2, does not internalize.

A point of concern is that most of the data in the present study are derived from heterologous (over)expression of the proteins in RBL-2H3, human embryonic kidney 293, and COS-7 cells. Furthermore, if the interaction of a GPCR with β-arrestin is weak, coimmunoprecipitation of receptor with β-arrestin may not be possible. For these reasons, one can argue that the question as to whether internalization of a GPCR is β-arrestin-independent (or not) can only currently be answered after analysis of receptor internalization in cells derived from mice deficient in either β-arrestin1, β-arrestin2, or both (Kohout et al., 2001). Lefkowitz and coworkers showed that β_2-adrenergic receptors do not internalize in the β-arrestin double-knockout cells, whereas 18% of angiotensin AT_{1A} receptor internalization remains in the absence of β-arrestins (Kohout et al., 2001). In contrast, internalization of the protease-activated receptor-1 (Paing et al., 2002) or formyl peptide receptor (Vines et al., 2003) is unchanged in the double-knockout cells.

Activation of BLT1 leads to receptor phosphorylation by GRKs (Gaudreau et al., 2002), but why does β-arrestin not redistribute to the plasma membrane and associate with agonist-activated phosphorylated BLT1? It is theoretically possible that the binding of β-arrestin to activated BLT1 is transient and escaped detection. As a result, the net effect might have been no discernible translocation of β-arrestin-GFP to the plasma membrane or coimmunoprecipitation with BLT1. Binding of β-arrestin to phosphorylated GPCRs is thought to occur through disruption of a "polar" core within β-arrestin by the highly charged receptor-attached phosphate moieties, which convert β-arrestin to a high-affinity receptor-binding state (Gray et al., 2003). Because the conserved DRY motif, located at the interface between the third transmembrane domain and a cytoplasmic loop of GPCRs, is required for receptor association of β-arrestin (Bennett et al., 2000), the authors suggest that the less-than-perfect DRY motif of BLT1 might be responsible for the inability of BLT1 to recruit β-arrestin. Although this may be an explanation, M_2 muscarinic acetylcholine receptors, which internalize in a β-arrestin-independent manner in HEK293 cells, carry the conserved DRY motif (van Koppen and Kaiser, 2003).

As the authors suggest, another adaptor protein could also bind to phosphorylated BLT1, such as AP-2. AP-2 has recently been found to mediate part of the internalization of the α_{1B}-adrenergic receptor (Diviani et al., 2003). This possibility seems less likely because BLT1 lacks the polyarginine motif that is required for AP-2–mediated internalization of the α_{1B}-adrenergic receptor (Diviani et al., 2003). On the other hand, GRK2 bound to BLT1 might act as an adaptor protein. GRK2 can directly interact with clathrin via a clathrin box, which is present in the carboxyl-terminal region of GRK2 and can mediate GPCR internalization in a β-arrestin–independent manner (Shiina et al., 2001). It is thus possible that BLT1/GRK2/clathrin interaction could be involved in the β-arrestin–independent, clathrin-dependent internalization of BLT1. However, the authors found that coexpression of dominant-negative GRK2 blocks internalization of BLT1 in RBL-2H3 cells. This suggests that if GRK2 acts as adaptor, kinase activity of GRK2 (or wild-type kinase conformation) should be required for clathrin interaction. This remains unknown.

A second question is how BLT1 desensitizes in the absence

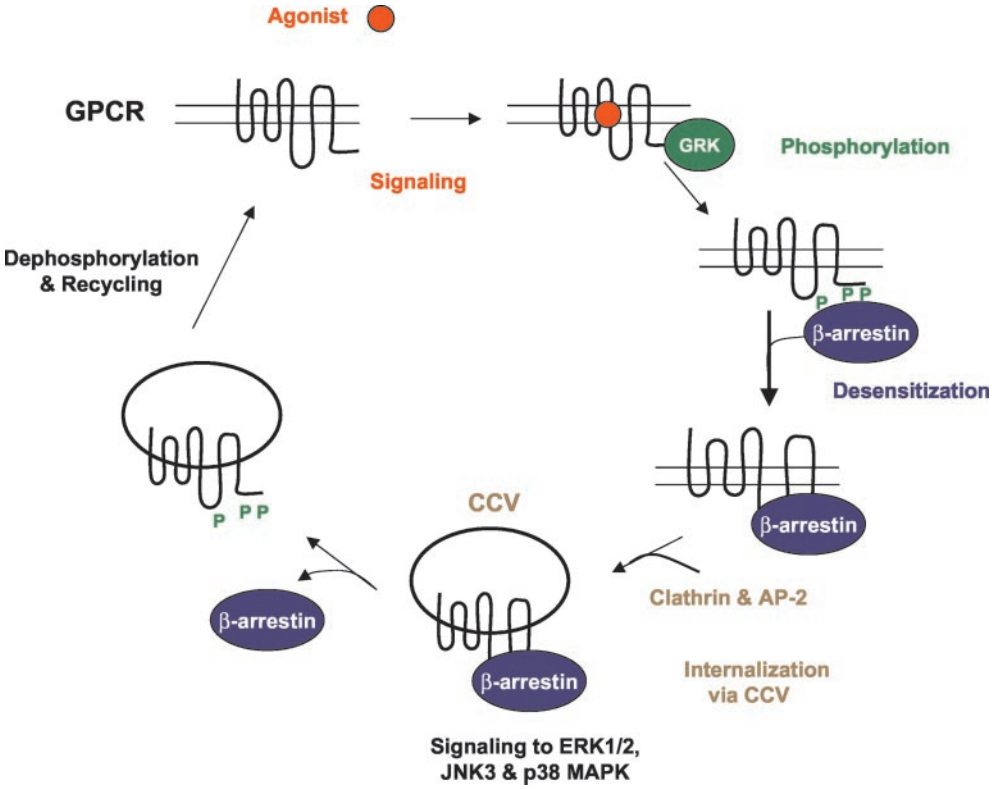

Fig. 1. Model of β-arrestin-regulated internalization of a prototypic GPCR. The binding of agonist to a GPCR induces signaling to heterotrimeric G proteins. Prolonged stimulation induces receptor phosphorylation by G protein-coupled receptor kinases (GRKs) as well as second messenger-activated kinases and others (not shown). Then, cytosolic β-arrestin interacts with the phosphorylated receptor, leading to uncoupling of the GPCR from the G proteins. β-Arrestin also interacts with clathrin and the clathrin adaptor complex AP-2 as well as other components of the endocytic machinery (not shown), leading to immobilization of the receptor in the clathrin-coated pit. Depending on the affinity of binding, β-arrestin may dissociate from the GPCR either before the clathrin-coated vesicle (CCV) pinches off from the plasma membrane or at a later stage during intracellular trafficking. β-Arrestin bound to the receptor in the CCV may also act as adaptor/scaffold for GPCR activation of extracellular signal-regulated kinase 1/2, JNK3, and p38 mitogen-activated kinases in the cytoplasm. After the release of β-arrestin, the GPCR is dephosphorylated by phosphatases and recycles back to the plasma membrane for another round of stimulation.

of apparent β-arrestin association. Again, we cannot exclude the possibility that there is transient interaction of BLT1 with β-arrestin, which leads to uncoupling of BLT1 from the heterotrimeric G proteins. It is of note that protease-activated receptor-1, which internalizes in a β-arrestin–independent manner, requires β-arrestin for receptor desensitization (Paing et al., 2002). It is also possible that BLT1 desensitization is mediated by second messenger-activated kinases, such as protein kinase C, because treatment with protein kinase C activators induces BLT1 desensitization (Gaudreau et al., 2002). Another possibility would be agonist-promoted loss of receptors at the plasma membrane. GRK2 is able to bind $G\alpha_q$, $G\beta\gamma$, and GPCRs (Lodowski et al., 2003; Willets et al., 2003; Dhami et al., 2004), thereby leading to suppression of phospholipase C-β activation. On the other hand, binding of another adaptor protein, such as AP-2, may sterically hinder interaction of BLT1 with heterotrimeric G proteins, although it is has not been shown that AP-2 is able to do this.

To summarize, the study by Chen et al. (2004) poses new possibilities regarding molecular mechanisms of β-arrestin–independent, clathrin-dependent GPCR internalization. We believe that final proof for β-arrestin–independent internalization of BLT1 will require confirmation in cells of β-arrestin double-knockout mice. Assuming this is the case, future studies should be directed at identifying adaptor proteins that are recruited to the phosphorylated receptors and defining their role in BLT1 desensitization. Generation of novel information on BLT1 internalization and desensitization will undoubtedly extend our understanding of regulation of the family of GPCRs, with more than 600 putative members.

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