Mini-Review

Heptahelical Receptor Signaling: Beyond the G Protein Paradigm

Randy A. Hall, Richard T. Premont, and Robert J. Lefkowitz

Howard Hughes Medical Institute, Department of Medicine and Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

HEPTAHELICAL receptors, so called because of their conserved structure featuring seven α-helical transmembrane spans, mediate physiological responses to a remarkably diverse array of stimuli. These include hormones, neurotransmitters, small peptides, proteins, lipids and ions, as well as sensory stimuli such as odorants, pheromones, bitter and sweet tastants, and photons. This superfamily of receptors contains >1,000 members, making it the largest class of cell surface molecules in the mammalian genome. Moreover, it was found recently that heptahelical receptors account for >5% of the total genes in the *Caenorhabditus elegans* genome (Bargmann, 1998), testifying to the importance of this family and demonstrating that the structure of these receptors has been highly conserved throughout evolution. For many years, this family of receptors has been referred to as G protein–coupled, a term based on the well documented paradigm that such receptors interact with and signal through heterotrimeric G proteins. Simply stated, this repeatedly validated paradigm is that when heptahelical receptors are stimulated with ligand, their intracellular regions undergo conformational changes, allowing the receptors to interact with G proteins. This association in turn causes conformational changes in the G proteins that facilitate GDP release and GTP binding, leading to dissociation of G_α and $G_{\beta\gamma}$ subunits. The activated G protein subunits then bind to and regulate various intracellular effectors.

During the past few years, however, several reports have appeared in the literature describing various physiological consequences of heptahelical receptor stimulation that, surprisingly, do not seem to be mediated by G protein activation. Concurrently, novel techniques for detecting protein–protein interactions such as yeast two-hybrid, phage display, and fusion protein overlays have revealed associations of heptahelical receptors with a variety of intracellular partners other than G proteins. This convergence of unexplained physiology and provocative protein–protein interactions has led increasingly to the realization that the mechanisms of heptahelical receptor signaling are more diverse than previously thought. This mini-review summarizes recent work on the subject of intracellular signaling by heptahelical receptors through means other than classical G protein pathways.

Arrestins and G Protein–coupled Receptor Kinases

Activated heptahelical receptors are phosphorylated by a family of G protein–coupled receptor kinases (GRKs).[1] Following phosphorylation, the receptors bind to another family of proteins called arrestins (Lefkowitz, 1998). The regions of the receptors that arrestins bind to, generally the third intracellular loop and the portion of the carboxyl-terminal tail closest to the membrane, are also primary determinants for G protein interaction. Arrestin binding to receptors thus results in desensitization of G protein–mediated signaling by preventing interaction of receptors with G proteins. An emerging view, however, is that the binding of arrestins to heptahelical receptors also initiates a new set of signaling pathways in addition to blocking those mediated by G protein activation.

It was proposed recently, for example, that β-arrestin can act as an adaptor protein to recruit the tyrosine kinase Src into a signaling complex organized around the β_2-adrenergic receptor (Luttrell et al., 1999). It is well known that stimulation of many heptahelical receptors can lead to the activation of MAP kinases, but the mechanisms involved have been difficult to define. While G protein activation is clearly necessary, activation of tyrosine kinases of the Src family is required in many cases as well (Luttrell et al., 1996). The most recent findings reveal that Src associates in cells with agonist-activated β_2-adrenergic receptors, as assessed by immunofluorescence and coimmunoprecipitation. The recruitment of cellular Src to β_2-adrenergic receptors is potentiated by overexpression of β-arrestin, and in vitro pull-down studies reveal a direct high-affinity association between Src and β-arrestin. β-Arrestin–mediated association of Src with β_2-adrenergic receptors is a key step in mitogenic signaling by these receptors, since inhibition of the binding of β-arrestin to either the β_2-adrenergic receptor or Src attenuates β_2-adrenergic activation of MAP kinase. These results indicate that the association of

Address correspondence to Robert J. Lefkowitz, Howard Hughes Medical Institute, Box 3821, Duke University Medical Center, Durham, NC 27710. Tel.: (919) 684-2974. Fax: (919) 684-8875. E-mail: lefko001@mc.duke.edu

1. *Abbreviations used in this paper:* GAP, GTPase-activating protein; GRK, G protein–coupled receptor kinase; mGluR, metabotropic glutamate receptor; NHERF, Na^+/H^+ exchanger regulatory factor.

arrestins with heptahelical receptors does not simply uncouple receptors from G protein pathways, but rather induces a switch in receptor signaling from classical second messenger–generating G protein–mediated pathways to other pathways such as those involving Src and leading to the activation of MAP kinase. Moreover, arrestins have also been found to interact with a number of cellular proteins involved in endocytosis such as clathrin heavy chain (Goodman et al., 1996), the clathrin adaptor AP-2 (Laporte et al., 1999), and NSF (*N*-ethylmaleimide sensitive fusion protein) (McDonald et al., 1999). These interactions represent potential mechanisms by which heptahelical receptors might directly regulate the cellular endocytic machinery. Thus, arrestins may well represent multifunctional adaptor proteins that mediate a number of aspects of heptahelical receptor signaling.

GRKs may also be signaling intermediates for heptahelical receptors rather than just proteins involved in receptor desensitization. Recently, it was found that GRK2 can associate with and phosphorylate tubulin (Carman et al., 1998; Haga et al., 1998; Pitcher et al., 1998). GRKs have also been shown to associate with actin (Freeman et al., 1998) and a novel ARF GTPase-activating protein (ARF GAP) called GIT1 (Premont et al., 1998). These findings illustrate at least two ways in which the recruitment of GRKs to activated heptahelical receptors may lead directly to cytoskeletal regulation or to modulation of other intracellular processes: (a) allosteric activation of GRKs by ligand-occupied receptors (Palczewski et al., 1991; Chen et al., 1993; Premont et al., 1994) may catalyze the phosphorylation of key nonreceptor substrates such as tubulin; and (b) GRKs may act as noncatalytic adaptors to recruit key signaling intermediates (e.g., an ARF GAP) into complex with the receptors at the plasma membrane.

SH2 Domain–containing Signaling Proteins

Several subtypes of heptahelical receptors have been proposed to organize SH2 domain–based signaling complexes in a manner analogous to that seen for receptor tyrosine kinases. The heptahelical angiotensin AT_1 receptor, for example, activates the Jak2 tyrosine kinase following stimulation with angiotensin II (Marrero et al., 1995). The mechanism underlying this effect involves Src-mediated tyrosine phosphorylation of the AT_1 receptor itself (Venema et al., 1998). It is interesting to speculate that this phosphorylation might result from β-arrestin–mediated recruitment of Src to the receptor, but at present this idea has not been tested. When Tyr319 on the AT_1 receptor carboxyl-terminal tail is phosphorylated, Jak2 coimmunoprecipitates with the AT_1 receptor in an agonist-dependent fashion; mutation of Tyr319 to Phe blocks coimmunoprecipitation of Jak2 with AT_1 receptors and also attenuates Jak2 activation mediated by angiotensin II stimulation (Ali et al., 1997). Originally, it was thought that Jak2 interaction with the AT_1 receptor was direct. Jak2 does not have an SH2 domain, however, so it was not clear how it could bind to the AT_1 receptor tail in a phosphotyrosine-dependent manner. Subsequent studies revealed that the Jak2/AT_1 receptor tail interaction can be blocked by antibodies to the SHP family of SH2 domain–containing tyrosine phosphatases (Marrero et al., 1998), indicating that SHP proteins probably act as adaptors to facilitate the association of Jak2 with the AT_1 receptor. It has also been shown that another SH2 domain–containing protein, phospholipase Cγ1, can be coimmunoprecipitated with the tyrosine-phosphorylated AT_1 receptor (Venema et al., 1998), although the significance of this interaction for downstream signaling by the receptor has not yet been clarified.

The β_2-adrenergic receptor is phosphorylated on tyrosine by the insulin receptor tyrosine kinase (Hadcock et al., 1992; Karoor et al., 1995; Valiquette et al., 1995; Baltensperger et al., 1996). Several tyrosines in the β_2-adrenergic receptor have been shown to be phosphorylated, and it has also been reported that the SH2 domain–containing adaptor protein Grb2 can associate with β_2-adrenergic receptors following phosphorylation of Tyr350/354 on the receptor (Karoor et al., 1998). It is not yet known, however, if this association mediates any downstream signaling by the β_2-adrenergic receptor. Nonetheless, given these provocative findings with the AT_1 and β_2-adrenergic receptors, a significant point of future interest will be to see if other heptahelical receptors may be tyrosine-phosphorylated and thus capable of hosting SH2 or PTB domain–based signaling complexes.

Small GTP-binding Proteins

Heptahelical receptor–mediated regulation of small GTP-binding proteins, such as Ras, Rab, Rho, and ARF, has been studied for years but has typically been viewed as a downstream consequence of heterotrimeric G protein activation (Buhl et al., 1995; Kozasa et al., 1998). Recently, however, it has been shown that activation of phospholipase D by certain heptahelical receptors, including M_3 muscarinic acetylcholine receptors and H_1 histamine receptors, is not blocked by inhibitors of heterotrimeric G protein pathways, such as pertussis toxin or phospholipase C inhibitors, but is sensitive to the ARF inhibitor brefeldin A and the Rho inhibitor C3 botulinum toxin (Mitchell et al., 1998). ARF and Rho can also be immunoprecipitated in an agonist-dependent fashion in association with M_3 muscarinic receptors and AT_1 angiotensin receptors. The receptors capable of binding ARF and Rho exhibit a conserved motif (N-P-x-x-Y) in their seventh transmembrane span. Mutation of this motif prevents association of the receptors with ARF and Rho and also alters receptor signaling to phospholipase D. While it is not clear at present if the association of ARF and Rho with the heptahelical receptors is direct, it is clear that these small GTP-binding proteins can form a complex with some heptahelical receptors and that formation of this complex can mediate signaling of these receptors to phospholipase D.

PDZ Domain–containing Proteins

The heptahelical receptor-binding proteins discussed so far (heterotrimeric G proteins, arrestins, GRKs, SH2 proteins, and small GTP-binding proteins) all bind to either the receptor third intracellular loop or the portion of the receptor tail nearest the plasma membrane. Many heptahelical receptors, however, have quite long intracellular carboxyl-terminal tails, suggesting that the distal portions of some receptor tails may also be capable of mediating

association with various intracellular signaling proteins. Moreover, the carboxyl-terminal tails of some heptahelical receptors terminate in variants of the T/S-x-V motif required for binding to PDZ domain–containing proteins such as PSD-95 (Kornau et al., 1995).

One example of a heptahelical receptor with a long intracellular tail is the β_2-adrenergic receptor. Overlay studies demonstrated that the tail of this receptor binds with very high affinity to a single protein in tissue extracts; subsequent purification and sequencing revealed this binding partner to be a PDZ domain–containing protein, the Na^+/H^+ exchanger regulatory factor (NHERF) (Hall et al., 1998a). NHERF binds not only to the β_2-adrenergic receptor tail in vitro, but also to the full-length β_2-adrenergic receptor in cells in an agonist-dependent fashion as assessed by immunofluorescence studies.

β_2-Adrenergic regulation of renal Na^+/H^+ exchange has long been known to be opposite of what would be expected from a G_s-coupled receptor. Activation of G_s-coupled receptors such as parathyroid hormone receptors increases cellular cyclic AMP, which in a PKA-dependent fashion facilitates the association of NHERF with renal Na^+/H^+ exchangers and thus leads to inhibition of Na^+/H^+ exchange (Weinman and Shenolikar, 1993). Activation of β_2-adrenergic receptors also increases cellular cyclic AMP, yet paradoxically leads to stimulation of renal Na^+/H^+ exchange (Bello-Reuss, 1980; Weinman et al., 1982). A point mutant of the β_2-adrenergic receptor with the final residue of the receptor changed from leucine to alanine, which cannot bind NHERF but which exhibits normal G protein coupling, inhibits the activity of the renal Na^+/H^+ exchanger in cells rather than stimulating it like the wild-type receptor (Hall et al., 1998a). These findings suggest that the ability of the β_2-adrenergic receptor to bind NHERF is critical for β_2-adrenergic regulation of renal Na^+/H^+ exchange in vivo.

Rhodopsin is another heptahelical receptor that has been found to associate with a PDZ domain–containing protein in a functionally relevant manner. Rhodopsin binds to InaD (Chevesich et al., 1997; Xu et al., 1998), a multi-PDZ domain scaffolding protein that also associates with a number of signaling intermediates involved in rhodopsin-initiated pathways, such as phospholipase Cβ, protein kinase C, and the TRP ion channel (Huber et al., 1996; Shieh and Zhu, 1996; Chevesich et al., 1997; Tsunoda et al., 1997; Xu et al., 1998). Mutations in InaD profoundly distort photon-induced rhodopsin signaling (Scott and Zuker, 1998). The physical association of rhodopsin and InaD has been demonstrated by coimmunoprecipitation and by in vitro fusion protein pull-down experiments (Chevesich et al., 1997; Xu et al., 1998), but it is not known at present if the association of InaD and rhodopsin in cells occurs constitutively or if instead it is promoted by photoactivation of rhodopsin. In any case, it seems that rhodopsin can facilitate the assembly of intracellular protein complexes involved in phototransduction via its interaction with InaD.

The interactions of PDZ domains with the carboxyl termini of their target proteins are quite specific (Songyang et al., 1997). As demonstrated by the β_2-adrenergic receptor point mutant, a change of a single amino acid can be enough to completely disrupt an otherwise high-affinity association. Only a small number of heptahelical receptors terminate in the carboxyl-terminal motif (S/T-x-L) required for high-affinity NHERF binding (Hall et al., 1998b). However, since the >50 known PDZ domain–containing proteins recognize diverse target motifs, it is probable that some of these proteins associate with specific heptahelical receptors in a functionally relevant manner. Signaling through PDZ domain–mediated associations may therefore be a feature common to many heptahelical receptors.

Polyproline-binding Proteins

Several heptahelical receptors exhibit polyproline regions on either their third intracellular loops or carboxyl-terminal tails. Polyproline regions are known to mediate binding to a variety of conserved protein domains such as SH3 domains, WW domains, and EVH domains (Pawson and Scott, 1997). Recently, several subtypes of heptahelical metabotropic glutamate receptor (mGluR) were shown to bind members of the Homer family of EVH domain–containing proteins through a polyproline region found in the mGluR tail region (Brakeman et al., 1997; Tu et al., 1998; Xiao et al., 1998). This binding has been shown in yeast two-hybrid studies, fusion protein pull-downs, and coimmunoprecipitation studies. Some members of the Homer family can dimerize, and are thus capable of linking mGluRs to other proteins with appropriate polyproline motifs. For example, Homer proteins can facilitate a functional interaction between mGluRs and endoplasmic reticulum–based inositol trisphosphate (IP3) receptors, which control intracellular calcium release. When the mGluR/Homer association is blocked, the ability of mGluRs to mobilize intracellular calcium is attenuated (Tu et al., 1998). These findings suggest that Homer is a key intermediate in mGluR regulation of intracellular calcium levels, and thus shed light on the puzzling observation made shortly after the cloning of the mGluRs that alternative splicing of the mGluR1 carboxyl-terminal tail results in profound differences in the ability of this receptor to mobilize intracellular calcium (Pin et al., 1992; Joly et al., 1995).

Another heptahelical receptor that can bind signaling proteins through a polyproline region is the dopamine D4 receptor, which contains a stretch of prolines in its third intracellular loop. This polyproline region in the D4 receptor can mediate in vitro binding to a number of SH3 domain–containing proteins, including Grb2 and Nck, as assessed by yeast two-hybrid and protein pull-down assays (Oldenhof et al., 1998). It is not clear at present, however, which polyproline-binding proteins are the relevant cellular partners for D4 receptors or for other polyproline-containing heptahelical receptors such as β_1-adrenergic receptors and M4 muscarinic receptors. Further work in this area should reveal which polyproline-binding proteins couple to which receptors in cells, as well as what the consequences of these interactions are for receptor signaling.

Unsolved Heptahelical Receptor Mysteries

Several heptahelical receptor binding partners have been identified for which no clear roles in downstream signaling have yet been demonstrated. Examples include the in-

teraction of Grb2 with the β_2-adrenergic receptor and dopamine D4 receptor, as described above, as well as the interaction of the β_2-adrenergic receptor and some α-adrenergic receptor subtypes with the α subunit of the eukaryotic initiation factor 2B (Klein et al., 1997), and the interaction of the bradykinin B2 receptor with endothelial nitric oxide synthase (Ju et al., 1998). The recent proliferation of techniques for detecting protein–protein interactions is likely to lead to an increase in the number of known binding partners for various heptahelical receptors. Each of these interactions will represent a new potential mechanism of heptahelical receptor signaling, although the true physiological significance of each interaction may not be immediately obvious.

While such lines of research are describing novel mechanisms by which heptahelical receptors may generate intracellular signals, other lines of research are describing physiological effects mediated by heptahelical receptors for which the molecular mechanisms are unknown. Genetic studies in invertebrates, in particular, have yielded a number of examples of heptahelical receptors mediating physiological actions through pathways that are apparently independent of G proteins. For instance, the cyclic AMP receptors of the slime mold *Dictyostelium discoideum* are heptahelical receptors that induce chemotaxis of undifferentiated *Dictyostelium* cells into an aggregated fruiting body. These chemotactic effects of *Dictyostelium* cyclic AMP receptor stimulation are known to be mediated through G protein activation (Devreotes, 1994). However, aggregated *Dictyostelium* cells undergo a number of cyclic AMP receptor–mediated transcriptional changes that are independent of G protein activation, since cells with G protein subunits deleted still exhibit these changes following stimulation by cyclic AMP (Milne et al., 1995; Schnitzler et al., 1995; Maeda et al., 1996; Jin et al., 1998). The mechanisms by which this class of heptahelical receptors might mediate G protein–independent effects, however, are completely unknown.

More genetic evidence for signaling by heptahelical receptors through means other than traditional G protein pathways comes from the study of a family of receptors known as *frizzled*. In many species, ranging from *C. elegans* to *Drosophila* to mammals, tissue polarity during development is regulated by the Wnt family of secreted proteins, which exert their effects on developing cells by binding to members of the *frizzled* family (Bhanot et al., 1996; Yang-Snyder et al., 1996; He et al., 1997). Activation of some *frizzled* family heptahelical receptors results in increases in cellular calcium that can be inhibited by modulators of G protein function such as pertussis toxin and GDP-β-S (Slusarski et al., 1997). Thus, it seems that *frizzled* receptors can couple to G proteins. However, genetic studies have identified a number of signaling intermediates downstream of *frizzled*, such as *dishevelled*, glycogen synthase kinase-3, β-catenin, and the product of the adenomatous polyopsis coli (APC) gene (Dale, 1998), and none of these proteins resemble known components of classical G protein signaling pathways.

Dishevelled is the most proximal *frizzled* signaling intermediate identified. It is not known if the interaction between *frizzled* and *dishevelled* is direct, but it is interesting to note that *dishevelled* contains a PDZ domain and many *frizzled* family members possess carboxyl-terminal motifs appropriate for PDZ domain association. Therefore, it is possible that members of the *frizzled* family may signal through direct coupling to PDZ domain–containing proteins like *dishevelled* in a manner analogous to the PDZ domain–mediated interaction of the β_2-adrenergic receptor with NHERF. Some components of *frizzled* signaling pathways have been identified as oncogenes in mammalian tissues (Kinzler and Vogelstein, 1996), emphasizing the importance of understanding *frizzled* signaling.

Another genetically identified heptahelical receptor that signals via unknown mechanisms is *smoothened*. This receptor is a relative of the *frizzled* family of receptors, and is a key mediator of *hedgehog* signaling (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). *Hedgehog*, a soluble protein first identified as a regulator of patterning during *Drosophila* development, binds to a cell surface receptor known as *patched* (Chen and Struhl, 1996; Stone et al., 1996), which leads to regulation of the activity of *smoothened* to exert control over cell proliferation and differentiation. Since *smoothened* is a heptahelical receptor, much attention has been focused on the possibility that it might couple to heterotrimeric G proteins, but at present there is no conclusive evidence for such coupling. Indeed, genetic studies have identified several key proteins, such as the serine/threonine kinase *fused* and the putative transcriptional factor *cubitus interruptus*, as intermediates in the *smoothened* signaling pathway; none of these proteins resemble known components of G protein signaling pathways (Ingham, 1998). Activating mutations in the mammalian homologue of *smoothened* have been identified recently as underlying causes of sporadic basal-cell carcinoma (Xie et al., 1998), revealing that *smoothened*, like *frizzled*, may be involved in carcinogenesis. The intracellular signaling mechanisms used by both *frizzled* and *smoothened* are thus of interest not just as novel examples of heptahelical receptor signaling, but also as potential points of clinical intervention in the treatment of some cancers.

Beyond the G Protein Paradigm

Over the past several years, evidence has emerged that heptahelical receptors can signal through associations with intracellular partners other than G proteins. In some cases, these partners are known receptor-interacting proteins, such as arrestins and GRKs, which were thought previously to be involved only in receptor desensitization. In other cases, they are novel partners such as NHERF or Homer, which were not known previously to interact with heptahelical receptors. For heptahelical receptors that seem to mediate physiological effects via unknown G protein–independent pathways, such as *frizzled* and *smoothened*, it might be useful to consider analogies with other heptahelical receptors for which the early steps of various G protein–independent signaling mechanisms have been elucidated. Some of these mechanisms are likely to be quite general: for example, arrestins and GRKs can bind to many heptahelical receptors, and arrestin- and GRK-mediated formation of signaling complexes may therefore be a feature common to many heptahelical receptors. Other mechanisms, such as the activation of small GTP-binding proteins or the formation of SH2-based signaling

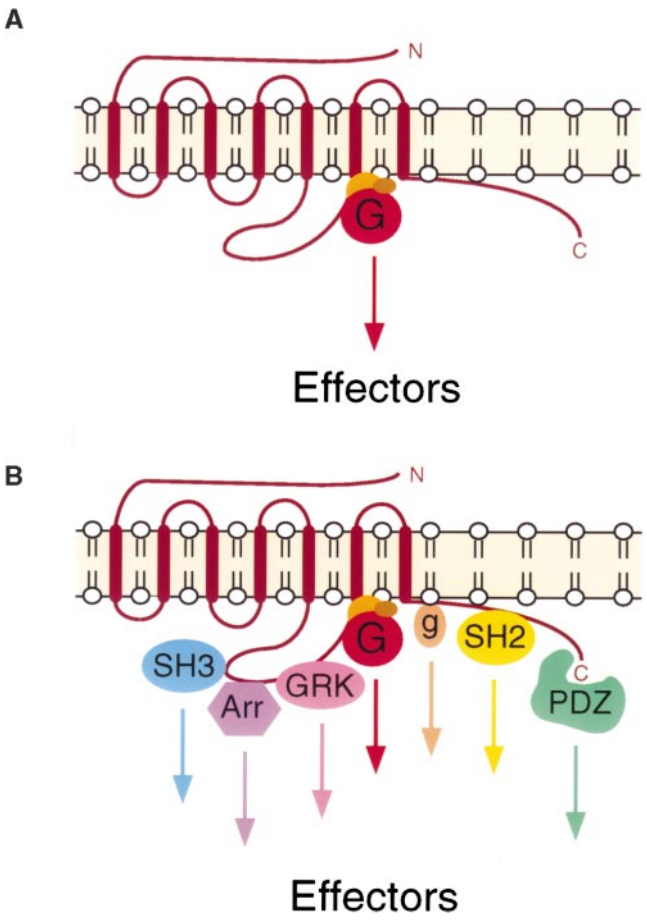

Figure 1. Schematic diagram of heptahelical receptor signaling. (A) The G protein paradigm. Following agonist binding, heptahelical receptors activate heterotrimeric G proteins (G), which then regulate the activity of specific cellular effectors. (B) Beyond the G protein paradigm. Following agonist binding, heptahelical receptors can associate with members of diverse families of intracellular proteins, including heterotrimeric G proteins (G), polyproline-binding proteins such as those containing SH3 domains (SH3), arrestins (Arr), G protein–coupled receptor kinases (GRK), small GTP-binding proteins (g), SH2 domain–containing proteins (SH2) and PDZ domain–containing proteins (PDZ). These interactions allow heptahelical receptors to initiate multiple intracellular signaling pathways, with each subtype of receptor likely coupled to a relatively unique set of effectors.

complexes organized around tyrosine-phosphorylated residues, may be relevant to a small number of heptahelical receptors but not to the majority. Still other mechanisms are likely to be highly receptor-specific: the binding of NHERF to the β_2-adrenergic receptor and the binding of Homer to metabotropic glutamate receptors, for example, depend on the presence of precise motifs that are likely to be found in few other heptahelical receptors, although other receptors are likely to contain slightly modified motifs that mediate binding to other specific PDZ or polyproline-binding domains.

There are >1,000 heptahelical receptors but only ~20 different heterotrimeric G proteins. Such an arrangement would seem to place limitations on the specificity of heptahelical receptor signal transduction, if G proteins were the only mediators of heptahelical receptor–initiated signaling. However, it now seems likely that each heptahelical receptor may activate its own relatively specific set of intracellular signaling pathways, including both G protein–dependent and G protein–independent mechanisms (Fig. 1). The net physiological effect of stimulation of a particular heptahelical receptor will thus reflect the sum of the various intracellular pathways it can activate, with some of the pathways being quite general, others being fairly specific, and some being unique to the individual receptor.

The near future is likely to yield a number of new examples of heptahelical receptor signaling through means other than classical G protein pathways. Some of these new receptor-initiated signaling pathways may be variations on a theme already seen in other heptahelical receptors, while others are likely to be completely novel. In any case, the old view of heptahelical receptors as simple G protein activators is currently being replaced by a new view of these receptors as complicated signal-transducing machines capable of directly coupling to a host of intracellular signaling pathways.

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