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## Role of G Protein–Coupled Receptor Kinase 2 and Arrestins in $\beta$ -Adrenergic Receptor Internalization

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*G protein–coupled receptors (GPCRs) mediate the action of messengers that are key modulators of the function, growth, and differentiation of cardiac and vascular cells. A general feature of GPCRs is the existence of complex regulatory mechanisms that modulate receptor responsiveness and underlie important physiologic phenomena such as signal integration and desensitization. The molecular mechanisms of desensitization have been investigated with the  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR) used as the main model system. Rapid regulation of  $\beta$ AR and other GPCRs appears to involve agonist-promoted receptor phosphorylation by G protein–coupled receptor kinases (GRKs). This is followed by binding of uncoupling proteins termed arrestins and transient receptor internalization, which plays a key role in resensitizing GPCR by allowing its dephosphorylation and recycling. Recent data indicate that, besides the uncoupling function, GRK2 and  $\beta$ -arrestin also directly participate in  $\beta_2$ AR sequestration, thus providing the trigger for its resensitization. A detailed knowledge of the role of GRKs and arrestins in  $\beta$ AR internalization would make their physiologic role in the modulation of cellular responses to messengers better understood. (Trends Cardiovasc Med 1998;8:234–240) © 1998, Elsevier Science Inc.*

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G protein-coupled receptors (GPCRs) mediate the actions of messengers such as catecholamines, endothelins, or angiotensin, which are key regulators of cardiovascular function and are also involved in cardiovascular development and in the control of the growth and remodeling of cardiovascular cells. Acting through different G proteins,  $\alpha$  and  $\beta$  adrenergic, angiotensin II AT1, or endothelin-1 receptors can modulate the intracellular levels of second messengers such as cAMP, diacylglycerol, and calcium; the activity of plasma membrane channels; and the triggering of mitogenic protein kinase cascades (Gudermann et al. 1996). Such receptor systems are important pharmacologic targets in the management of chronic heart failure, angina pectoris or hypertension (Cohn 1996). Agonist stimulation also leads to receptor desensitization and the modulation of signal transduction efficacy in the face of acute or sustained activation. Desensitization of GPCRs is clinically relevant in several cardiovascular diseases (Brodde and Michel 1992, Bristow 1993, Castellano and Böhm 1997). Recent work in the  $\beta$ AR system indicates that G protein-coupled receptor kinases (GRKs) and arrestins are critical components of the regulatory network triggered by agonists and suggests that changes in their expression or functionality may have important physiological consequences.

### • Mechanisms of $\beta$ AR Desensitization

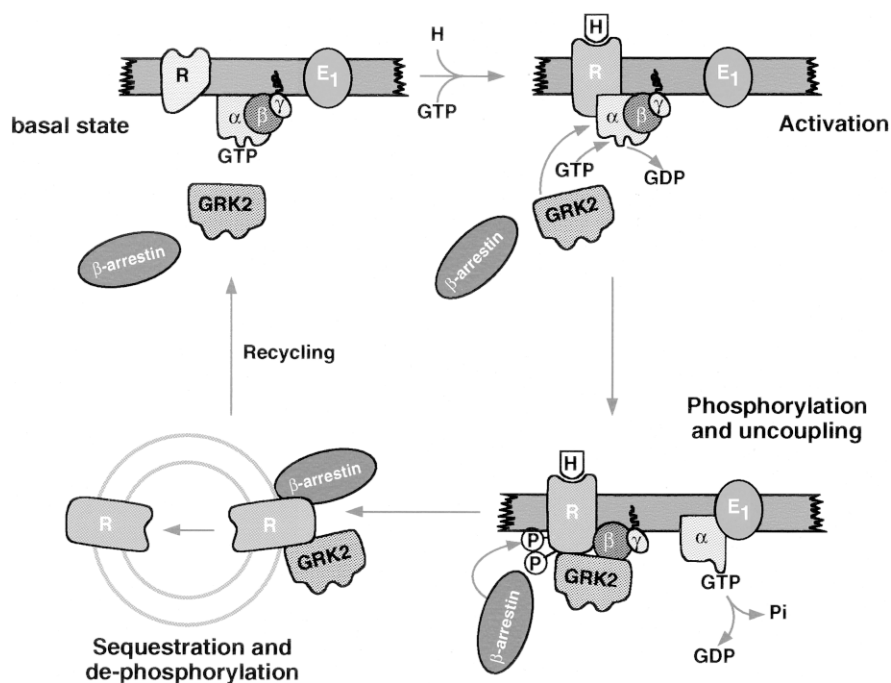
Rapid, short-term  $\beta$ AR desensitization is due to functional uncoupling from G proteins as a consequence of receptor phosphorylation [see Böhm et al. (1997), Chuang et al. (1996), and Lohse et al. (1996), for recent reviews]. Two types of kinases have been reported to alter receptor function: (a) specific kinases able to phosphorylate GPCRs only when occupied by agonists, termed the G protein-coupled receptor kinases, and (b) second messenger-activated protein kinases [such as protein kinase A (PKA) or protein kinase C (PKC)], which phosphorylate the receptor on sites different from GRKs. Receptor regulation by GRKs appears to be more rapid than that mediated by PKA or PKC and desensitizes the subsequent response of the activated receptor only (homologous

desensitization), whereas phosphorylation by PKA or PKC may attenuate the response to multiple distinct agonists operating through different receptors (heterologous desensitization) (Chuang et al. 1996).

The essential steps of homologous desensitization are depicted in Figure 1. Initial experiments with the  $\beta$ AR indicated that the presence of agonists promotes, in addition to G protein activation, the translocation to the plasma membrane of the  $\beta$ -adrenergic receptor kinase ( $\beta$ ARK), a serine/threonine kinase that specifically phosphorylates the agonist-occupied form of the receptor.  $\beta$ ARK was the first cloned member (Benovic et al. 1989) of the GRK family and is now also termed GRK2. Six GRKs have been cloned to date (GRK 1–6), and most of them (with the exception of

GRK1 and GRK4) appear to be expressed in multiple tissues and cell lines. The GRKs share a central catalytic domain and differ in both an N-terminal domain of largely unknown function and a C-terminal domain that contains specific determinants for membrane attachment (Sterne-Marr and Benovic 1995, Böhm et al. 1997, Lohse et al. 1996, Palczewski 1997). Recent data suggest that the binding of  $G\beta\gamma$  and lipids to the C-terminal domain of GRK2 synergistically enhances agonist-dependent receptor phosphorylation and that both types of molecules are required for effective membrane localization of the kinase, in addition to activated receptor domains (Pitcher et al. 1995).

GRK2-mediated phosphorylation in the C-terminal cytosolic tail of the  $\beta$ AR allows interaction of the receptor with



**Figure 1.** A model for the internalization of  $\beta$ -adrenergic and other G protein-coupled receptors. Ligand (H) binding to the receptor (R) simultaneously leads to G protein activation and effector modulation and to the triggering of termination and desensitization mechanisms. Receptor activation of G proteins results in the exchange of GDP for GTP by the G protein  $\alpha$  subunit, followed by the dissociation of the activated  $G\alpha$  subunit from the  $G\beta\gamma$  complex. Both  $G\alpha$  and  $G\beta\gamma$  complexes can interact with plasma membrane effectors (such as  $E_1$ ) to generate intracellular signals. Transduction is terminated by GTP hydrolysis by  $G\alpha$ . In parallel, the agonist-occupied receptor promotes the translocation to the membrane of a G protein-coupled receptor kinase (GRK2), a serine/threonine kinase that specifically phosphorylates the activated form of the receptor. The targeting of GRK2 to the membrane appears to be facilitated by its interaction with free  $G\beta\gamma$  subunits. The phosphorylated receptor is recognized by the cytosolic protein  $\beta$ -arrestin, that prevents any further coupling between the receptor and the G protein. The uncoupled receptor is removed from the plasma membrane by internalization via the clathrin-coated vesicles endocytic pathway. GRK2 and  $\beta$ -arrestin colocalize with the  $\beta$ AR in the internalization vesicles and may act as adaptors of the receptor with the endocytic machinery. The sequestered receptor is dephosphorylated in early endosomes and recycled back to the plasma membrane (resensitization).

additional regulatory proteins, the  $\beta$ -arrestins (Sterne-Marr and Benovic 1995, Böhm et al. 1997), which block signal transduction (see Figure 1). The uncoupled receptors are subsequently removed from the plasma membrane in a process termed internalization or sequestration [see Koenig and Edwardson (1997), for a review]. The internalization compartments of  $\beta$ AR have been identified as early endosomes (von Zastrow and Kobilka 1992). Eventually, receptors are dephosphorylated and recycled back to the plasma membrane. It should be stressed that similar desensitization mechanisms appear to operate for GPCRs other than the  $\beta$ AR. It has been reported that GRK2 is able to phosphorylate a variety of receptors ( $\alpha$  and  $\beta$  adrenergic subtypes, several muscarinic subtypes, AT II, substance P, fMLP receptors, and so on) in an agonist-dependent manner. In addition, other members of the GRK family (such as GRK3 and GRK5) and other  $\beta$ -arrestin homologues are also able to modulate the  $\beta$ AR and different GPCRs [see Chuang et al. (1996) and Palczewski (1997), for additional references].

In summary, receptor activation does not only lead to G protein and effector modulation but to the recruitment of cytosolic regulatory proteins and to changes in the subcellular receptor distribution. Recent and current research in this field is aimed toward a better understanding of the functional role of receptor internalization, of the mechanisms of sequestration, and of the interactions leading to the assembly and disassembly of these regulatory macromolecular complexes.

#### • **Receptor Internalization Plays a Key Role in $\beta$ AR Resensitization**

The functional relevance of agonist-induced receptor internalization has been a controversial issue. Receptor sequestration may contribute to desensitization by physically separating the receptors from G proteins. Several lines of evidence suggest, however, that internalization does not play a role in desensitization. First, the rate of receptor internalization is usually too slow to account for desensitization (Koenig and Edwardson 1997); second, inhibitors of endocytosis do not affect receptor desensitization. Instead, agonist-promoted receptor

sequestration appears to contribute to functional resensitization of the cellular response to messengers. Inhibition of  $\beta_2$ AR internalization with sucrose or concanavalin A results in impaired receptor resensitization (Yu et al. 1993). Similar results are obtained by inhibition of phosphatase activity with caliculyn A (Pippig et al. 1995), suggesting that receptor internalization is a key step for receptor resensitization by allowing the dephosphorylation and recycling of functional receptors back to the plasma membrane. More recently, Krueger et al. (1997) identified a G protein-coupled receptor phosphatase (GRP), a member of the PP2A phosphatase family, which colocalizes with the  $\beta_2$ AR in endosomes during agonist-promoted receptor internalization. This phosphatase dephosphorylates the receptors in acidic environments such as those found in early endosomes. These findings suggest that the low intraluminal pH of the endosome vesicles induces conformational changes in the receptor that can be recognized by the GRP (Krueger et al. 1997). The identification of acidification as a mechanism regulating  $\beta$ AR dephosphorylation would explain the requirement for receptor internalization in endosomes for resensitization. The dynamic balance between receptor uncoupling and resensitization would therefore determine the overall desensitization process.

#### • **Molecular Mechanisms of Receptor Internalization**

The molecular mechanisms underlying agonist-stimulated receptor internalization still remain unclear and are currently the objective of active research. Two main issues are being addressed: (a) the identification of sequence motifs or receptor domains involved in the interaction with the endocytic machinery and (b) the potential relationship between agonist-promoted receptor phosphorylation and uncoupling and the triggering of sequestration.

The role of different receptor domains in internalization has been investigated on different mutant and chimeric receptors. Although domains modulating endocytosis in several GPCRs have been described, a common endocytic motif has not been identified. Truncation of the C-terminal tail may reduce or increase the rate of internalization. Serine- and

threonine-rich regions are critical for agonist-induced sequestration of several receptors, but not for all types of GPCR [reviewed by Böhm et al. (1997) and Koenig and Edwardson (1997)]. The presence of a proline-rich region in the third loop of the  $\beta_1$ AR appears to be responsible for its poor internalization compared with that of the  $\beta_2$ AR, which lacks this structural domain (Green and Liggett 1994). It has also been suggested that a NPX<sub>(2-3)</sub>Y motif near the seventh transmembrane domain could be critical for GPCR internalization. This motif is highly conserved in most GPCRs and is similar to the endocytic motif present in low-density lipoprotein (LDL) or insulin receptors. Mutations of this Tyr residue markedly reduces internalization of  $\beta_2$ AR (Barak et al. 1994) and angiotensin AT1A receptor (Hunyady et al. 1995). It has been shown, however, that the NPLY sequence in the  $\beta_2$ AR is also involved in G protein coupling and receptor phosphorylation and that the effect of the Tyr mutation on sequestration can be rescued by increased GRK2 and  $\beta$ -arrestin expression (see the following section). In addition, mutations in this motif do not affect the endocytosis of other members of the GPCR superfamily (Böhm et al. 1997). This Tyr sequence appears to be important for the conformation of the receptor required for signaling and subsequent internalization, but it is not a universal endocytic motif for GPCR.

#### • **Role of Receptor Phosphorylation in Agonist-Promoted Internalization**

The potential relationship between the phosphorylation of GPCR by GRK2 and receptor internalization, suggested by the facts that both processes are agonist dependent, are triggered by similar concentrations of agonist, and display a sequential temporal frame, has remained controversial. C-terminal truncation of the thrombin receptor or mutation of its phosphorylation sites by GRKs, as well as the removal of the C-terminal tail of the AT1AR, strongly impaired their agonist-dependent internalization (Shapiro et al. 1996). On the other hand, early studies using  $\beta_2$ AR mutants lacking phosphorylation residues for GRK2 seemed to indicate that phosphorylation was not required for internalization (Bouvier et

al. 1988). A variety of recent studies, however, suggest a role for GRK2-mediated phosphorylation in  $\beta$ AR and GPCR sequestration. Tsuga et al. (1994) showed that GRK2 overexpression facilitates the sequestration of m2 muscarinic receptors at low agonist concentrations, whereas a dominant negative mutant of GRK2 attenuates internalization. Similar data were recently obtained for  $\beta_2$ AR agonist-dependent sequestration (Ruiz-Gómez and Mayor 1997); GRK2 overexpression increases the rate and extent of receptor internalization, particularly at low concentrations of agonist (also see the next section). Ferguson et al. (1995) likewise have reported that the failure of the mutant receptor  $\beta_2$ AR Y326A to be sequestered in response to stimulation was a result of defective phosphorylation by GRK2. Overexpression of this kinase can thus rescue the mutant  $\beta_2$ AR Y326A phenotype, this effect being strictly dependent on the presence of phosphorylation sites for GRK2, but not for PKA. Moreover, a dominant-negative GRK2 mutant was able to inhibit both phosphorylation and internalization of a native  $\beta_2$ AR. Similar results were obtained with GRK3, GRK4, and GRK5 and 6 (Ménard et al. 1996), suggesting that the rescue of the Y326A mutant phenotype by these different kinases was a consequence of their ability to phosphorylate the mutant receptor. These data strongly suggest that receptor phosphorylation by GRKs contributes to the achievement and/or maintenance of a conformational state required for endocytosis. Such conformational change would enhance the interaction with intracellular adaptor proteins involved in sequestration or would unmask receptor domains interacting with the endocytic machinery (Koenig and Edwardson 1997).

- **Arrestins (and GRK2 ?) as Molecular Adaptors in Agonist-Promoted Receptor Internalization**

As just described, receptor phosphorylation by GRK2 allows the interaction of the receptor with members of the arrestin family. It has recently been demonstrated that the nonvisual arrestins play a role in agonist-induced sequestration and might act as adaptorlike molecules in this process. Overexpression of either

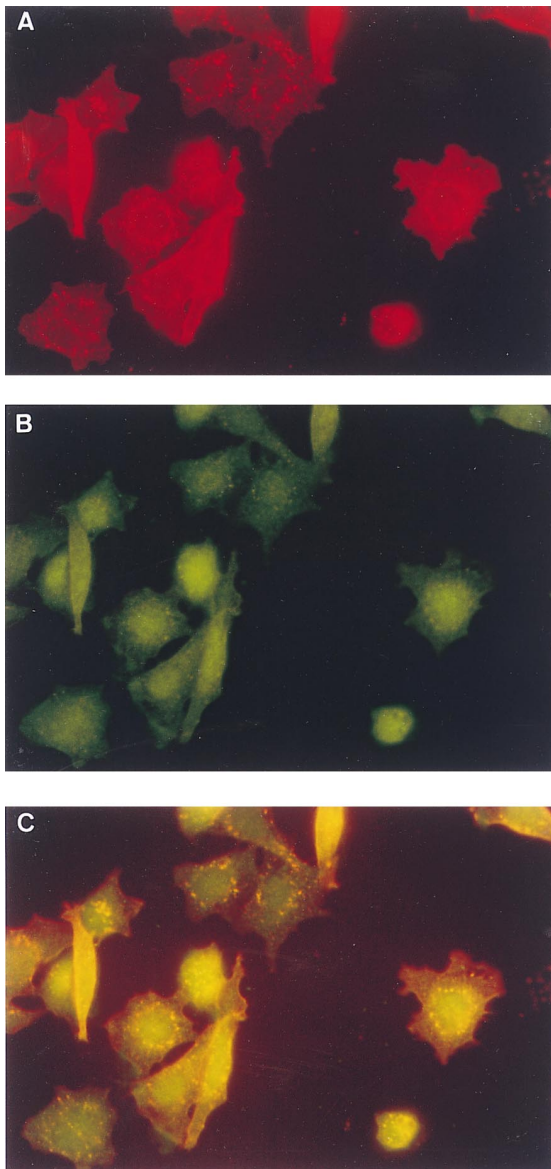
$\beta$ -arrestin1 or  $\beta$ -arrestin2 rescues the  $\beta_2$ AR Y326A internalization-defective phenotype. Interestingly,  $\beta$ -arrestin overexpression does not rescue the impaired phosphorylation of the  $\beta_2$ AR mutant, and its effect is observed even when the receptor sites phosphorylated by GRK are mutated, suggesting the existence in the  $\beta_2$ AR of  $\beta$ -arrestin interaction domains different from the phosphorylated residues (Ferguson et al. 1996). Moreover, dominant-negative  $\beta$ -arrestin mutants ( $\beta$ -arrestin1 V53D and  $\beta$ -arrestin2 V54D) block wild-type  $\beta_2$ AR internalization in the presence of overexpressed GRK2. These results put forward the arrestins as potential adaptor proteins involved in receptor trafficking. In this regard, it has been described that nonvisual arrestins can target GPCR for dynamin-dependent endocytosis via clathrin-coated vesicles (Zhang et al. 1996). Both dynamin and  $\beta$ -arrestin mutants strongly inhibit  $\beta_2$ AR internalization but do not affect angiotensin AT1 receptor sequestration, which is targeted to nonclathrin-coated vesicles by an unknown mechanism. However,  $\beta$ -arrestin1 overexpression increases the extent of AT1AR sequestration by inducing a dynamin-dependent pathway similar to that utilized by the  $\beta_2$ AR. These data suggest a "plasticity" in the choice of endocytic pathways, so the final mechanism by which a given receptor is internalized will be determined not only by the receptor structure and conformation but also by the cellular content of adaptor proteins such as  $\beta$ -arrestins.

Goodman et al. (1996) have provided evidence that arrestins are able to bind in vitro to clathrin, the major structural protein of coated pits.  $\beta$ -arrestin colocalized with  $\beta_2$ AR and clathrin following agonist stimulation in cultured cells, suggesting that the  $\beta$ -arrestin-clathrin interaction occurs in intact cells in the presence of activated receptor. The  $\beta$ -arrestin binding to clathrin is based on ionic and hydrophobic interactions between their carboxy- and amino-terminal domains, respectively (Krupnick et al. 1997, Goodman et al. 1997).

In this context, the fact that GRK2 phosphorylation promotes agonist-induced sequestration and that the expression of wild-type or dominant-negative GRK2 enhances or decreases internalization, respectively, may be explained by a facilitation of the binding of ar-

restins to the phosphorylated receptors. The mechanism of  $\beta$ AR internalization may be more complex, however. We have recently reported (Ruiz-Gómez and Mayor 1997) that GRK2 also colocalizes with  $\beta_2$ AR in endosomes following agonist stimulation. Double immunofluorescence analysis in cells stably transfected with an epitope-tagged  $\beta_2$ AR and GRK2 shows extensive colocalization of  $\beta_2$ AR and the kinase in intracellular vesicles upon receptor stimulation (Figure 2). Such pattern of subcellular distribution is clearly different from that observed under basal conditions ( $\beta_2$ AR at the plasma membrane and GRK2 either soluble or associated with plasma or microsomal membranes [Ruiz-Gómez and Mayor (1997) and data not shown]). Similar results are obtained using confocal microscopy or subcellular fractionation procedures. These data indicate that GRK2 not only translocates to the plasma membrane in response to receptor activation but shares endocytic mechanisms with the  $\beta_2$ AR, and they suggest that macromolecular complexes including receptor, GRK2, and arrestins are present in the initial steps of internalization (see Figure 1). This might suggest that the kinase plays a direct role in sequestration, in addition to facilitating the binding of  $\beta$ -arrestins by receptor phosphorylation. The presence of GRK2 may contribute to a correct conformation of the various receptor domains involved in sequestration, as a consequence of its interaction with cytoplasmic receptor regions other than the phosphorylation sites. Alternatively, direct interaction of GRK2 with yet unidentified proteins of the endocytic machinery may occur. Likewise, the presence of GRK2 in the endosomes may indicate that the endocytic system plays a role in the recycling of the kinase after translocation to the plasma membrane upon receptor stimulation (thus regulating the complex intracellular trafficking of GRK2) or serves other unknown cellular functions (Ruiz-Gómez and Mayor 1997).

Taken together, these recent data raise a number of key questions: Are GRK2 and  $\beta$ -arrestin bound (simultaneously or not) to different domains of the  $\beta_2$ AR or do they interact with other components of the endocytic vesicles? Do these proteins also participate in the internalization of GPCRs that are endocytosed by clathrin- and dynamin-independent



**Figure 2.** Colocalization of epitope-tagged  $\beta_2$ AR with GRK2 in internalization vesicles. HEK-293 cells stably transfected with epitope-tagged  $\beta_2$ AR and GRK2 were cultured on glass coverslips and incubated for 10 min at 37°C with 10  $\mu$ M isoproterenol as described (Ruiz-Gómez and Mayor 1997). After fixation and permeabilization, receptors were localized by immunofluorescence using M1 monoclonal anti-FLAG antibody detected with a Texas Red-conjugated antimouse antibody (*red*; **A**), and GRK2 distribution was assessed with the specific polyclonal Ab FP1 antibody visualized with a fluorescein-conjugated antirabbit antibody (*green*; **B**).  $\beta_2$ AR and GRK2 colocalized in intracellular vesicles, yielding a yellow color when superimposed (**C**).

pathways, such as the AT1AR or the thrombin receptor? How are the complexes formed by the receptor, GRK2, arrestin, and components of the endocytic machinery assembled and disassembled? How and where in the cell takes place the dissociation of GRK2 and, particularly, of  $\beta$ -arrestin, which would be required for the action of phosphatases in the endosomes and subsequent receptor recycling and resensitization?

- **GRK2 and  $\beta$ -Arrestins Synergistically Regulate  $\beta_2$ AR Internalization**

The active participation of GRK2 and arrestins in GPCR internalization suggests that the rate and extent of seques-

tration of a given receptor may vary depending on the cellular content of these proteins. In fact, the extent of sequestration of  $\beta_2$ AR varies from cell type to cell type. Ménard et al. (1997) have recently established a model that provides a direct correlation between the steady-state  $\beta_2$ AR sequestration and the product of total endogenous GRK2 and  $\beta$ -arrestin concentrations, rather than with the concentrations of either one alone. Similar results have been obtained for the m2 muscarinic receptor (Schlador and Nathanson 1997) and the CCR5 chemokine receptor (Aramori et al. 1997). In their model, the sequestration kinetics is regulated not only by the GRK or  $\beta$ -arrestin concentrations but also by the affinity of the receptor for each protein. Both physiologic or pathologic changes

in the levels or activity of GRK2 and  $\beta$ -arrestins would therefore modify the rate and extent of receptor internalization.

- **Concluding Remarks and Physiologic Implications**

The functional relationship between  $\beta$ AR phosphorylation and uncoupling by GRK2 and  $\beta$ -arrestins and the subsequent process of receptor internalization opens exciting research fields and may shed new light on the physiologic functions of these regulatory proteins. The key role played by internalization as a requisite for receptor dephosphorylation and resensitization indicates that GRK2 and  $\beta$ -arrestin perform a dual role in the modulation of  $\beta$ -adrenergic receptors and other GPCRs; they both uncou-

ple the receptor from the signaling machinery and provide the trigger for its resensitization (Ménard et al. 1997).

Internalization of  $\beta$ AR and other GPCRs appears to involve a variety of complex mechanisms. No general endocytic motifs have been detected, although alteration of different receptor domains can markedly alter their ability to internalize in response to agonist. It appears that proper sequestration requires the concerted participation of multiple cytoplasmic domains (Jockers et al. 1996), allowing receptor recognition by regulatory proteins such as GRK2 and arrestins and/or other unknown components of the endocytic machinery. A detailed knowledge of the role of GRK2 and arrestins in the internalization process, and of their interactions with other cellular proteins, is needed to ascertain whether they are necessary and sufficient as adaptors for receptor internalization or whether they play a facilitatory role by promoting a more efficient coupling of the activated receptor to a limiting endocytic factor.

One important emerging issue is that the cellular or tissue complement of GRKs and arrestins may influence the rate and extent of GPCR desensitization and resensitization (Ménard et al. 1997). This means that in a given tissue, different GPCR would be desensitized and resensitized at different rates, depending on their relative affinity for GRKs and arrestins and on the particular receptor-to-GRK-to-arrestin expression ratio. For a given receptor, such as the  $\beta$ AR, it implies that its regulation may vary from one tissue to another, depending on GRK and arrestin expression patterns. More interestingly, physiologic or pathologic changes in the activity or expression levels of these proteins would modulate the desensitization and resensitization patterns of GPCRs. It is worth noting that several mechanisms leading to the regulation of GRK2 activity and of GRK2 and arrestin expression have been identified (García-Higuera and Mayor 1994, Chuang et al. 1996, Lohse et al. 1996). It is tempting to suggest that changes in the expression or function of these proteins may affect the efficacy of signal transduction systems and underlie physiopathologic processes. Marked desensitization of  $\beta$ AR is a major mechanism in congestive heart failure, and increased GRK2 expression has been re-

cently reported in the failing human heart (Ungerer et al. 1993). GRK2 levels are also altered in mouse models of cardiac hypertrophy (Choi et al. 1997), in rat models of hypothyroidism (P. Penela and F. Mayor, in preparation), and in certain hypertensive patients (Gros et al. 1997). It has also been shown that cardiac contractility can be decreased or enhanced in transgenic animals overexpressing GRK2 or a GRK2 inhibitory construct, respectively, thus confirming a key role for this kinase in regulating cardiac function (Koch et al. 1995). Moreover, the disruption of the GRK2 gene results in marked myocardial hypoplasia and embryonic death in mice, indicating a crucial role for this kinase in cardiac cell growth and differentiation (Jaber et al. 1996). It should be stressed that alterations in GRKs or arrestins may affect the function of receptor systems other than the  $\beta$ AR that are related to cardiovascular physiology, such as  $\alpha$ 1-adrenergic, angiotensin, or endothelin receptors.

In summary, given the crucial participation of GRK2 and  $\beta$ -arrestins in the uncoupling and internalization of  $\beta$ AR and other GPCRs, the identification of the signals and mechanisms governing their expression and function in different cell types of the cardiovascular system is of great interest and may help to understand the etiology of diverse cardiovascular malfunctions involving GPCR.

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