

Evolving Concepts in G Protein-Coupled Receptor Endocytosis: The Role in Receptor Desensitization and Signaling

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Abstract—G protein-coupled receptors (GPCRs) are seven transmembrane proteins that form the largest single family of integral membrane receptors. GPCRs transduce information provided by extracellular stimuli into intracellular second messengers via their coupling to heterotrimeric G proteins and the subsequent regulation of a diverse variety of effector systems. Agonist activation of GPCRs also initiates processes that are involved in the feedback desensiti-

zation of GPCR responsiveness, the internalization of GPCRs, and the coupling of GPCRs to heterotrimeric G protein-independent signal transduction pathways. GPCR desensitization occurs as a consequence of G protein uncoupling in response to phosphorylation by both second messenger-dependent protein kinases and G protein-coupled receptor kinases (GRKs). GRK-mediated receptor phosphorylation promotes the binding of β -arrestins, which not only uncouple receptors from heterotrimeric G proteins but also target many GPCRs for internalization in clathrin-coated vesicles. β -Arrestin-dependent endocytosis of GPCRs involves the direct interaction of the carboxyl-terminal tail domain of β -arrestins with both β -adaptin and clathrin. The focus of this review is the current and

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evolving understanding of the contribution of GRKs, β -arrestins, and endocytosis to GPCR-specific patterns of desensitization and resensitization. In addition to their role as GPCR-specific endocytic adaptor proteins, β -arrestins also serve as molecular scaffolds that foster the formation of alternative, heterotri-

meric G protein-independent signal transduction complexes. Similar to what is observed for GPCR desensitization and resensitization, β -arrestin-dependent GPCR internalization is involved in the intracellular compartmentalization of these protein complexes.

I. Introduction

G protein-coupled receptors (GPCRs²) constitute a superfamily of seven transmembrane spanning proteins that respond to a diverse array of sensory and chemical stimuli, such as light, odor, taste, pheromones, hormones, and neurotransmitters. GPCRs transduce the information provided by these stimuli into intracellular second messengers that are interpreted as meaningful signals by the cell. This process involves the coupling of agonist-activated GPCRs to a wide variety of effector systems via their interaction with heterotrimeric guanine nucleotide binding proteins (G proteins). The binding of agonist to a GPCR selects for a receptor conformation state that promotes the exchange of GDP for GTP on the G protein α -subunit and is presumed to allow the dissociation of the G protein $G\alpha$ - and $G\beta\gamma$ -subunits (Neer, 1995; Surya et al., 1998). Subsequently, the activated $G\alpha$ - and $G\beta\gamma$ -subunits positively and/or negatively regulate the activity of effector enzymes and ion channels (reviewed by Neer et al., 1995; Gautam et al., 1998). Agonist activation of a GPCR not only results in the G protein-dependent activation of effector systems, but also sets in place a series of molecular interactions that allows for: 1) feedback regulation of G protein coupling, 2) receptor endocytosis, and 3) signaling through G protein-independent signal transduction pathways (Lefkowitz, 1993; Ferguson et al., 1996a; Ferguson and Caron, 1998; Krupnick and Benovic, 1998; Hall et al., 1999; Luttrell et al., 1999a; Schoneberg et al., 1999). Thus, in the relatively few years since the cloning of the first GPCRs (Nathans and Hogness; 1983; Dixon et al., 1986), work done in a large number of laboratories has made it apparent that the functional activity of GPCRs extends far beyond the traditional model of: receptor \rightarrow G protein \rightarrow effector.

GPCR activity represents a coordinated balance between molecular mechanisms governing receptor signaling, desensitization, and resensitization. Receptor de-

sensitization, the waning of GPCR responsiveness to agonist with time, represents an important physiological "feedback" mechanism that protects against both acute and chronic receptor overstimulation. GPCR desensitization also acts to filter information from multiple receptor inputs into an integrated and meaningful biological signal through second messenger protein kinase-dependent phosphorylation and inactivation of weaker receptor-mediated signals. However, GPCR desensitization can also significantly limit the therapeutic usefulness of many receptor agonists.

Three families of regulatory molecules are known to contribute to the GPCR desensitization process: second messenger-dependent protein kinases, G protein-coupled receptor kinases (GRKs) and arrestins (reviewed by Lefkowitz, 1993; Ferguson et al., 1996a; Ferguson and Caron, 1998; Krupnick and Benovic, 1998). As will be documented in the present review, it is now recognized that the same regulatory molecules that contribute to agonist-stimulated receptor desensitization (GRKs and β -arrestins), initiate and regulate GPCR endocytosis, intracellular trafficking, and resensitization (e.g., Tsuga et al., 1994; Ferguson et al., 1995, 1996b; Zhang et al., 1997; Oakley et al., 1999).

In addition to signaling via heterotrimeric G proteins, it is now recognized that GPCRs act as scaffolds promoting the formation and compartmentalization of G protein-independent signal transduction complexes. A growing number of proteins have been identified that bind GPCRs and either couple GPCRs to G protein-independent signal transduction pathways or alter G protein specificity and agonist selectivity. The list of GPCR interacting proteins now includes: GRKs (Benovic et al., 1991), arrestins (Lohse et al., 1990a), calmodulin (Minakami et al., 1997; Thomas et al., 1999; Wang et al., 1999), calycon (Lezcano et al., 2000), A kinase-anchoring protein (AKAP) (Fraser et al., 2000), ATRAP (Daviet et al., 1999), tubulin (Ciruela et al., 1999), receptor activity modulating proteins (McLatchie et al., 1998), Homer (Brakeman et al., 1997), Janus kinase 2 (Marrero et al., 1995), PDZ domain-containing proteins (e.g., NHERF, RGS12), (Hall et al., 1998; Snow et al., 1998) SH3 domain-containing adaptor molecules (e.g., Grb2, Nck, c-Src, and endophilin) (Oldenhof et al., 1998; Tang et al., 1999; Cao et al., 2000), and small G proteins (Mitchell et al., 1998). Although this list of GPCR-interacting proteins is expanding rapidly, the global impact of these protein interactions on GPCR signaling has yet to be precisely determined. The potential contribution of

² Abbreviations: GPCR, G protein-coupled receptor; AKAP, A kinase-anchoring protein; α_2 AR, α_2 -adrenergic receptor; AT_{1A}R, angiotensin II type 1A receptor; β -ARK, β -adrenergic receptor kinase; β_2 AR, β_2 -adrenergic receptor; CXCR2, interleukin-8 receptor B; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; GnRH, gonadotropin-releasing hormone; G protein, heterotrimeric guanine nucleotide binding protein; GRK, G protein-coupled receptor kinase; mAChR, muscarinic acetylcholine receptor; MAPK, mitogen-activated protein kinase; PAR, protease-activated receptor; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; RGS, regulator of G protein signaling.

these interactions to GPCR signaling has been reviewed previously (Bockaert and Pin; 1999; Hall et al., 1999).

The present review will focus on our current, yet evolving, understanding of the molecular mechanism(s) involved in GPCR endocytosis, as well as the contribution of receptor endocytosis to the regulation of GPCR signaling. The identification of the molecular mechanisms underlying GPCR endocytosis has progressed rapidly in recent years. However, as new information becomes available regarding the multitude of potential molecular interactions between GPCRs, their regulatory proteins and the cellular endocytic machinery, it is becoming clear that GPCR endocytosis is regulated by a myriad of complex determinants. Although many of the molecular mechanisms first described for the β_2 -adrenergic receptor (β_2 AR) might apply equally well to other GPCRs, this is more likely to be an exception rather than the rule. Thus, it can be anticipated that the diversity in GPCR structure/function will lead to important differences in the intracellular trafficking patterns of distinct GPCR subtypes. Therefore, the challenge awaiting researchers in the field will be to understand the reasons for observed differences in GPCR subtype regulation. Consequently, this review will not only focus on well established paradigms of GPCR regulation, but will also discuss the role of GPCR endocytosis in shifting the traditional paradigms for GPCR regulation and signaling.

II. G Protein-Coupled Receptor Desensitization

The exposure of GPCRs to agonists often results in a rapid attenuation of receptor responsiveness. This process, termed desensitization, is the consequence of a combination of different mechanisms. These mechanisms include the uncoupling of the receptor from heterotrimeric G proteins in response to receptor phosphorylation (e.g., Bouvier et al., 1988; Hausdorff et al., 1989; Lohse et al., 1990a,b), the internalization of cell surface receptors to intracellular membranous compartments (e.g., Hermans et al., 1997; Trejo and Coughlin, 1998; Oakley et al., 1999; Anborgh et al., 2000), and the down-regulation of the total cellular complement of receptors due to reduced receptor mRNA and protein synthesis, as well as both the lysosomal and plasma membrane degradation of pre-existing receptors (e.g., Doss et al., 1981; Hadcock and Malbon 1988; Valiquette et al., 1990, 1995; Jockers et al., 1999; Pak et al., 1999). The time frames over which these processes occur range from seconds (phosphorylation) to minutes (endocytosis) and hours (down-regulation). The extent of receptor desensitization varies from complete termination of signaling, as observed in the visual and olfactory systems, to the attenuation of agonist potency and maximal responsiveness, such as observed for the β_2 AR (Pippig et al., 1995; Zhang et al., 1997; Sakmar, 1998). The extent of receptor desensitization is regulated by a number of factors that

include receptor structure and cellular environment (Jockers et al., 1996; Aramori et al., 1997; Menard et al., 1997; Barlic et al., 1999). Since GPCR endocytic mechanisms are intimately linked to the molecular events that contribute to the desensitization of GPCR responsiveness, a clear understanding of these desensitization processes is required.

Traditionally, GPCR desensitization has been characterized by events that contribute to the uncoupling of receptors from their heterotrimeric G proteins. Thus, for the purpose of this review, the term desensitization refers solely to the uncoupling of GPCRs from G protein-mediated signaling pathways. However, GPCR signaling can also be terminated at the level of the heterotrimeric G protein. For example, a family of proteins, termed regulators of G protein signaling (RGS) act to increase the rate of hydrolysis of GTP bound to both G_i and G_q α -subunits, thereby dampening signaling via G_i - and G_q -regulated signaling pathways (reviewed by Dohlman and Thorner, 1997; Siderovski et al., 1999). The recent demonstration that RGS12 interacts with the carboxyl-terminal PDZ domain binding motif of the interleukin-8 receptor B (CXCR2) suggests that the regulation of G protein signaling by RGS proteins may also involve direct interactions with the receptor (Snow et al., 1998).

A. Protein Kinase Phosphorylation

The most rapid means by which GPCRs are uncoupled from heterotrimeric G proteins is through the covalent modification of the receptor as a consequence of phosphorylation by intracellular kinases. It is generally accepted that both second messenger-dependent protein kinases [e.g., cAMP-dependent protein kinase (PKA) and protein kinase C (PKC)] and GRKs phosphorylate serine and threonine residues within the intracellular loop and carboxyl-terminal tail domains of GPCRs (reviewed by Lefkowitz, 1993; Ferguson et al., 1996a; Ferguson and Caron, 1998; Krupnick and Benovic, 1998). GRK family members selectively phosphorylate agonist-activated receptors, thereby promoting the binding of cytosolic cofactor proteins called arrestins, which sterically uncouple the receptor from heterotrimeric G protein (Benovic et al., 1987; Lohse et al., 1990b, Pippig et al., 1993). In contrast, second messenger-dependent protein kinases not only phosphorylate agonist-activated GPCRs, but also indiscriminately phosphorylate receptors that have not been exposed to agonist (Hausdorff et al., 1989; Lohse et al., 1990a). Thus, agonist-independent phosphorylation is a property that has generally been ascribed only to second messenger-dependent protein kinases and not GRKs (Lefkowitz, 1993). Nevertheless, it is now recognized that GPCRs spontaneously isomerize to an activated conformation in the absence of agonist, which suggests that GRKs may also contribute to the regulation of basal GPCR activity (Pei et al., 1994; Rim and Oprian, 1995). Second messenger-dependent

protein kinases are also thought to represent the predominant mechanisms by which GPCR desensitization is achieved at low agonist concentrations. However, in young hypertensive patients, elevated GRK2 protein levels are correlated with enhanced β_2 AR desensitization in response to low levels of circulating catecholamines (Gros et al., 1997). This observation demonstrates the need to re-evaluate the relative contributions of second messenger-dependent protein kinases and GRKs to receptor/G protein uncoupling at low agonist concentrations. Moreover, second messenger-dependent protein kinase and GRK activities may not be independent from each other since, in the olfactory system, inhibition of either kinase family results in the complete abolition of olfactory receptor desensitization (Schleicher et al., 1993; Boekhoff et al., 1994). Thus, the relative contributions and mechanisms by which second messenger-dependent protein kinases and GRKs regulate GPCR desensitization are not fully understood and may be more complex than originally envisaged. GRKs also contain amino-terminal RGS domains, suggesting that they may not only regulate GPCR signaling at the level of the receptor, but also regulate the activity of the G protein as well (Carman et al., 1999; Sallèse et al., 2000). The ability of GRKs to serve as RGS-like proteins may account for the phosphorylation-independent desensitization of the parathyroid hormone receptor responsiveness in response to GRK protein overexpression (Dicker et al., 1999).

1. *Second Messenger-Dependent Protein Kinases.* The second messenger-dependent protein kinases, PKA and PKC, are phosphotransferases that catalyze the transfer of the γ -phosphate group of ATP to serine and threonine residues contained within specific amino acid consensus sequences of proteins. Second messenger-dependent protein kinase are activated in response to GPCR-stimulated increases in intracellular second messengers such as cAMP, Ca^{2+} , and diacylglycerol and participate in GPCR signaling by mediating the phosphorylation of downstream target proteins. However, these kinases also feedback phosphorylate GPCRs at phosphorylation consensus sites within their intracellular

loops and carboxyl-terminal tail domains. For example, β_2 AR-activated PKA activity leads to receptor desensitization in response to PKA-mediated phosphorylation of at least one of two PKA consensus sites within the receptor (Bouvier et al., 1988; Yuan et al., 1994; Moffet et al., 1996). One site is found within the G protein-binding domain of the third intracellular loop of the β_2 AR and the other site is found within the proximal region of the β_2 AR carboxyl-terminal tail (Bouvier et al., 1988; Yuan et al., 1994; Moffett et al., 1996). It is proposed that covalent modification of the β_2 AR at only the PKA site within the third intracellular loop domain contributes to receptor/G protein coupling (Yuan et al., 1994). However, phosphorylation of the PKA site within the β_2 AR carboxyl-terminal tail occurs subsequent to the depalmitoylation of cysteine residue 341, suggesting that this site contributes to the agonist-dependent desensitization of β_2 AR responsiveness (Moffet et al., 1996). Two recent studies suggest that PKA-mediated phosphorylation of the β_2 AR involves the direct and constitutive association of the AKAP (AKAP79/150 and AKAP250) with the receptor (Fraser et al., 2000; Lin et al., 2000). PKC activation leads to the phosphorylation and desensitization of many G_i - and G_q -linked GPCRs (e.g., Diviani et al., 1997; Liang et al., 1998; Tang et al., 1998). Nonetheless, second messenger-dependent protein kinase-mediated mechanisms of receptor desensitization have received less attention than GRK-mediated mechanisms of receptor desensitization.

2. G Protein-Coupled Receptor Kinases.

a. *The G Protein-Coupled Receptor Kinase Family.* The GRK family of kinases is comprised of seven family members that share significant sequence homology (reviewed by Premont et al., 1995; Stoffel et al., 1997) (Table 1). Each of the GRKs share a similar functional organization with a central catalytic domain, an amino-terminal domain that is thought to be important for substrate recognition and that contains an RGS-like domain, and a carboxyl-terminal domain that contributes to the plasma membrane targeting of the kinase (Fig. 1). The members of the GRK family can be subdivided into three groups based on sequence and func-

TABLE 1
Characteristics of GRK family members

Family Name	Size (kDa)	Polypeptide Variants	Tissue Distribution ^a	Covalent Modification	Activators	Inactivators
GRK1 (rhodopsin kinase)	63	N.D.	Retina (ROS) > pineal	Farnesylation	Polycations	Recoverin
GRK2 (β ARK1)	79	N.D.	<i>pbl</i> > <i>cx</i> > <i>h</i> > <i>lu</i> > <i>k</i>	N.D.	$G\beta\gamma$, PIP ₂ , PKC, c-Src	MAPK
GRK3 (β ARK2)	80	N.D.	<i>olf</i> > <i>b</i> > <i>s</i> > <i>h</i> > <i>lu</i> > <i>k</i>	N.D.	$G\beta\gamma$, PIP ₂	N.D.
GRK4	66	Four	<i>t</i> >>> <i>b</i>	Palmitoylation	N.D.	N.D.
GRK5	68	N.D.	<i>h</i> , <i>lu</i> > <i>sk</i> > <i>b</i> , <i>l</i> > <i>k</i>	N.D.	Polycations, PIP ₂	PKC, calmodulin
GRK6	66	Yes	<i>b</i> , <i>sk</i> >> <i>h</i> , <i>lu</i> , <i>k</i> > <i>l</i>	Palmitoylation	Polycations	N.D.
GRK7	62	N.D.	Retina (cone)	N.D. (Farnesylation?)	N.D.	N.D.

N.D., not determined.

^a As determined by mRNA expression: *b*, brain; *cx*, cerebral cortex; *h*, heart; *l*, liver; *lu*, lung; *k*, kidney; *olf*, olfactory tubercle; *pbl*, primary blood leukocytes; *s*, spleen; *sk*, skeletal muscle; *t*, testes. PIP₂, phosphatidylinositol 4,5-bisphosphate; ROS, rod outer segment.

References used: Arriza et al., 1992; Inglese et al., 1992; Pitcher et al., 1992, 1995, 1999; Benovic and Gomez, 1993; Dawson et al., 1993; Kunapuli and Benovic, 1993; Parruti et al., 1993a; Kunapuli et al., 1994; Premont et al., 1994, 1996; Stoffel et al., 1994; Touhara et al., 1994; Chuang et al., 1995; Winstel et al., 1996; Stoffel et al., 1998; Weiss et al., 1998; Iacovelli et al., 1999; Sarnago et al., 1999; Elorza et al., 2000.

TABLE 2
Characteristics of arrestin family members

Family Name	Size (a.a.)	Polypeptide Variant (a.a.)	Tissue Distribution ^a	Substrate	Phosphorylation	Function
Visual arrestin (S antigen) <i>bovine</i>	404	396, 370 (p ⁴⁴)	<i>r</i> > <i>pin</i> ; <i>cb</i> = <i>pbl</i> p ⁴⁴ . <i>ros</i> >> <i>ris</i> ^b	Rho > β ₂ AR > m2 mAChR	PKC Ca ²⁺ -calmodulin	Desensitization
Cone arrestin (C- or X-arrestin) <i>human</i>	388	N.D.	> <i>pin</i> > <i>pit</i> , <i>lu</i>	N.D.	N.D.	Desensitization
β-Arrestin1 <i>rat</i>	418	410	<i>b</i> > <i>hip</i> > <i>bs</i> , <i>s</i> , <i>o</i> > <i>h</i> >> <i>lu</i> , <i>k</i> > <i>pit</i> , <i>sk</i> > <i>l</i>	β ₂ AR > m2 mAChR >> Rho	MAPK	Desensitization Endocytosis Signaling
β-Arrestin 2 ^c (arrestin3) <i>rat</i>	410	399	<i>s</i> > <i>hip</i> > <i>b</i> > <i>o</i> , <i>bs</i> > <i>pit</i> , <i>h</i> , <i>l</i> >> <i>k</i> >> <i>lu</i> > <i>sk</i>	β ₂ AR, m2 mAChR >> Rho	Casein kinase II	Desensitization Endocytosis Signaling

N.D., not determined; a.a., amino acids.

^a As determined by mRNA expression: *b*, brain; *bs*, brain stem; *c*, cone photoreceptors; *cb*, cerebellum; *cx*, cerebral cortex; *h*, heart; *hip*, hippocampus; *hyp*, hypothalamus; *i*, intestine; *l*, liver; *lu*, lung; *k*, kidney; *o*, ovary; *pin*, pinealocytes; *pit*, pituitary; *pbl*, primary blood leukocytes; *r*, retina; *ros*, rod outer segment; *ris*, rod inner segment; *s*, spleen; *sk*, skeletal muscle; *t*, testes.

^b As determined by immunofluorescence.

^c Very abundant in human mononuclear leukocytes.

References used: Yamaki et al., 1987; Sagi-Eisenberg et al., 1989; Lohse et al., 1990a; Yamada et al., 1990; Attramadal et al., 1992; Parruti et al., 1993b; Sterne-Marr et al., 1993; Craft et al., 1994; Smith et al., 1994; Gurevich et al., 1995; Ferguson et al., 1996b; Weiss et al., 1998; Kim et al., 1999; Lin et al., 1999; Luttrell et al., 1999; Barlic et al., 2000; DeFea et al., 2000.

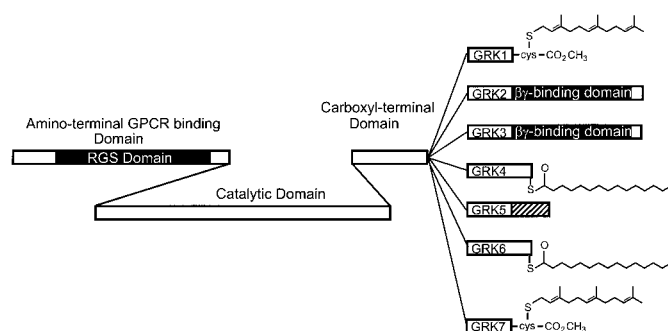


FIG. 1. Schematic representation of the domain architecture for GRK1–GRK7. The amino-terminal GPCR-binding domain of GRK1–GRK7 contains a conserved RGS domain (Carman et al., 1999). The plasma membrane targeting of each of the GRKs is mediated by distinct mechanisms that involves their carboxyl-terminal domains. GRK1 and GRK7 are farnesylated at CAAX motifs in their carboxyl termini (Inglese et al., 1992). The carboxyl-terminal domains of GRK2 and GRK3 contain a βγ-subunit binding domain that exhibits sequence homology to a pleckstrin homology domain (Pitcher et al., 1992; Touhara et al., 1994). The GRK5 carboxyl-terminal domain contains a stretch of 46 basic amino acids that mediate plasma membrane phospholipid interactions (Kunapuli et al., 1994; Premont et al., 1994). GRK4 and GRK6 are palmitoylated at cysteine residues (Stoffel et al., 1994, 1998). Figure adapted from Stoffel et al., 1997.

tional homology: 1) GRK1 (rhodopsin kinase) (Shichi and Somers, 1978) and GRK7 (a new candidate cone opsin kinase) (Weiss et al., 1998); 2) GRK2 (β-adrenergic receptor kinase 1, βARK1) (Benovic et al., 1986) and GRK3 (β-adrenergic receptor kinase 2, βARK2) (Benovic et al., 1991); and 3) GRK4 (Premont et al., 1994; Salles et al., 1994), GRK5 (Kunapuli and Benovic, 1993), and GRK6 (Benovic and Gomez, 1993).

b. Targeting and Regulation. In unstimulated cells, GRK1–3 are localized to the cytosol and translocate to bind their substrates in response to the agonist activation of their plasma membrane-bound receptor targets. For GRK1, the light-activated association of the kinase with the plasma membrane is facilitated by the post-translational farnesylation of its carboxyl-terminal

CAAX motif (Inglese et al., 1992). The activity of GRK1, but not GRK2–6, can be regulated by the calcium sensor protein recoverin (Iacovelli et al., 1999). Although GRK2 and GRK3 are not isoprenylated, the plasma membrane translocation of these kinases is regulated in part by their association with the βγ-subunit of heterotrimeric G proteins (Pitcher et al., 1992; Boekhoff et al., 1994). The association of GRK2 and GRK3 with G protein βγ-subunits is mediated by a 125 amino acid βγ-subunit-binding domain in the carboxyl termini of the kinases that bears striking sequence homology with pleckstrin homology domains (Koch et al., 1993; Touhara et al., 1994) (Fig. 1). Membrane translocation of endogenous GRK2 can be impaired by the overexpression of the carboxyl-terminal βγ-binding domain of GRK2, which presumably acts to sequester free G protein βγ-subunits (Koch et al., 1993). The expression of the βγ-binding domain has been used to block GRK-mediated desensitization in both in vitro cell culture systems and in vivo using transgenic mice (Koch et al., 1993, 1995; Dicker et al., 1999). The targeting of GRK2 and GRK3 to the plasma membrane is also influenced by phosphatidylinositol 4,5-bisphosphate binding to the carboxyl-terminal pleckstrin homology domain of the kinases (Pitcher et al., 1995a). Recently, it was demonstrated that mitogen-activated protein kinase (MAPK) phosphorylation of the GRK2 carboxyl-terminal domain decreased the efficacy of the kinase toward GPCR substrates (Pitcher et al., 1999; Elorza et al., 2000). In contrast, GRK2 activity and plasma membrane translocation are potentiated in response to serine phosphorylation by both PKC and tyrosine phosphorylation by c-Src (Chuang et al., 1995; Winstel et al., 1996; Sarnago et al., 1999). Consequently, GRK2 activity seems to be regulated by a complex series of protein phosphorylation events.

In the absence of GPCR activation by agonist, GRK4, GRK5, and GRK6 all exhibit substantial membrane localization. Both GRK4 and GRK6 are palmitoylated on carboxyl-terminal cysteine residues (Stoffel et al., 1994; Premont et al., 1996; Stoffel et al., 1998) (Fig. 1). The palmitoylation of these kinases seems essential for their plasma membrane localization since only the palmitoylated form of these kinases is isolated from membrane fractions (Stoffel et al., 1994; Premont et al., 1996; Stoffel et al., 1998). Moreover, palmitoylated GRK6 is 10-fold more active at phosphorylating β_2 AR than the nonpalmitoylated GRK6 (Stoffel et al., 1998). Since protein palmitoylation is a reversible post-translational protein modification, dynamic regulation of the palmitoylation state of GRK4 and GRK6 may have important effects on the functional activity of these kinases.

GRK5 association with the plasma membrane is thought to be mediated by the electrostatic interaction between 46 highly basic amino acid residues contained within the carboxyl terminus of the kinase and plasma membrane phospholipids (Kunapuli et al., 1994; Premont et al., 1994) (Fig. 1). The activity of the GRK5 enzyme is not only influenced by autophosphorylation of serine and threonine residues in the carboxyl terminus of the kinase, but also by the binding of membrane phospholipids (Kunapuli et al., 1994). Unlike GRK2, PKC-mediated phosphorylation reduces GRK5 activity (Chuang et al., 1996). In addition, calmodulin associates directly with the amino-terminal domain of GRK5 and not only reduces the ability of the kinase to bind both receptor and phospholipids, but also inhibits the activity of the kinase by stimulating autophosphorylation of serine and threonine residues that are distinct from those involved in the activation of the kinase (Pronin and Benovic, 1997; Pronin et al., 1997; Iacovelli et al., 1999). Since the activation of PKC, Ca^{2+} -calmodulin, and/or phospholipid metabolism is stimulated by some, but not all GPCRs, it is likely that GPCR subtype differences in the stimulation of GRK5 activity will be observed.

c. Site of Action. GRKs phosphorylate GPCRs at both serine and threonine residues localized within either the third intracellular loop or carboxyl-terminal tail domains. Some GPCRs, for example the α_2 -adrenergic receptor (α_2 AR) and m2 muscarinic acetylcholine receptor (mAChR), have short carboxyl-terminal tails containing relatively few serine and threonine residues, but have enlarged third intracellular loop domains containing multiple serine and threonine residues. In contrast, receptors such as rhodopsin and the β_2 AR have relatively short third intracellular loops but have long carboxyl-terminal tails containing several serine and threonine residues. Mutation of all of the serine and threonine residues within either the carboxyl-terminal tail of the β_2 AR or the third intracellular loop of the m2 mAChR abolishes GRK-mediated phosphorylation of these receptors (Bouvier et al., 1988; Nakata et al., 1994). Al-

though no distinct GRK phosphorylation consensus motifs have been identified, localization of acidic amino acid residues proximal to the site of phosphorylation seems to favor GRK2-mediated phosphorylation (Onorato et al., 1991; Chen et al., 1993).

The stoichiometry of GRK phosphorylation differs, depending upon the GPCR studied. Nonetheless, whereas GRKs phosphorylate receptors on many sites *in vitro*, it is thought that, at least in the case of rhodopsin, receptor desensitization requires only the initial phosphorylation event (Ohguro et al., 1993). In addition, high-affinity binding of arrestins to rhodopsin and β_2 AR requires GRK phosphorylation to a stoichiometry of only 2 mol of phosphate per mole of receptor *in vitro* (Gurevich et al., 1995). However, mutation of the primary GRK-phosphorylated residues on the β_2 AR identified *in vitro* (Fredericks et al., 1996) did not prevent GRK-mediated β_2 AR desensitization in cells (Seibold et al., 1998). Thus, the primary sites of GRK phosphorylation identified *in vitro* may not necessarily represent the GRK-phosphorylated residues *in vivo*. Alternatively, the phosphorylation of secondary GRK phosphorylation sites may compensate for the loss of the primary site for GRK-mediated phosphorylation. It is also becoming apparent that the GRK-mediated phosphorylation of clusters of serine and threonine residues in the carboxyl-terminal tails of some receptors may regulate the stability of receptor/arrestin complexes (Oakley et al., 1999). Thus, it is likely that difference in the stoichiometry of GRK-mediated phosphorylation of GPCR subtypes may underlie observed differences in the intracellular trafficking and signaling of desensitized receptors (see *Section III.B.*).

3. Other Kinases. In addition to serving as substrates for PKA, PKC, and GRK phosphorylation, GPCRs have been shown to serve as substrates for phosphorylation by other protein kinases. Casein kinase 1a-mediated phosphorylation of the third intracellular loop domain of the m3 mAChR occurs in response to agonist activation of the receptor (Tobin et al., 1997; Budd et al., 2000). Moreover, casein kinase 1a-mediated phosphorylation of the m3 mAChR was blocked by either the expression of a catalytically inactive casein kinase 1a mutant or a peptide corresponding to the third intracellular loop domain of the m3 mAChR (Budd et al., 2000). Nonetheless, the functional consequence of casein kinase 1a receptor phosphorylation remains to be fully elucidated (Budd et al., 2000). The presence of casein kinase phosphorylation consensus motifs within the intracellular loop and carboxyl-terminal tail domains of many GPCRs makes these observations particularly intriguing.

There is evidence that tyrosine kinase-mediated GPCR phosphorylation may influence the activity of some GPCRs. Mutagenesis of tyrosine residues in the carboxyl-terminal tail of the μ opioid receptor reduced the agonist stimulated down-regulation of the receptor (Pak et al., 1999). This effect could be mimicked using

the tyrosine kinase inhibitor genistein (Pak et al., 1999). Insulin-stimulated tyrosine phosphorylation of the β_2 AR has also been reported (Valiquette et al., 1995). However, whereas carboxyl-terminal tyrosine residues contributed to β_2 AR down-regulation, these residues were not the substrates for insulin-promoted tyrosine phosphorylation (Valiquette et al., 1990, 1995). Tyrosine phosphorylation of the bradykinin B_2 receptor in response to agonist can be prevented by genistein treatment and seems to contribute to receptor signaling leading to arachidonic acid release (Jong et al., 1993). The identity of the tyrosine kinase(s) mediating the phosphorylation of these receptors is unknown.

B. The Arrestins

GRK-mediated phosphorylation of either rhodopsin or the β_2 AR was not sufficient to promote the full inactivation of these GPCRs; full inactivation required an additional component or "arresting agent". The identification of an arresting protein was first made in rod outer segments where a 48-kDa protein, now called visual arrestin, was demonstrated to bind light-activated rhodopsin (Pfister et al., 1985). Subsequently, a visual arrestin-like protein, β -arrestin1, was identified as a cofactor required for GRK2-mediated β_2 AR desensitization in vitro (Benovic et al., 1987). The cloning of β -arrestin1 revealed 59% sequence homology with visual arrestin (Lohse et al., 1990a). The role of arrestins in regulating the desensitization of GPCRs has been demonstrated in intact cells (Pippig et al., 1995; Zhang et al., 1997), in the *Drosophila* photosystem in vivo (Dolph et al., 1993), and in mice either lacking visual arrestin or β -arrestin2 (Xu et al., 1997; Bohn et al., 1999). The mechanism(s) by which arrestins contribute to GPCR desensitization involves both the physical uncoupling of GPCRs from heterotrimeric G proteins (visual arrestins and β -arrestins) and the targeting of GPCRs for endocytosis (β -arrestins) (see Sections II.B.2. and III.B.1.).

1. The Arrestin Family. To date, four arrestin family members have been identified (Table 1). The members of the arrestin family can be divided into two groups based on sequence homology, function, and tissue distribution: 1) visual arrestin (S antigen) (Shinohara et al., 1987; Yamaki et al., 1987) and cone arrestin (X-arrestin or C-arrestin) (Murakami et al., 1993; Craft et al., 1994) and 2) β -arrestins (β -arrestin1 and β -arrestin2) (Lohse et al., 1990a; Attramadal et al., 1992). Visual arrestin is a major protein constituent of rod outer segments and is localized primarily to the retina with low expression in the pineal gland (Smith et al., 1994). C-Arrestin is highly enriched in both retina and pineal gland, but is localized primarily within cone photoreceptors in the retina (Craft et al., 1994). The β -arrestins are ubiquitously expressed outside the retina, but are predominantly localized in neuronal tissues and in the spleen (Attramadal et al., 1992). In the rat central nervous system β -arrestin2 is more abundant than β -arrestin1 (Attramadal et al., 1992). The evaluation of β -arrestin1

and β -arrestin2 protein expression in the brain reveals extensive, heterogenous neuronal labeling (Attramadal et al., 1992). β -Arrestin protein is found in several neuronal pathways and immunoelectron microscopy reveals that β -arrestins are concentrated at neuronal synapses along with GRKs (Arriza et al., 1992; Attramadal et al., 1992). Thus, these proteins are ideally localized to modulate neuronal function. A third family of arrestin proteins might exist, since partial cDNA clones for D- and E-arrestin have been reported (Craft et al., 1994). However, although the mRNAs for D- and E-arrestin are expressed in a broad range of tissues, there is still question whether full-length D- and E-arrestin proteins truly exist (Craft et al., 1994).

Alternative splice variants have been identified for visual arrestin, β -arrestin1, and β -arrestin2. Bovine visual arrestin is expressed as a 404 amino acid residue protein, as well as two polypeptide variants, one for which the last 35 amino acid residues are replaced by an alanine residue (p44) and another that lacks residues 338–345 encoded by exon 13 (Yamaki et al., 1987, 1990; Smith et al., 1994). The p44 visual arrestin variant is specifically localized to the rod outer segment and is severalfold more potent an inhibitor of rhodopsin signal transduction than the long form (Palczewski et al., 1994). Thus, the carboxyl-terminal domain of visual arrestin does not seem to be important for binding to rhodopsin. This observation has been confirmed using β -arrestin truncation mutants (Gurevich, 1998). However, it is the β -arrestin carboxyl-terminal domain that distinguishes these arrestin isoforms from visual arrestins (Fig. 2, A and B). Similar to visual arrestin, at

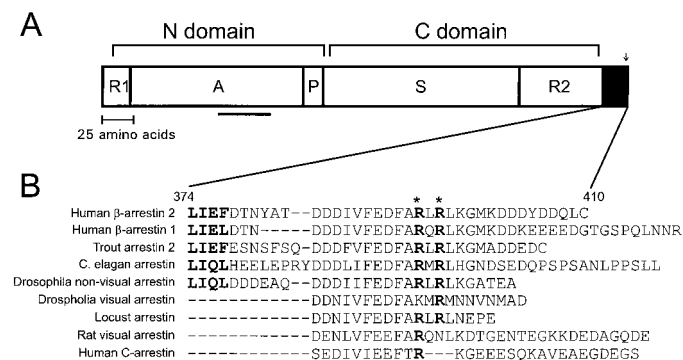


FIG. 2. Molecular architecture of arrestins. Panel A, the arrestin regulatory domains identified by the solution of the visual arrestin crystal structure (Hirsch et al., 1999) and mutagenesis studies (Gurevich et al., 1995). R1, amino terminal regulatory domain (residues 1–24); A, receptor activation domain (residues 24–180); P, phosphate sensor domain (residues 163–182); S, secondary receptor-binding domain (residues 180–330); and R2, carboxyl-terminal regulatory domain (residues 330–404). Underlined is the proline-rich region conserved in mammalian β -arrestin1 and β -arrestin2 but not visual arrestins (Luttrell et al., 1999). The arrow points to serine residue 412, which is phosphorylated by MAPK (Lin et al., 1999). The black box highlights the clathrin- and β -adaptin-binding domains that are conserved among nonvisual arrestins. Panel B, sequence alignment of visual and nonvisual arrestins from different species. The alignment highlights the functional differences between visual versus nonvisual arrestins that arise as the consequence of the clathrin (bold) and β -adaptin (bold and asterisk) binding domains among nonvisual arrestins (Krupnick et al. 1997; Laporte et al., 2000).

least two alternatively spliced forms of β -arrestin1 and β -arrestin2 are expressed (Parruti et al., 1993b; Sterne-Marr et al., 1993). The variant form of β -arrestin1 involves the insertion of eight amino acid residues between amino acids 333 and 334, and the variant form of β -arrestin2 involves the insertion of 11 amino acid residues between amino acids 361–362 (Parruti et al., 1993b; Sterne-Marr et al., 1993). Although the existence of alternatively spliced variant β -arrestin isoforms increases the potential number of functionally distinct β -arrestins, there are no reported differences in the functional activity of the β -arrestin variants. However, considering the limited number of arrestins and the preponderance of GPCR subtypes, it is likely that receptor specificity is governed by discrete differences in receptor structure and tissue-specific arrestin protein expression patterns rather than by a multitude of different arrestin isoforms.

2. Receptor Binding. Arrestins preferentially bind to agonist-activated and GRK-phosphorylated GPCRs as opposed to second messenger protein kinase-phosphorylated or nonphosphorylated receptors (Lohse et al., 1990a, 1992). In vitro, the affinity of β -arrestin binding to the β_2 AR is increased 10- to 30-fold by GRK phosphorylation (Lohse et al., 1992), and this selectivity is even more pronounced for visual arrestin binding to rhodopsin (Gurevich et al., 1995). GRK phosphorylation occurs within either the third intracellular loop domain (e.g., m2 mAChR and α_{2A} AR) or the carboxyl-terminal tails of receptors (e.g., rhodopsin and β_2 AR) (Bouvier et al., 1988; Nakata et al., 1994; Eason et al., 1995; Brannock et al., 1999). Thus, to bind and interdict the signaling of multiple distinct GPCR subtypes, arrestins must exhibit the capacity to recognize and bind multiple receptor domains and conformations. This idea is supported by the following observations: 1) the interaction of visual arrestin with rhodopsin can be blocked by synthetic peptides representing the first and third intracellular loops of rhodopsin (Krupnick et al., 1994); 2) β -arrestin can be coimmunoprecipitated with the third intracellular loop domains of the m3 mAChR, α_{2A} AR, and 5-hydroxytryptamine_{2A} receptor (Wu et al., 1997; Gelber et al., 1999); and 3) the β_2 AR carboxyl-terminal tail is not absolutely required for β -arrestin binding (Ferguson et al., 1996b). Gurevich et al. (1995) examined the ability of several arrestins to bind to various functional forms of rhodopsin, β_2 AR, and m2 mAChR. Although each of the arrestin isoforms demonstrated preference for binding to the GRK-phosphorylated agonist-activated form of the receptors, there was also substantial binding to phosphorylated nonactivated receptors, as well as agonist-activated nonphosphorylated receptors (Gurevich et al., 1995). This suggests that, depending on the GPCR isoform studied, agonist-independent β -arrestin association may be observed (Anborgh et al., 2000). The clear exception is visual arrestin, which binds selectively to only GRK-phosphorylated and light-activated

rhodopsin (Gurevich et al., 1995). In addition, the site of phosphorylation within the rhodopsin carboxyl-terminal tail appears critical for visual arrestin-dependent quenching of rhodopsin activity (Brannock et al., 1999).

The recent solution of the crystal structure for arrestin, together with mutagenesis studies, has provided further insight into the molecular events involved in arrestin binding to phosphorylated light-activated rhodopsin (Gurevich et al., 1995; Granzin et al., 1998; Vishnivetskiy et al., 1999; Hirsch et al., 1999). With respect to receptor binding, mutagenesis studies revealed that the molecular structure of visual arrestin can be divided into three functional and two regulatory domains (Gurevich et al., 1995). The functional domains include: a receptor activation recognition domain (amino acid residues 24–180), a secondary receptor binding domain (amino acids 180–330), and a phosphate sensor domain (amino acid residues 163–182). The regulatory domains are comprised of an amino-terminal regulatory domain (amino acid residues 1–24) and a carboxyl-terminal regulatory domain (amino acid residues 330–404) (Fig. 2A). The solution of the visual arrestin crystal structure is consistent with these observations and reveals that visual arrestin is comprised of two major functional domains that are each constructed from a seven-stranded β sandwich (Granzin et al., 1998; Hirsch et al., 1999). The two domains, the N domain (amino acid residues 8–180) and C domain (amino acid residues 188–362), respectively, comprise the activation recognition and secondary receptor binding domains originally identified by mutational analysis (Fig. 2A). The carboxyl-terminal tail of visual arrestin (amino acids 372–404) is connected to the C domain by a flexible linker, and the carboxyl-terminal tail forms various interactions with parts of the arrestin N and C domains to regulate their structure. The phosphate sensor domain, identified by mutagenesis, constitutes a polar core that in the basal state is embedded between the N and C domains and forms the fulcrum of the arrestin molecule. It is likely that this core structure is highly conserved among arrestin isoforms. Residues from both the amino-terminal and carboxyl-terminal regulatory domains (Asp-30 and Arg-382) also contribute to the polar core of visual arrestin. It is predicted from both mutagenesis studies and the crystal structure for visual arrestin that the interaction of the carboxyl-terminal tail with the polar core stabilizes the basal state structure of visual arrestin (Gurevich et al., 1995; Hirsch et al., 1999). However, in response to receptor binding, the phosphorylated receptor tail invades the polar core, thereby disrupting polar residues and releasing the arrestin carboxyl-terminal tail. This leads to the reorientation of the N and C domains along the fulcrum formed by the polar core facilitating the formation of a receptor-arrestin complex (Hirsch et al., 1999). This model is consistent with the observation that the p44 visual arrestin isoform exhibits greater affinity for rhodopsin and that the mutation of

polar residues within the polar core of the visual arrestin protein results in arrestin mutants able to bind non-phosphorylated rhodopsin (Palczewski et al., 1994; Vishnivetskiy et al., 1999). Thus, it can be concluded that the conformation of free arrestin has evolved to resist agonist- and phosphorylation-independent interactions with receptors. This may be further ensured by the formation of arrestin oligomers (Schubert et al., 1999).

III. G Protein-Coupled Receptor Internalization

An important aspect of GPCR activity and regulation is the internalization or sequestration of agonist-activated receptors into the intracellular membrane compartments of the cell. GPCR internalization has become the subject of intensive investigation over the past several years (reviewed by Sterne-Marr and Benovic, 1995; Ferguson et al., 1996a; Bohm et al., 1997a; Ferguson and Caron, 1998; Krupnick and Benovic, 1998). Consequently, a large volume of data has accumulated regarding the mechanisms regulating the endocytosis of a wide variety of different GPCRs. These studies have revealed GPCR domains involved in receptor endocytosis, some of the molecular intermediates that regulate GPCR endocytosis, and the potential for GPCRs to internalize by multiple endocytic mechanisms. In addition, whereas the molecular mechanism(s) involved in the initiation of GPCR endocytosis are best characterized for the β_2 AR, recent studies using other GPCRs have revealed important diversity in the patterns of GPCR endocytosis and intracellular trafficking. Therefore, the following sections will review the current understanding of the mechanism(s) involved in the initiation and regulation of GPCR endocytosis, how differences in GPCR structure affect the formation of endocytic complexes, and how these complexes contribute to distinct GPCR signaling and intracellular trafficking patterns.

The concept that GPCRs are lost from the cell surface following agonist activation originated from the observation that β -adrenergic agonist treatment resulted in a loss of β -adrenergic receptor recognition sites on the surface of frog erythrocytes (Chuang and Costa, 1979). Subsequently, cell surface versus internalized β_2 AR binding sites were discriminated from one another either by differential sedimentation on a sucrose gradient or by using hydrophobic and hydrophilic β -adrenergic ligands (Harden et al., 1980; Staehelin and Simons, 1982). Internalized receptors were found in a "light vesicular" fraction, whereas cell surface receptors were found in a "heavy vesicular" plasma membrane fraction (Harden et al., 1980). Similarly, internalized β_2 AR were accessible to hydrophobic, but not hydrophilic, adrenergic ligands (Staehelin and Simons, 1982). More recently, the subcellular redistribution of cell surface β_2 AR in response to agonist activation was demonstrated by immunocytochemical staining of epitope-tagged receptors (von Zastrow and Kobilka, 1992), as well as in real time

in living cells using a green fluorescent protein (GFP)-tagged β_2 AR (Barak et al., 1997a). Similar experiments have now been performed for several GPCRs (e.g., Tarasova et al., 1997; Schulein et al., 1998; Barlic et al., 1999; Bevan et al., 1999; Doherty et al., 1999; Drmotá et al., 1999; Liu et al., 1999; Bremmes et al., 2000). The rate at which GPCRs internalize seems to be receptor specific. For example, the A1 adenosine receptor internalizes quite slowly ($t_{1/2} = 90$ min) when compared with the A3 adenosine receptor ($t_{1/2} = 19$ min) (Ferguson et al., 2000). These kinetic differences suggest that GPCR internalization can be mediated by multiple endocytic mechanisms and/or that structural heterogeneity between receptor subtypes modulates their relative affinities to bind endocytic adaptor proteins.

A. Molecular Mechanisms Involved in G Protein-Coupled Receptor Endocytosis

1. *Role of G Protein-Coupled Receptor Kinase and β -Arrestin Proteins.* Sibley et al. (1986) were the first to suggest that receptor phosphorylation might be involved in GPCR endocytosis. However, when this hypothesis was tested, using β_2 AR mutants lacking sites for both second messenger-dependent protein kinase- and GRK-mediated receptor phosphorylation, no significant differences were observed between the internalization of wild-type and mutant β_2 ARs (Bouvier et al., 1988; Hausdorff et al., 1989). A similar result was obtained when permeabilized A431 cells were treated with PKA and GRK inhibitors (Lohse et al., 1990b). This led to a commonly held view that receptor phosphorylation did not contribute to GPCR endocytosis.

Despite the fact that phosphorylation was originally not considered to play a role in the internalization of the β_2 AR, there was growing evidence that phosphorylation might be involved in the endocytosis of other GPCRs, such as the m2 mAChR (Moro et al., 1993). Early experiments demonstrated that the internalization of the m2 mAChR was reduced by the mutation of serine and threonine residues within the third intracellular loop domain of the receptor (Moro et al., 1993). Subsequently, Tsuga et al. (1994) demonstrated that the overexpression of wild-type GRK2 enhanced both the rate and maximal extent of m2 mAChR internalization, whereas a dominant-negative GRK2 mutant (K220W) impaired both the phosphorylation and the internalization of the receptor in COS7 cells. However, whereas catalytically inactive GRK2 dominant-negative mutants blocked m2 mAChR in COS7 cells, they had no effect on m2 mAChR internalization in BHK-21 and HEK 293 cells (Tsuga et al., 1994; Pals-Rylaarsdam et al., 1995). Thus, these experiments provided the first indication that differences in the cellular context in which dominant-negative mutants are used could result in discordant observations. Subsequently, it was determined that GRK2 protein expression levels vary from cell type to cell type, with lowest levels of GRK2 protein expression found in

COS7 cells and substantially higher levels found in cells derived from the hematopoietic system (e.g., COS7 < HEK 293 cells < RBL-2H3 cells) (Aramori et al., 1997; Menard et al., 1997; Barlic et al., 1999). Therefore, the effectiveness of dominant-negative proteins may be dependent on the level of GRK protein expressed in the particular cell line utilized for experimentation.

The observation that GRK2 phosphorylation played a role in the internalization of the m2 mAChR, but that β_2 AR mutants lacking sites for GRK2 phosphorylation internalized normally, led to speculation that GRK2-dependent internalization was peculiar to G_i -coupled receptors (Tsuga et al., 1994). However, a role for GRK-mediated phosphorylation in the internalization of the G_s -coupled β_2 AR was eventually demonstrated using an internalization-defective β_2 AR-Y326A mutant (Ferguson et al., 1995). The β_2 AR-Y326A mutant was not only internalization-defective, but also did not serve as a substrate for GRK-mediated phosphorylation. The overexpression of GRK2 not only promoted the internalization of this receptor mutant, but re-established GRK-mediated phosphorylation of the mutant receptor. The GRK expression-dependent rescue of β_2 AR-Y326A internalization required intact sites for GRK2 phosphorylation, indicating that receptor phosphorylation rather than GRK2 association per se was required to allow β_2 AR internalization in HEK 293 cells. Wild-type β_2 AR phosphorylation and internalization were reduced by the overexpression of a catalytically inactive GRK2 mutant. The role of GRK2-mediated phosphorylation in facilitating GPCR internalization has now been confirmed for multiple other receptors, e.g., the AT_{1A}R (Smith et al., 1998), endothelin A receptor (Bremmes et al., 2000), D2 dopamine receptor (Itokawa et al., 1996), follitropin receptor (Lazari et al., 1999), and monocyte chemoattractant protein-1 receptors (Franci et al., 1996) CCR-5 (Aramori et al., 1997) and CXCR1 (Barlic et al., 1999).

The contribution of other GRK family members to GPCR internalization has not been the subject of intense investigation and their role in promoting GPCR internalization remains less clear. For example, the agonist-promoted internalization of the β_2 AR-Y326A mutant was facilitated by GRK2, GRK3, GRK5, and GRK6, but not by GRK4, which increased receptor internalization in the absence of agonist (Menard et al., 1996). The GRK-mediated phosphorylation of the follitropin receptor was blocked by the expression of dominant-negative mutants of both GRK2 and GRK6 (Lazari et al., 1999). However, the internalization of the follitropin receptor was blocked only by the dominant-negative GRK2 mutant (Lazari et al., 1999). The role of different GRK isoforms in facilitating the internalization of mAChR subtypes seemed to be even more complicated (Tsuga et al., 1998a). The internalization of human m2-m5, but not m1 mAChR, was increased by GRK2 overexpression (Tsuga et al., 1998a). However, the ability of GRK4,

GRK5, and GRK6 to promote the internalization of mAChR subtypes differed for each receptor subtype tested and the cell line used (Tsuga et al., 1998a).

Depending on the GPCR studied, GRK-mediated phosphorylation is not absolutely required for internalization (Bouvier et al., 1988; Hausdorff et al., 1989; Ferguson et al., 1995). Rather, it seems that phosphorylation stabilizes a conformation state required to promote the interaction of GPCRs with some other cellular element that directly promotes the internalization of the receptor. Consequently, β_2 AR mutants lacking sites for GRK-mediated phosphorylation must be able to interact with an endocytic adaptor protein, even in the absence of phosphorylation. In fact, GRK-phosphorylation increases the affinity of the β_2 AR to bind β -arrestins, which in addition to uncoupling receptors from heterotrimeric G proteins, act as endocytic adaptor proteins targeting GPCRs for internalization via clathrin-coated vesicles (Ferguson et al., 1996b; Zhang et al., 1996) (Fig. 3). When overexpressed, both β -arrestin1 and β -arrestin2 ameliorate the internalization defect of the β_2 AR-Y326A even in the absence of GRK-mediated phosphorylation (Ferguson et al., 1996b). Furthermore, β -arrestins facilitate the endocytosis of β_2 ARs lacking either carboxyl-terminal tails or putative sites for GRK phosphorylation. Thus, depending on the level of β -arrestin protein expression and β -arrestin binding affinity, GRK-mediated phosphorylation may be dispensable for some GPCR subtypes (Menard et al., 1997). However, for most receptors, including the β_2 AR, a synergistic relationship exists between GRK-mediated phosphorylation and β -arrestin binding (Ferguson et al., 1996b).

As mentioned previously, the relationship between GRK-mediated phosphorylation and β -arrestin binding is likely different for each GPCR subtype. This may explain the myriad of related, but dissimilar, observations that have been reported in the literature regarding the relative importance of GRKs and β -arrestins in the

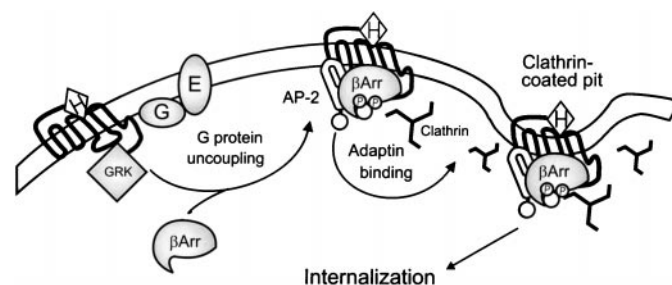


FIG. 3. Molecular mechanisms involved in the GRK- and β -arrestin-dependent desensitization and internalization of GPCRs. GPCR activation leads to GRK-dependent phosphorylation of intracellular serine and threonine residues that facilitate the translocation and binding of β -arrestin proteins to the receptor. β -arrestins, via their association with the β_2 -adaptin subunit of the AP-2 heterotetrameric adaptor complex, target GPCRs to clathrin-coated pits (Ferguson et al., 1996b; Zhang et al., 1996; Barak et al., 1997b; Laporte et al., 1999). In addition to their association with β_2 -adaptins, β -arrestins also bind clathrin (Goodman et al., 1996). The GPCR is subsequently internalized via clathrin-coated vesicles. AP-2, AP-2 heterotetrameric adaptor complex; β Arr, β -arrestin; H, hormone; P, phosphate group.

internalization of different GPCR subtypes. For example, the internalization of two chemokine receptors, CCR-5 and CXCR1, in HEK 293 cells requires the overexpression of both GRK and β -arrestin proteins (Aramori et al., 1997; Barlic et al., 1999). In contrast, whereas the internalization of the m2 mAChR seems dependent on GRK-mediated phosphorylation, the internalization of this receptor subtype does not seem to require β -arrestin, depending on the cellular system in which the receptor is expressed (Tsuga et al., 1994; Schlador and Nathanson, 1997; Vogler et al., 1999; Werbonat et al., 2000). There are also examples of receptors that do not serve as substrates for GRK and β -arrestin proteins and do not internalize in response to agonist activation (Jockers et al., 1996). Thus, differences in GPCR structure-activity relationships likely play an equally important role in regulating differences in the patterns of GPCR endocytosis as they do for regulating differences in agonist and G protein coupling specificity.

In addition to apparent differences in GPCR structure-activity relationships with regard to GRK and β -arrestin binding, GPCR endocytosis is also regulated by both the context of agonist activation and the cellular milieu in which a receptor is expressed. There are several excellent examples that illustrate this point. 1) Etorphine, but not morphine, stimulates μ -opioid receptor phosphorylation and internalization in HEK 293 cells (Zhang et al., 1998). However, GRK2, but not β -arrestin overexpression, allows both phosphorylation and internalization of the μ -opioid receptor in response to morphine. This observation indicates that different receptor agonists stabilize distinct receptor conformations that are able to discriminate between G protein coupling and GRK phosphorylation. This may explain why some peptide receptor antagonists are observed to stimulate receptor internalization (Roettger et al., 1997; Bhowmick et al., 1998). 2) The extent of agonist-promoted β_2 AR internalization was different, depending on the cell line in which it was tested (Menard et al., 1997). For example, the maximal extent of β_2 AR internalization in different cell lines correlates nicely with the levels of GRK and β -arrestin protein expression (Menard et al., 1997). It is possible that GPCR structure may have evolved to match the levels of GRK and β -arrestin protein expression in the cells in which they are normally expressed in vivo. For example, CXCR1 is effectively internalized in neutrophil-like RBL-2H3 cells, but does not internalize in HEK 293 cells (Barlic et al., 1999). The difference between these cell lines is that RBL-2H3 cells express substantially high levels of GRK2 and β -arrestin2 protein than HEK 293 cells (Barlic et al., 1999). 3) The endocytosis of GPCR subtypes differs depending on the complement of β -arrestin protein isoforms expressed by a particular cell. It was recently reported that there are striking differences in the ability of different arrestin isoforms to bind different GPCRs at the plasma membrane (Oakley et al., 2000). Therefore, the ability of

a specific GPCR to internalize in a particular cell type may be dictated by the complement of β -arrestin isoforms expressed in the cell. Consequently, the endogenous complement of GRK and β -arrestin proteins expressed in immortalized cell culture systems, such as HEK 293 cells, may not accurately reflect protein expression levels that will be observed in the diverse physiological environments in which a particular GPCR subtype may be expressed such as in hematopoietic, cardiac or neuronal cells. Therefore, potentially important differences in the physiological regulation of different GPCR subtypes that occur in vivo will require the examination of endocytosis patterns under conditions of varied GRK and β -arrestin protein expression levels.

2. Clathrin and β -Adaptin Interactions. The first indication that β -arrestins specifically target GPCRs for endocytosis via clathrin-coated vesicles came from experiments testing the effects of β -arrestin and dynamin dominant-negative mutants on the internalization of the β_2 AR and AT_{1A}R (Zhang et al., 1996). Dynamin is a large GTPase that is involved in the pinching off of clathrin-coated vesicles from the plasma membrane (Damke et al., 1994). The expression of a dynamin mutant (K44A) lacking GTPase activity effectively blocked both β_2 AR internalization and β -arrestin-stimulated AT_{1A}R internalization (Zhang et al., 1996). Furthermore, Goodman et al. (1996) demonstrated that both β_2 ARs and β -arrestins were colocalized with clathrin in clathrin-coated pits. The idea that β -arrestins specifically target GPCRs for endocytosis via clathrin coated vesicles has been corroborated by recent studies showing that β -arrestins interact directly with components of the endocytic machinery involved in the formation of clathrin-coated pits (Goodman et al., 1996; Laporte et al., 1999, 2000). β -Arrestins bind to both the clathrin heavy chain and the β_2 -adaptin subunit of the heterotetrameric AP-2 adaptor complex (Goodman et al., 1997; Laporte et al., 1999, 2000).

β -Arrestins bind with high affinity and stoichiometry to purified clathrin in vitro (Goodman et al., 1996). β -Arrestin2 binds clathrin with approximately 6-fold higher affinity than β -arrestin1 (Goodman et al., 1996). Visual arrestin, while structurally related to the β -arrestins, does not promote β_2 AR internalization and does not bind to clathrin (Goodman et al., 1996). The clathrin β -arrestin binding domain is localized to residues 89–100 of the amino-terminal globular region in the terminal domain of the clathrin heavy chain that lies at the distal end of each clathrin triskelion (Goodman et al., 1997). The β -arrestin domain involved in clathrin binding is localized to amino acid residues 373–377 in the carboxyl terminus of β -arrestin2 (Krupnick et al., 1997) (Fig. 2). Mutation of the residues within this region of β -arrestin2 substantially reduced clathrin cage binding without altering binding to phosphorylated rhodopsin (Krupnick et al., 1997). However, the mutation of the amino residues within this domain to alanine residues did not

affect the ability of the mutant β -arrestin2 to facilitate β_2 AR internalization in COS7 cells (Laporte et al., 1999). The ability of β -arrestins to bind to clathrin led to the proposal that β -arrestins, rather than the AP-2 adaptor complex, had evolved as GPCR-specific clathrin adaptors allowing GPCR endocytosis via clathrin-coated vesicles (Goodman et al., 1996, 1997; Krupnick et al., 1997). However, unlike observed for the AP-2 adaptor complex, β -arrestins do not promote clathrin coat assembly (Goodman et al., 1997). More recently, Laporte et al. (1999, 2000) demonstrated that, in addition to binding clathrin, β -arrestins also bind to the β_2 -adapting subunit of the heterotetrameric AP-2 adaptor complex and that this interaction is essential for β_2 AR internalization.

The heterotetrameric AP-2 adaptor complex consists of four subunits: two large 100 kDa subunits (α -adapting and β_2 -adapting), one medium size 50 kDa subunit (μ_2), and one small 17 kDa subunit (σ_2) (reviewed by Kirchhausen, 1999). When AP-2 complexes are examined by electron microscopy they appear as two smaller lobes (ears) attached to a brick-like structure. The two ears of the AP-2 adaptor complex are formed by the α - and β_2 -subunits. Each of the AP-2 adaptor complex subunits is ascribed a distinct function. The α -adapting subunit binds to clathrin, dynamin, and Eps15 (Goodman and Keen, 1995; Wang et al., 1995; Benmerah et al., 1996). The β_2 -adapting subunit is essential for clathrin coat formation and interacts with both clathrin and dileucine motifs (Kirchhausen, 1999). The μ_2 -subunit recognizes tyrosine-based internalization signals such as the NPXY (where N, P, and Y are asparagine, proline and tyrosine residues and X can be any amino acid) and Ypp ϕ (where Y is a tyrosine residue, ϕ is a residue with a bulky hydrophobic side chain, and p is a polar residue) motifs. Ypp ϕ was first identified in the carboxyl terminus of the transferrin receptor and is used more broadly as an endocytic motif than the NPXY motif (Kirchhausen, 1999).

The β -arrestin domain involved in binding to the β_2 -adapting subunit of the AP-2 adaptor complex is also localized to the carboxyl termini of β -arrestin1 and β -arrestin2 (Laporte et al., 1999) (Fig. 2, A and B). In particular, two arginine residues (R394 and R396) in β -arrestin2 mediate binding to β_2 -adapting in vitro (Laporte et al., 2000) (Fig. 2B). The binding of β -arrestins to β_2 -adapting is independent of clathrin binding (Laporte et al., 2000). In cells, the association of β_2 -adapting with β -arrestins is stimulated by receptor activation and β_2 -adapting-GFP is recruited to the plasma membrane in response to β_2 AR activation (Laporte et al., 1999). Accordingly, it is possible that GPCR activation may lead to the nucleation of clathrin-coated pits (Laporte et al., 1999). Moreover, whereas β_2 AR/ β -arrestin complexes lacking the β -arrestin clathrin binding motif redistributed to coated pits, receptor/ β -arrestin complexes lacking the β_2 -adapting binding site did not (Laporte et al., 2000). Thus, β -arrestin interactions with the AP-2 com-

plex, rather than with clathrin, are necessary for the initial targeting of receptors to coated pits (Laporte et al., 2000) (Fig. 3). Consequently, it seems that GPCR endocytosis is mediated by the same molecular intermediates that have been previously described to involved in the endocytosis of receptor tyrosine kinases (reviewed by Kirchhausen, 1999), except that GPCRs seems to use a common proximal intermediate, β -arrestin. The role of β -arrestin in GPCR endocytosis may be analogous to the function of Eps15 in coated pit formation and the endocytosis of both transferrin and the EGF receptor (Benmerah et al., 1998, 1999). Although there is no doubt that β -arrestins bind clathrin directly, the consequence of the association between β -arrestin and clathrin with regards to regulating GPCR internalization requires additional investigation.

3. β -Arrestin Regulation. In response to GPCR activation, cytosolic β -arrestin proteins translocate to the plasma membrane and then subsequently redistribute to clathrin-coated pits bound to receptors (Barak et al., 1997b). The extent of β -arrestin translocation recapitulates the dose-response curves for agonist-stimulated receptor-G protein coupling and internalization (Zhang et al., 1999). However, the mechanism underlying this receptor-mediated response remains unclear. It is not known whether β -arrestin translocation is a passive process involving diffusion-dependent interactions with agonist-activated phosphorylated receptors or whether β -arrestin translocation is signal-driven. However, feedback regulation of β -arrestin activity may occur, particularly in the case of β -arrestin1. β_2 AR activation leads to the feedback phosphorylation of β -arrestin1 on serine residue 412 by extracellular signal-regulated kinases (ERKs) (Lin et al., 1998, 1999). The endocytic function of β -arrestin1 is reported to depend on the phosphorylation/dephosphorylation state of β -arrestin1 (Lin et al., 1998). β -Arrestin1 recruited to the plasma membrane-bound receptor is apparently rapidly dephosphorylated, whereas cytoplasmic β -arrestin1 is primarily phosphorylated (Lin et al., 1998). Consequently, the mutation of serine residue 412 to an aspartic acid residue in β -arrestin1 creates a β -arrestin mutant that functions as a dominant-negative with respect to β_2 AR endocytosis (Lin et al., 1998). However, this mutation has no effect on β_2 AR desensitization (Lin et al., 1998). Moreover, β -arrestin1 dephosphorylation does not seem to be required for β -arrestin1 translocation to the plasma membrane (Oakley et al., 2000). Nonetheless, β -arrestin1 dephosphorylation seems to increase clathrin association (Lin et al., 1999). It remains unknown whether ERK-mediated phosphorylation contributes to the regulation of β -arrestin1/ β -adapting interactions. Interestingly, serine residue 412 is not conserved in β -arrestin2. Therefore, ERK-mediated phosphorylation either occurs at a different site, and/or β -arrestin2 activity is regulated by an alternative mechanism. The answer to this question will be important, because β -arrestin2 is con-

sidered to play a predominant role in the endocytosis of most GPCRs (Oakley et al., 2000; Santini et al., 2000).

β -Arrestin activity seems to be regulated by phosphoinositides, in particular IP_6 (Gaidarov and Keen, 1999; Gaidarov et al., 1999). The β -arrestin phosphoinositide-binding site is localized to amino acid residues 233–251 of β -arrestin2. The mutation of basic residues within this domain significantly reduces the phosphoinositide binding to β -arrestin2 and produces a β -arrestin mutant that does not support β_2AR internalization in COS1 cells. Furthermore, the β -arrestin mutant defective in phosphoinositide binding did not concentrate at clathrin-coated pits, but was still recruited to the plasma membrane in response to β_2AR activation. Taken together, these observations suggest that phosphoinositide binding may contribute to the recruitment of receptor/ β -arrestin complexes to clathrin-coated pits. However, the mechanism by which this is achieved remains to be determined.

4. Alternative G Protein-Coupled Receptor Endocytic Pathways. The precise mechanism(s) by which all GPCRs internalize remains a controversial topic. It is now thought that not all GPCRs necessarily internalize in a β -arrestin- and clathrin-dependent manner. This idea has arisen from two initial experimental observations. First, when expressed in different cell types, the internalization profiles for some, but not all GPCRs, is different (e.g., Zhang et al., 1996). In COS7 cells, which express relatively little GRK and β -arrestin protein, the maximal extent of $AT_{1A}R$ internalization is virtually indistinguishable from that observed in HEK 293 cells (Zhang et al., 1996; Menard et al., 1997). In contrast, the maximal extent of β_2AR internalization in response to agonist activation is markedly lower in COS7 cells (Zhang et al., 1996; Menard et al., 1997). Second, the internalization of some GPCRs is less sensitive to the effects of dominant-negative β -arrestin and dynamin mutants (Zhang et al., 1996; e.g., Vogler et al., 1999). In the case of the $AT_{1A}R$ and m2 mAChR, the coexpression of either dominant-negative β -arrestin or dynamin mutants does not block agonist-stimulated internalization (Zhang et al., 1996; Vogler et al., 1999), whereas β -arrestin stimulated $AT_{1A}R$ internalization was blocked completely by dominant-negative dynamin (Zhang et al., 1996). Furthermore, GIT-1, a GTPase-activating protein for the ADP ribosylation factor family of small GTP-binding proteins, only affects the function of G protein-coupled receptors that are internalized through the clathrin-coated pit pathway in a β -arrestin- and dynamin-sensitive manner (Claing et al., 2000). Together, these observations have led to the suggestion that an alternative endocytic mechanism may account for the internalization of some GPCRs, at least in the presence of dominant-negative inhibitors of clathrin-mediated endocytosis. However, the molecular identity of these alternative pathway(s) continues to elude characterization.

Although the internalization of the β_2AR is reported to be caveolin-dependent in A431 cells, the putative β_2AR caveolin-binding motif is found within the extracellular portion of the seventh transmembrane spanning domain of the β_2AR (Raposo et al., 1989; Watson and Arkininstall, 1994; Couet et al., 1997). The putative caveolin binding motifs found within other members of both the β -adrenergic receptor and mAChR families are also found within the extracellular portion of the seventh transmembrane spanning receptor domain (Watson and Arkininstall, 1994). The caveolin binding motif in the endothelin A receptor, which internalizes in a β -arrestin-dependent manner, is localized to the first extracellular loop (Watson and Arkininstall, 1994; Bremmes et al., 2000). Therefore, these putative caveolin-binding motifs are not appropriately localized to receptor domains that would facilitate interactions with caveolin.

Although both the $AT_{1A}R$ and m2 mAChR seem to internalize normally in the presence of dominant-negative β -arrestin and dynamin mutants, the internalization of both these GPCR subtypes seem to be dependent on receptor phosphorylation (Smith et al., 1998; Tsuga et al., 1998b). Furthermore, the internalization of both GPCRs is facilitated by the coexpression of GRK and β -arrestin proteins (Zhang et al., 1996; Schlador and Nathanson, 1997). Thus, these receptors retain the capacity to internalize in a β -arrestin- and clathrin-dependent manner, suggesting the possibility that, in the absence of dominant-negative inhibitors, the receptors normally internalize via clathrin-coated vesicles. This is a particularly important question since the overexpression of dynamin mutants is reported to induce the upregulation of alternative endocytic pathways, such as pinocytosis (Damke et al., 1995).

The idea that the $AT_{1A}R$ and m2 mAChR normally internalize via clathrin-coated vesicles is supported by two observations. First, the mutation of all three dynamin GTPase domains creates a dynamin dominant-negative mutant that blocks the internalization of both the $AT_{1A}R$ and m2 mAChR (Werbonat et al., 2000). Second, the dynamin I-K44A mutant traps β -arrestin bound $AT_{1A}R$ s in coated pits and blocks the trafficking of β -arrestin with the receptor to endosomes (Anborgh et al., 2000). A potential explanation for these apparently incongruous observations is provided by the observation that functionally and biochemically distinct subpopulations of clathrin-coated pits might exist (Cao et al., 1998). As a consequence, GPCRs may be targeted to compositionally distinct membrane vesicles that exhibit differences in their sensitivity to the limited battery of dominant-negative mutants currently used by researchers in the field. Regardless, these observations do not rule out the existence of an alternative GPCR endocytic pathway. However, they do suggest that, under physiological conditions, the predominant pathway for GPCR endocytosis is β -arrestin- and dynamin-dependent.

5. Receptor Determinants for Endocytosis. The identification of receptor-specific determinants for GPCR internalization has been the subject of intense investigation but has not yielded the identity of a conserved GPCR-specific endocytosis consensus motif. Rather it seems that multiple receptor domains contribute to GPCR-specific differences in the rate and extent of receptor internalization. In fact, many of the receptor domains that are necessary for G protein coupling are also important for GPCR internalization. Although the mediators of GPCR endocytosis (e.g., β -arrestin) and G proteins likely interact at similar sites at the intracellular face of the receptor, these processes are functionally distinguishable (Mahan et al., 1985; e.g., Moro et al., 1993; Barak et al., 1994; Hunyady et al., 1995). Perhaps the best evidence that G protein coupling is not required for receptor endocytosis is provided by the observation that in S49 murine lymphoma cell lines, which either lack $G_s\alpha$ or have point mutations preventing receptor/G protein interactions, β_2 AR internalization in response to agonist is normal (Mahan et al., 1985).

Several intracellular receptor domains and conserved motifs have been identified as potential determinants for receptor endocytosis. Although each of these domains and motifs are involved in the internalization of some receptors, they are not required for the internalization of all GPCRs. For example, the NP(X)_{2,3}Y motif found at the intracellular face of the seventh transmembrane domain of many GPCRs was originally considered to serve as a GPCR-specific endocytosis motif due the similarity between this motif and the tyrosine-based NPXY internalization signal (Barak et al., 1994; Kirchhausen, 1999). However, whereas the mutation of the tyrosine residue in the NP(X)_{2,3}Y motif reduced the internalization of the β_2 AR and neurokinin 1 receptor, the same mutation did not reduce the internalization of either the AT_{1A}R or the gastrin-releasing peptide receptor (Barak et al., 1994; Hunyady et al., 1995; Slice et al., 1994; Bohm et al., 1997b). Further characterization of the NP(X)_{2,3}Y motif revealed that it serves as a critical determinant of receptor conformations required for normal receptor interactions with agonist, G proteins, and GRKs (Barak et al., 1995; Ferguson et al., 1995).

Depending on the GPCR tested, regions of the second and third intracellular loop domains contribute to GPCR internalization. In the case of the m2 mAChR, the determinants for internalization are localized to a serine/threonine rich region within the large third intracellular loop of the receptor (Moro et al., 1993). These amino acid residues presumably represent the GRK phosphorylation sites that are involved in promoting the endocytosis of this GPCR subtype (Tsuga et al., 1998b). In addition to the third intracellular loop, the conserved DRYXXV/IXXPL sequence found within the second intracellular loop domain also serves as an internalization determinant for some GPCRs. In particular, mutation of the leucine residue within this motif reduces the internal-

ization of both the m1 mAChR and gonadotropin-releasing hormone (GnRH) receptor (Moro et al., 1994, Arora et al., 1995). Mutation of the DRS sequence in the GnRH receptor to DRY substantially increases the relative internalization of the GnRH receptor (Arora et al., 1995). However, the second intracellular loop also contributes to the regulation of ligand binding affinity, G protein coupling, and receptor stability (e.g., Arora et al., 1995; Alewijnse et al., 2000). Therefore, similar to what is observed for the NP(X)_{2,3}Y motif, the effects of mutating this motif on receptor sequestration are not easily distinguishable from effects on other receptor properties. Furthermore, although the DRY motif within the *N*-formyl peptide receptor contributes to β -arrestin binding, mutants defective in β -arrestin binding are still able to internalize in response to agonist (Bennett et al., 2000).

Numerous studies have examined the role of GPCR carboxyl-terminal tails and putative sites for GRK phosphorylation in regulating agonist-stimulated GPCR internalization. These studies have provided a broad spectrum of different results, highlighting the complex diversity in receptor structure-function relationships as they pertain to receptor endocytosis. For example, despite the fact that the internalization of the β_2 AR is β -arrestin-dependent, neither the truncation of the β_2 AR carboxyl-terminal tail nor the mutation of all potential GRK phosphorylation sites prevents β_2 AR internalization (Hausdorff et al., 1989; Ferguson et al., 1995, 1996b). In contrast, whereas the internalization of the AT_{1A}R is not absolutely dependent on β -arrestin, truncation of the carboxyl-terminal tail or the mutation of potential sites for GRK-dependent phosphorylation prevents AT_{1A}R internalization (Thomas et al., 1995; Zhang et al., 1996; Smith et al., 1998). In other cases (e.g., CCR5 and GnRH receptor), GPCR internalization is β -arrestin-dependent and requires an intact carboxyl-terminal tail domain (e.g., Aramori et al., 1997; Blomenrohr et al., 1999). Furthermore, both positive and negative regulators of agonist-stimulated internalization are found within the carboxyl-terminal tail and third intracellular loop domains of the parathyroid hormone receptor and m2 mAChR, respectively (Huang et al., 1995; Lee et al., 2000). A dileucine motif within the transmembrane proximal domain of the β_2 AR carboxyl-terminal tail is also important for β_2 AR internalization (Gabilondo et al., 1997). In contrast, a carboxyl-terminal tail dileucine motif negatively regulates lutropin/choriogonadotropin receptor internalization because the mutation of the dileucine motif increased the agonist-stimulated internalization of the receptor (Nakamura and Ascoli, 1999). Therefore, the mutation of similar motifs in different GPCRs can result in diametrically opposed effects.

Taken together, the structure-activity studies and mutational analyses of intracellular GPCR domains and motifs indicate that GPCR internalization is regulated

by the interaction of multiple intracellular domains and residues with either β -arrestins and/or other endocytic adaptor proteins. This point is best illustrated by the study of Jockers et al. (1996), where all of the intracellular domains of the β_3 AR had to be substituted with the equivalent domains from the β_2 AR to establish a β_2 AR-like internalization phenotype. Consequently, it is becoming increasingly apparent that the agonist-stimulated GPCR conformation allowing the association of endocytic adaptor proteins can be achieved by multiple mechanisms that are GPCR subtype-specific. Furthermore, the relative requirement for GRK-mediated phosphorylation to allow GPCR internalization varies with each individual receptor. Thus, from a structure-function standpoint, GPCRs should be considered as bipartite integral membrane proteins that consist of an extracellular ligand-binding domain and an intracellular protein-binding domain. Similar to what is observed for ligand binding, the substrate affinity and specificity of the protein-binding domains will be GPCR subtype-specific and will involve multiple intracellular receptor domains.

B. Biological Role of G Protein-Coupled Receptor Internalization

Our understanding of the biological consequences of GPCR endocytosis is expanding rapidly. Several recent studies indicate that, whereas GPCR activation, desensitization, and internalization may involve conserved mechanisms, observed differences in GPCR responsiveness may involve diverse patterns of GPCR trafficking between intracellular membrane compartments. These differences will have profound effects on both the duration and extent of GPCR desensitization, as well as both the rate and mechanism by which GPCR resensitization is achieved. In addition, it is now recognized that GPCR endocytosis contributes to the coupling of desensitized GPCRs to G protein-independent signaling pathways, as well as the compartmentalization of signaling complexes. The following sections will review our current understanding of the role of endocytosis in GPCR desensitization, resensitization and signaling.

1. Endocytosis and G Protein-Coupled Receptor Desensitization. GPCR internalization was originally considered to be a primary mechanism of receptor desensitization due to the spatial uncoupling of the receptor from its effector system (Sibley and Lefkowitz, 1985). However, this hypothesis subsequently fell out of favor for three reasons. 1) Since receptor desensitization proceeds more rapidly than receptor endocytosis, it is expected that the majority of internalized GPCRs will be pre-desensitized as the consequence of protein kinase phosphorylation. 2) Pharmacological treatments that blocked GPCR internalization, such as hypertonic sucrose and concanavalin A, did not alter the β_2 AR desensitization profile (Yu et al., 1993; Pippig et al., 1995). 3) It was observed that internalization played a role in the resensitization of GPCR responsiveness (Yu et al., 1993; Pippig et al., 1995; Zhang et al., 1997). Recently, with the advent of new techniques to examine GPCR intracellular trafficking patterns, protein-protein interactions and recycling, the field has come full circle and has now begun to recognize the important role of GPCR internalization in regulating differences in the patterns of GPCR desensitization.

In response to agonist-stimulation, many GPCRs are internalized but are not recycled back to the cell surface (Hermans et al., 1997; Trejo and Coughlin, 1999; Groarke et al., 1999; Oakley et al., 1999; Zhang et al., 1999; Anborgh et al., 2000; Bremnes et al., 2000). In some cases, internalized GPCRs, such as the protease-activated receptors (PAR) and endothelin B receptor, are predominantly targeted to lysosomes for degradation (Trejo and Coughlin, 1999; Bremnes et al., 2000). In the case of protease-activated receptors, the carboxyl-terminal tail regulates targeting to lysosomes (Trejo and Coughlin, 1999). For other GPCRs, such as the $AT_{1A}R$, internalized receptors are not necessarily sorted to lysosomes, but may be retained within the endosomal compartment (Zhang et al., 1999; Anborgh et al., 2000). As a consequence, by virtue of the fact that some receptors do not recycle, they will mediate transient responses to agonist. For example, endothelin A receptors are efficiently recycled and mediate persistent responses to endothelin, whereas the endothelin B receptors are targeted to lysosomes and mediate only transient responses to endothelin (Bremnes et al., 2000).

Several recent studies have used chimeric receptor constructs to examine differences in the intracellular sorting patterns of GPCRs (Trejo and Coughlin, 1999; Oakley et al., 1999; Anborgh et al., 2000). These studies have revealed that specific determinants within the cytoplasmic tails of GPCRs determine whether GPCRs are either recycled back to the plasma membrane or are retained within the intracellular compartment of the cell and/or targeted to lysosomes (Innamorati et al., 1998; Trejo and Coughlin, 1999; Oakley et al., 1999; Anborgh et al., 2000). In the case of the V2 vasopressin receptor, a carboxyl-terminal serine cluster was demonstrated to prevent receptor recycling (Innamorati et al., 1998). Further characterization of V2 vasopressin receptor trafficking revealed that, like many peptide receptors (e.g., $AT_{1A}R$, neurokinin 1 receptor, thyrotropin-releasing hormone receptor, PAR2, and neurotensin receptor), the V2 vasopressin receptor internalized as a complex with β -arrestin in endocytic vesicles (Dery et al., 1999; Groarke et al., 1999; McConalogue et al., 1999; Oakley et al., 1999; Zhang et al., 1999; Anborgh et al., 2000). The capacity of both the V2 vasopressin receptor and $AT_{1A}R$ to internalize with β -arrestin bound was conferred by their carboxyl-terminal tail domains (Oakley et al., 1999; Zhang et al., 1999; Anborgh et al., 2000). The same cluster of serine residues that prevented V2 vasopressin receptor recycling was required for the internalization of

β -arrestin with the receptor (Innamorati et al., 1998; Oakley et al., 1999). GPCRs that do not internalize with β -arrestin bound, such as the β_2 AR, lack this cluster of serine residues in the appropriate context (Oakley et al., 1999). This has led to the hypothesis that β -arrestin interactions with the carboxyl-terminal tails of GPCRs dictate the rate of receptor recycling and resensitization (Oakley et al., 1999).

Although β -arrestin internalization is well correlated with the inhibition of GPCR recycling, exceptions exist. For example, the neurokinin 1 receptor, which internalizes complexed with β -arrestin, is efficiently recycled back to the cell surface and is effectively resensitized (Grady et al., 1995; McConalogue et al., 1999). Therefore, multiple patterns of GPCR desensitization and resensitization exist. These include: 1) GPCRs that do not internalize in response to agonist [e.g., β_3 AR (Jockers et al., 1996)]. 2) GPCRs that are internalized without β -arrestin bound, and that are dephosphorylated and recycled back to the cell surface [e.g., β_2 AR (Oakley et al., 1999; Anborgh et al., 2000)]. 3) GPCRs that are internalized with β -arrestin bound, but that are dephosphorylated and rapidly recycle back to the cell surface after the dissociation of β -arrestins in the intracellular compartment of the cell [e.g., neurokinin 1 receptor (Grady et al., 1996; McConalogue et al., 1999)]. 4) GPCRs that are internalized with β -arrestin bound and are either retained in endosomes and/or targeted to lysosomes [e.g., AT_{1A} R and PAR (Trejo and Coughlin, 1999; Dery et al., 1999; Anborgh et al., 2000)] (Fig. 4A). Although β -arrestin seems to play a central role in regulating the intracellular trafficking properties of GPCR, it is likely

that the manifestation of distinct intracellular trafficking properties also involves other components of the endocytic machinery.

2. Endocytosis and G Protein-Coupled Receptor Resensitization. The physiological importance of receptor resensitization in the maintenance of normal tissue homeostasis is obvious since prolonged or irreversible receptor desensitization would leave a cell unable to respond appropriately to extracellular stimuli. Just as GPCR desensitization provides a mechanism protecting cells against receptor overstimulation, GPCR resensitization protects cells against prolonged receptor desensitization. The mechanism by which the resensitization of many GPCRs is achieved is thought to be the agonist-stimulated internalization of receptors to an intracellular membrane compartment (endosomes) enriched in a GPCR-specific phosphatase activity (Fig. 4A). GPCRs dephosphorylated in endosomes are subsequently recycled back to the cell surface in the preligand-exposed state (Fig. 4A). The rate at which GPCRs traffic from the plasma membrane to the endosomal compartment and back again varies, depending on the GPCR subtype tested, but at a minimum takes several minutes. In contrast, GPCR desensitization occurs within seconds to minutes. Thus, it is clear that the processes involved in GPCR resensitization are less efficient than GPCR desensitization. This accounts for the observation that many GPCR agonists have limited long-term therapeutic usefulness and that GPCR resensitization does not adequately compensate for increased GRK-mediated receptor desensitization observed in both congestive heart failure and hypertension (Ungerer et al., 1993; Gros et al., 1997).

The concept that internalization is important for receptor resensitization arose from several early observations. 1) β_2 ARs isolated from "light vesicular" membrane fractions (endosomes) exhibited lower stoichiometry of phosphorylation than β_2 ARs isolated from the plasma membrane (Sibley et al., 1986). 2) The "light vesicular" membrane fractions were enriched in a GPCR-specific phosphatase activity (Sibley et al., 1986; Pitcher et al., 1995b). 3) Pharmacological treatments, such as concanavalin A and hypertonic sucrose, that blocked receptor internalization also blocked resensitization without affecting either receptor G protein-coupling or desensitization (Pippig et al., 1995; Garland et al., 1996; Hasbi et al., 2000). 4) Internalization-defective mutants were identified that signaled and desensitized, but were not resensitized (Barak et al., 1994). The requirement of internalization for GPCR resensitization has been demonstrated for the β_2 AR (Pippig et al., 1995), m3 mAChR (Edwardson and Szekeres, 1999), neurokinin 1 receptor (Garland et al., 1996), delta opioid receptor (Hasbi et al., 2000), mu opioid receptor (Wolf et al., 1999), C5a anaphylatoxin receptor (Giannini and Boulay, 1995), cholecystokinin receptor (Lutz et al., 1993), and endothelin A receptor (Bremnes et al., 2000).

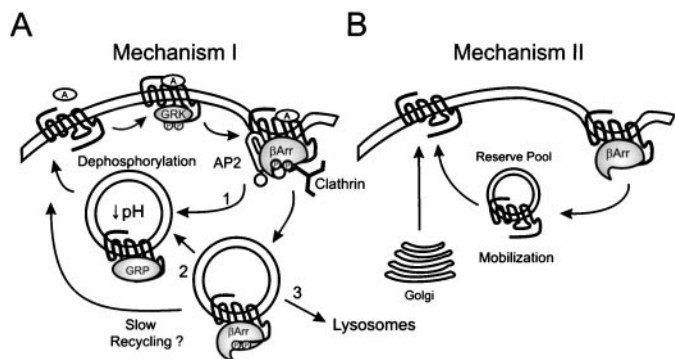


FIG. 4. β -Arrestin-dependent regulation of GPCR trafficking and the mechanisms involved in GPCR resensitization. Panel A, mechanism I: GPCR resensitization is achieved by the clathrin-dependent internalization of GPCRs to endosomes, and the receptors are subsequently dephosphorylated and recycled back to the cell surface (1). Alternatively, GPCRs that internalize with β -arrestin either dissociate from β -arrestin in the intracellular compartment, and are both dephosphorylated and recycled (2) or are retained within large core vesicles and/or are targeted for degradation in lysosomes (3) (Krueger et al., 1997; Trejo and Coughlin, 1998; Oakley et al., 1999; Zhang et al., 1999; Anborgh et al., 2000). Panel B, mechanism II: resensitization of GPCRs that are internalized with β -arrestin bound and do not recycle is mediated by the mobilization of a reserve pool of intracellular receptors and/or de novo receptor synthesis in the Golgi apparatus (Shapiro et al., 1996; Shapiro and Coughlin, 1998; Anborgh et al., 2000). A, agonist; AP-2, AP-2 heterotetrameric adaptor complex; β Arr, β -arrestin; P, phosphate group.

As a consequence of their role in GPCR endocytosis, β -arrestins not only play an important role in regulating GPCR desensitization, but also resensitization. For example, in COS7 cells that express relatively low levels of β -arrestin protein when compared with other cell lines (Menard et al., 1997), β_2 AR resensitization does not occur in COS7 cells unless β -arrestins are overexpressed (Zhang et al., 1997). These observations indicate that, due to differences in GRK and β -arrestin protein expression levels, the relative capacity of an individual GPCR to be resensitized might differ depending on the cell type and tissue in which it is expressed. Furthermore, the relative capacity of a GPCR to become resensitized may be depend upon the relative protein expression levels of other regulators of intracellular trafficking.

The internalization of GPCRs to an intracellular membrane compartment is considered to be required for dephosphorylation by a GPCR-specific phosphatase (Pitcher et al., 1995b). GPCR resensitization not only requires the β -arrestin-dependent targeting of receptors to clathrin-coated pits and their subsequent endocytosis to endosomes, but also requires that the receptors release bound β -arrestin, become dephosphorylated, sorted, and recycled back to the cell surface. The majority of the studies examining the molecular mechanisms underlying the trafficking and dephosphorylation of GPCRs have focused on the β_2 AR as a model GPCR. These studies have been facilitated by the use of GFP-tagged β_2 AR, β -arrestin, and RabGTPases. In addition to β -arrestins, the internalization of the β_2 AR, as well as the D2 dopamine receptor, is dependent on the small GTPase Rab5 (Iwata et al., 1999; Seachrist et al., 2000). Rab5 not only regulates the movement and fusion of β_2 AR-bearing vesicles to/with early endosomes, but also regulates the formation of β_2 AR-bearing vesicles at the cell surface (Seachrist et al., 2000) (Fig. 5). Furthermore, although β -arrestins are required for β_2 AR internalization and colocalize with the receptor in clathrin-coated pits (Zhang et al., 1999), β -arrestin does not internalize with the β_2 AR in endocytic vesicles (Zhang et al., 1999; Anborgh et al., 2000). The exclusion of β -arrestin from endocytic vesicles likely facilitates β_2 AR dephosphorylation by allowing phosphorylated receptors to be exposed to the GPCR-specific phosphatase (Anborgh et al., 2000). Although rhodopsin is not internalized, visual arrestin dissociation is also required for rhodopsin dephosphorylation in rod outer segments (Palczewski et al., 1989). Furthermore, the association of the phosphatase with the β_2 AR requires the acidification of the receptor in the endosomal compartment (Krueger et al., 1997). The dephosphorylation of the β_2 AR occurs as the receptor transits between Rab5- and Rab4-positive endosomal compartments (Seachrist et al., 2000) (Fig. 5). Rab4 also regulates the recycling of the β_2 AR back to the plasma membrane and is required for β_2 AR resensitization (Seachrist et al., 2000) (Fig. 5). Although the endothelin A receptor has also been localized to Rab5-positive en-

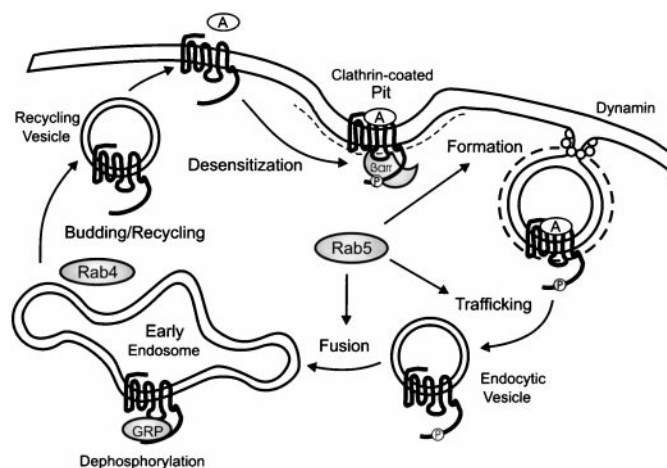


FIG. 5. Model depicting the regulation of β_2 AR internalization, trafficking, dephosphorylation, and recycling. Agonist activation of the β_2 AR results in β -arrestin (β Arr)-mediated desensitization and targeting to clathrin-coated pits. Rab5 contributes to the formation of endocytic vesicles, the trafficking of vesicles to early endosomes, and the fusion of endocytic vesicles with early endosomes. The β_2 AR is dephosphorylated by a G protein-coupled receptor specific phosphatase (GRP) as the receptor transits from the Rab5- to Rab4 positive compartments of early endosomes. The budding and/or recycling of β_2 AR-bearing recycling vesicles is regulated by Rab4 (Seachrist et al., 2000). A, agonist; P, phosphate group. Figure reprinted with permission from Seachrist et al. (2000).

dosomes (Bremnes et al., 2000), little is known about the role of Rab5 and other RabGTPases in the intracellular trafficking of other GPCRs.

GPCR internalization and dephosphorylation in endosomes is not the only mechanism by which GPCR resensitization is achieved. Protease-activated receptors are unique members of the GPCR superfamily that are irreversibly activated by a proteolytic mechanism, then internalized and degraded in lysosomes (Coughlin, 1999). Consequently, resensitization of PAR responses requires an alternative mechanism of resensitization (Fig. 4B). The resensitization of PAR-mediated cellular responses is mediated in two ways: 1) the de novo synthesis of new receptors and 2) the maintenance of an intracellular reserve of naïve receptors that can be mobilized to replace degraded receptors (Shapiro et al., 1996; Shapiro and Coughlin, 1998). This intracellular PAR pool is maintained by the agonist-independent movement of receptors between the cell surface and an intracellular membrane compartment (Shapiro et al., 1996; Shapiro and Coughlin, 1998). Although agonist-independent GPCR internalization is a phenomenon that is not well accepted, it is also observed for the $AT_{1A}R$ and cholecystokinin receptor A (Hein et al., 1997; Tarasova, 1997; Anborgh et al., 2000). In the case of the $AT_{1A}R$, β -arrestin association with the receptor in the absence of agonist seems to be responsible for the agonist-independent loss of cell surface receptor (Anborgh et al., 2000). Therefore, β -arrestin-dependent GPCR internalization may regulate GPCR resensitization by two distinct mechanisms.

3. *Endocytosis and G Protein-Coupled Receptor Signaling.* MAPKs, such as ERK1 and ERK2, are acti-

vated by a wide variety of GPCRs (reviewed by van Biesen et al., 1996; Luttrell et al., 1999a). GPCR-mediated activation of MAPKs via G_{i-} , G_{q-} , and G_{o-} mediated pathways has been studied extensively (van Biesen et al., 1996; Luttrell et al., 1999a). In the case of G_{i-} mediated signals, the $G\beta\gamma$ -subunits are involved in the activation of a Src family tyrosine kinase followed by the subsequent tyrosine phosphorylation of the same downstream adaptor proteins used by receptor tyrosine kinases (Scheme 1) (van Biesen et al., 1995) and seems to involve transactivation of tyrosine kinase receptors such as the EGF receptor (Prenzel et al., 1999; Pierce et al., 2000).



Scheme 1

The activation of MAPK by the G_s -coupled $\beta_2\text{AR}$ involves the PKA phosphorylation-dependent switch of $\beta_2\text{AR}$ G protein coupling to the activation of G_i rather than G_s (Daaka et al., 1997).

Recently, it was observed that one additional mechanism by which MAPKs were activated by GPCRs involved both endocytosis and β -arrestin proteins (Daaka et al., 1998; Luttrell et al., 1999b; Vogler et al., 1999; Barlic et al., 2000). Using β -arrestin and dynamin dominant-negative inhibitors of internalization, both $\beta_2\text{AR}$ - and m1 mAChR-mediated activation of MAPK was demonstrated to require clathrin-coated vesicle-mediated endocytosis (Daaka et al., 1998; Vogler et al., 1999). The plasma membrane-delineated event involved in this process was the tyrosine phosphorylation of Shc and Raf kinase activation by Ras (Daaka et al., 1998). The internalization of this complex was required for subsequent MAPK activation (Daaka et al., 1998).

Consistent with the concept that GPCRs activate MAPKs by multiple converging mechanisms, many GPCRs were observed to mediate MAPK activation in manner that was independent of their internalization (Blaukat et al., 1999; Budd et al., 1999; DeGraff et al., 1999; Li et al., 1999; Schramm and Limbird, 1999; Whistler and von Zastrow, 1999). In addition, Whistler and von Zastrow (1999) reported that MAPK activation by an internalization-deficient mu opioid receptor was inhibited by a dynamin dominant-negative mutant. These data were interpreted to suggest that dynamin played a unique role in signal transduction that was independent of its function in clathrin-coated vesicles-mediated endocytosis (Whistler and von Zastrow, 1999). However, a recent study indicated that both $\alpha_2\text{AR}$ and $\beta_2\text{AR}$ activation of MAPK required the transactivation and internalization of the epidermal growth factor receptor (EGFR) (Pierce et al., 2000). Whereas EGFR internalization was required for MAPK activation, neither EGFR internalization nor MAPK activation required GPCR internal-

ization. Consequently, the requirement of clathrin-mediated endocytosis for MAPK activation was separated from the need for GPCR internalization. Taken together, these observations indicate that GPCR-stimulated activation of MAPK is mediated by multiple overlapping mechanisms, all of which may involve endocytosis.

In addition to contributing to the regulation of GPCR desensitization, internalization and resensitization, β -arrestins contribute to the assembly of signaling protein complexes (Luttrell et al., 1999b; Barlic et al., 2000; DeFea et al., 2000). β -Arrestins are essential for the agonist-stimulated formation of a protein complex containing the $\beta_2\text{AR}$, β -arrestin, and c-Src (Luttrell et al., 1999b). The formation of this complex is dependent on the ability of β -arrestin to directly associate with c-Src. The β -arrestin/c-Src interactions involve the association of the β -arrestin amino terminus with the Src homology 1 domain of c-Src (Miller et al., 2000). β -Arrestin mutants defective in their ability to interact with c-Src also effectively blocked $\beta_2\text{AR}$ -mediated activation of MAPK (Luttrell et al., 1999b). As a consequence, β -arrestins can couple the termination of receptor-G protein coupling with the initiation of alternative signal transduction cascades in which the desensitized receptor and β -arrestin act as scaffolds.

An indication of the physiological role of β -arrestin signaling via Src family tyrosine kinases comes from a recent study examining the role of tyrosine kinase activation in the degranulation of neutrophils (Barlic et al., 2000). The internalization of the chemokine receptor CXCR1 is not only β -arrestin-dependent (Barlic et al., 1999), but CXCR1-mediated granule release and tyrosine kinase activation is inhibited by a β -arrestin dominant-negative mutant (P91G-P121E) that does not bind to c-Src kinase (Luttrell et al., 1999b; Barlic et al., 2000). Furthermore, the activation of the c-Src family tyrosine kinases, Hck and c-Fgr, in primary human neutrophils was dependent on the formation of a complex with endogenously expressed β -arrestins in neutrophils (Barlic et al., 2000) (Fig. 6A). β -Arrestin mediated tyrosine kinase activation, receptor internalization, and the subsequent redistribution of β -arrestin/Hck complexes to granules were required for chemokine-induced granule exocytosis. These studies provide evidence that β -arrestin-mediated signaling is physiologically relevant to normal GPCR function in a physiological setting.

In addition to interacting with Src family tyrosine kinases, β -arrestins were recently shown by DeFea et al. (2000) to interact directly with both Raf-1 kinase and MAPK in response to the activation of the G_q -coupled PAR2. Gel filtration experiments demonstrated that PAR2 activation resulted in the formation of a multiprotein complex containing the receptor, β -arrestin, Raf, and MAPK (Fig. 6B). As a consequence, the activation of wild-type PAR2 prevented the translocation of MAPK to the nucleus and by virtue of the fact that MAPK was retained within the cytosol prevented cell proliferation.

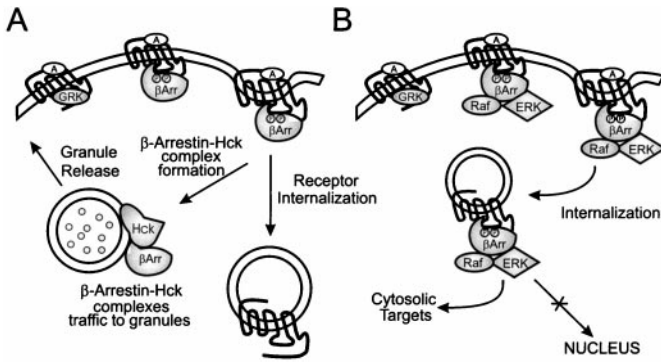


FIG. 6. β -Arrestin-dependent scaffolding of signaling complexes. A, role of β -arrestins in the regulation of c-Src family tyrosine kinase activity in response to chemokine receptor activation in neutrophils. CXCR1 activation leads to the β -arrestin-dependent formation of a complex involving the receptor, β -arrestin, and tyrosine kinase (e.g., Hck). The complex is redistributed to endocytic vesicles and may or may not lead to the activation of Ras, Raf, and MAPK pathway in neutrophils. Regardless, β -arrestin-mediated tyrosine kinase activation and redistribution to granules is required for the tyrosine kinase-dependent exocytosis of granules (Barlic et al., 2000). B, β -Arrestin acts as a scaffold for the formation of GPCR complexes with Raf-1 kinase and ERK (MAPK). The β -arrestin-dependent recruitment and internalization of these complexes in endocytic vesicles lead to ERK activation. However, the compartmentalization of the β -arrestin/Raf/ERK complex in vesicles prevents the translocation of activated ERK to the nucleus. As a consequence, ERK activity is limited to cytosolic targets (DeFea et al., 2000). A, agonist; β Arr, β -arrestin; P, phosphate group.

In contrast, a PAR2 mutant defective in β -arrestin binding stimulated MAPK activation and cell proliferation. Under these conditions, the receptor failed to form a complex with both Raf and MAPK via β -arrestin, and MAPK was able to translocate to the nucleus in response to receptor activation. These observations suggest that, due to their role as endocytic adaptor proteins, β -arrestins not only regulate the formation of signal transduction complexes, but also regulate the intracellular compartmentalization of these complexes. Consequently, similar to what is observed for GPCR desensitization, differences in β -arrestin-mediated internalization and trafficking of GPCRs may determine distinct patterns of MAPK activation and the mitogenic potential of GPCR activation. However, the relative role for β -arrestins as endocytic versus scaffolding proteins in GPCR-mediated activation of MAPK signaling remains to be determined.

IV. Conclusions

Taken together, the information summarized in this review highlights the complex inter-relationship between mechanisms involved in GPCR desensitization, internalization, resensitization, and mitogenic signaling. In particular, it is now clear that β -arrestins contribute to the regulation of each of these processes. Furthermore, the activation of distinct GPCR subtypes is translated into diverse receptor-specific patterns for GPCR desensitization, resensitization and signaling. This diversity is modulated, at least in part, by variations in GPCR structure that in turn lead to differences

in the ability of a particular GPCR to interact with intracellular regulatory proteins. Although, this concept is newer, it is really no different than the idea of either ligand-binding specificity or G protein-coupling specificity.

Although our understanding of the mechanisms involved in the regulation of GPCR responsiveness have evolved considerably over the past several decades, it is likely that we have only scratched the surface of the multiple protein-protein interactions and signal transduction pathways that are stimulated in response to GPCR activation. As stated in the introduction to this review, one of the challenges facing researchers in the field will be to begin to examine how GPCR subtype differences in the patterns of endocytosis translate into physiological differences in GPCR activity. These differences in GPCR trafficking patterns will certainly provide new and exciting opportunities for the development of strategies to therapeutically manipulate GPCR function in diseases associated with altered GPCR signaling, such as hypertension and congestive heart failure. It is likely that the development of novel therapeutic agents that specifically target the activity of GPCR regulatory proteins, such as GRKs and β -arrestins, will provide flexible approaches to specifically treat distinct aspects of GPCR dysfunction. For example, the development of specific GRK inhibitors may prevent GPCR desensitization associated with long-term agonist treatment or may even avoid the need to use receptor agonists. Furthermore, it may be possible to develop inhibitors that discriminate between β -arrestin endocytosis and signaling functions.

It is now apparent that GPCR activity and function is regulated by an incredible variety of mechanisms. These mechanisms act at the level of GPCR ligand specificity, G protein activation, and effector regulation. One of the most exciting developments in the field of GPCR endocytosis is the recent awareness that GPCR desensitization and endocytosis can act as molecular switches coupling GPCRs to alternative signal transduction pathways. β -Arrestins not only function in the molecular switch required for GPCR desensitization and internalization, but also act as scaffolds to transduce and compartmentalize the alternative signals. Furthermore, β -arrestins distinguish themselves from visual arrestins by virtue of their ability to act as adaptor proteins. β -Arrestins interact with a wide variety of endocytic and signaling proteins that now include: clathrin (Goodman et al., 1996), β -adaptin (Laporte et al., 1999), c-Src (Luttrell et al., 1999b), Hck (Barlic et al., 2000), c-Fgr (Barlic et al., 2000), MAPKs (DeFea et al., 2000), and Raf (DeFea et al., 2000). The field of GPCR regulation is likely to remain exciting with the expectation for the characterization of novel roles for GPCR endocytosis and the identification of additional β -arrestin-interacting proteins in the future.

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