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REGULATION OF ADENYLYL CYCLASE–COUPLED β-ADRENERGIC RECEPTORS

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CONTENTS

INTRODUCTION

In diverse biological systems the intensity of a response often wanes over time despite the continued presence of a stimulus of constant intensity. This phenomenon is referred to as desensitization, refractoriness, or toler-

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ance. In the realm of clinical therapeutics, desensitization to the actions of various drugs limits their efficacy and duration of action (Lefkowitz et al 1980).

One of the most useful in vitro model systems for studying the molecular mechanisms of desensitization is the hormone-responsive adenylyl cyclase system (Lefkowitz et al 1980). This system is prototypic of many biological systems in which a receptor is coupled via a guanine nucleotide regulatory protein (in this case G_s) to a biochemical effector (the adenylyl cyclase) (Gilman 1987). The most thoroughly studied example has been regulation of the adenylyl cyclase–coupled β-adrenergic receptor, which mediates the stimulatory effects of adrenaline and related agents on the enzyme.

STRUCTURE OF HORMONE-SENSITIVE ADENYLYL CYCLASE

The structure of receptor-coupled adenylyl cyclase systems has recently been reviewed (Gilman 1987). The past five years have witnessed the growing appreciation, not only of the widespread analogies among the three components of a wide variety of transmembrane signaling systems [including hormone-responsive adenylyl cyclase, light-responsive cGMP phosphodiesterase (Stryer 1986), and hormone-responsive phospholipase C], but also of the actual homology between the respective components in these systems.

The β_2-adrenergic receptor and rhodopsin are prototypic of the receptor components of such systems (Dohlman et al 1987b; Lefkowitz & Caron 1988). Their deduced amino acid sequences and proposed topographical organization within the plasma or discal membrane are shown in Figure 1. Each is a glycoprotein with sites for N-linked glycosylation near the amino terminus. There are seven presumed membrane-spanning domains and interconnecting loops. Each has a serine- and threonine-rich cytoplasmic carboxyl terminus, which, as discussed below, may be a locus of regulatory phosphorylation. There are a number of amino acid identities between the β_2-adrenergic receptor and rhodopsin when their sequences are aligned; this includes 24% of the residues if one considers only the presumed membrane-spanning regions. However, the similarities are even higher when one compares the β_2-adrenergic receptor with, for example, the muscarinic cholinergic receptors ($\sim$30%) or with the other members of the adrenergic receptor family (β_1, α_2, α_1 $\sim$40–70%). The membrane-spanning regions of these receptors are thought to form a ligand-binding pocket, whereas various regions of the cytoplasmic loops may be involved in coupling to the guanine nucleotide regulatory proteins.

Guanine nucleotide regulatory proteins (G proteins) are a family of

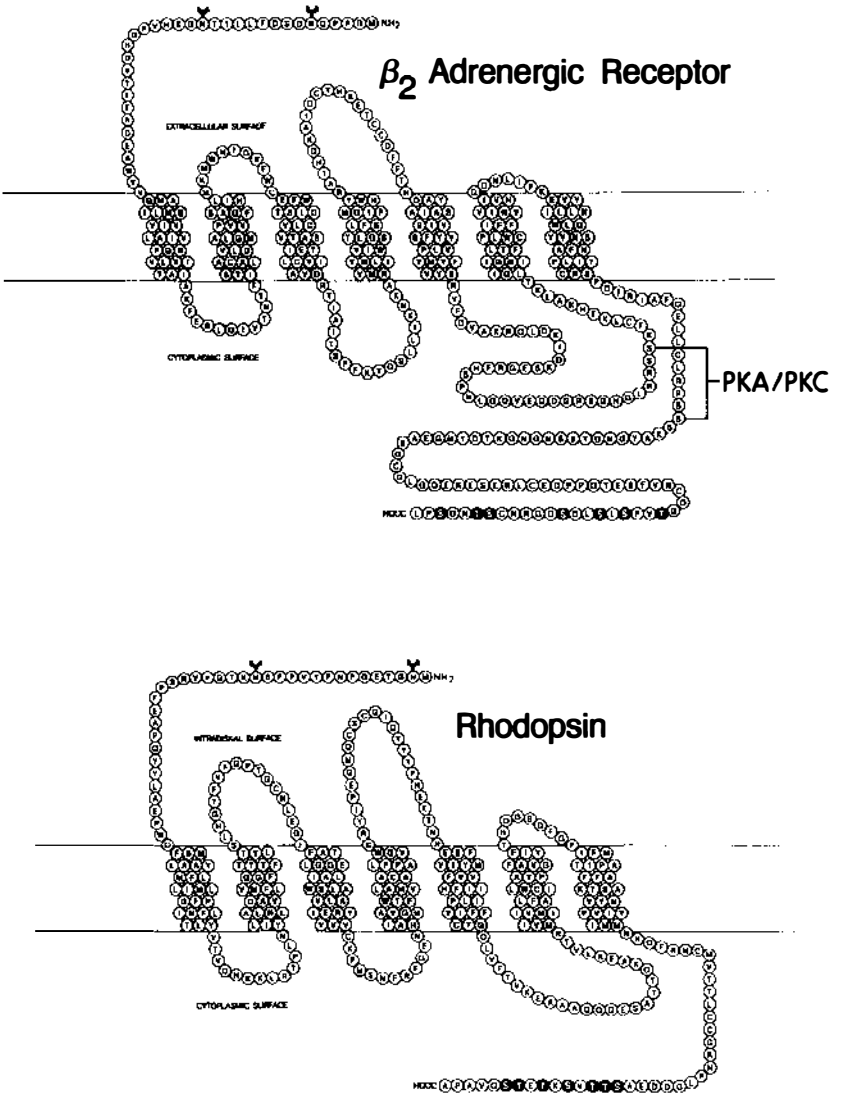

Figure 1 Proposed structure and membrane topology of hamster β_2-adrenergic receptor and bovine rhodopsin. The likely sites of phosphorylation by the cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) on the β_2-adrenergic receptor are indicated. The solid circles represent the serine- and threonine-rich region in the carboxyl terminus of each receptor, which may serve as sites for β-adrenergic receptor kinase or rhodopsin kinase phosphorylation.

homologous heterotrimeric proteins involved in coupling diverse cellular receptors to different effectors (Gilman 1987). Two such proteins are involved in the adenylyl cyclase system. G_s, the stimulatory guanine nucleotide regulatory protein, mediates the effects of stimulatory receptors, such as the β-adrenergic receptors, whereas G_i (the inhibitory guanine nucleotide regulatory protein) mediates the effects of inhibitory receptors, such as the α_2-adrenergic receptor. The G proteins consist of α subunits ($M_r \sim 39{,}000$–$45{,}000$) that interact with the receptors and bind and hydrolyze GTP, and β ($M_r \sim 35{,}000$) and γ ($M_r \sim 5{,}000$–$10{,}000$) subunits. While details of the mechanisms by which such G proteins function remain controversial, agonist occupancy of the receptor in the adenylyl cyclase system triggers conformational changes that promote GTP binding to G_s and subsequent dissociation into $\alpha_s \cdot$GTP and $\beta\gamma$ subunits (α_s is the α subunit of G_s). $\alpha_s \cdot$GTP appears to directly activate adenylyl cyclase; the activation cycle is terminated by the slow hydrolysis of GTP (Gilman 1987).

Less is currently known about the molecular properties of adenylyl cyclase. It has been purified by affinity chromatography and appears to consist of a single glycoprotein chain of $M_r \sim 150{,}000$ (Gilman 1987). All of these components (receptor, G protein, cyclase) have been reconstituted in phospholipid vesicles and have been shown to be sufficient to produce hormone-responsive adenylyl cyclase activity (Cerione et al 1984, May et al 1985).

PATTERNS OF DESENSITIZATION

Two major patterns of desensitization generally referred to as homologous and heterologous, have been distinguished (Lefkowitz et al 1980). To understand the difference between these two forms of refractoriness, consider any system in which essentially the same biological response can be elicited by several different classes of activators (e.g. an adenylyl cyclase system sensitive to β-adrenergic agents, prostaglandins, and glucagon). In homologous desensitization, only the subsequent response to the desensitizing hormone is attenuated; the efficacy of other hormone activators is unimpaired. For example, stimulation by the β-agonist isoproterenol diminishes responsiveness to β-adrenergic stimulation, while prostaglandins and glucagon continue to activate normally. Conversely, heterologous desensitization occurs when incubation with one agonist attenuates the response to multiple agonists operating through distinct receptors. In some instances the pattern of unresponsiveness of adenylyl cyclase in heterologous desensitization may be so broad as to include decreased sensitivity to activators that bypass the receptors, for example $(AlF_4)^-$

(aluminum tetrafluoride) and guanine nucleotides, both of which directly activate G_s.

HETEROLOGOUS DESENSITIZATION

Heterologous desensitization of adenylyl cyclase occurs in many tissues and cell types and involves multiple mechanisms. In many cell types it occurs in addition to homologous desensitization, thus complicating analysis. Heterologous desensitization usually displays a slower onset than homologous desensitization, which suggests that it represents an adaptive response to relatively prolonged stimulation. In several systems, cAMP analogues have been shown to induce heterologous desensitization (reviewed in Clark 1986), which suggests that this event itself is cAMP mediated. Evidence exists that supports modification of each of the individual components of the adenylyl cyclase system in various forms of heterologous desensitization. Undoubtedly, multiple mechanisms contribute simultaneously to the development of heterologous desensitization in certain situations. Nonetheless, for purposes of this discussion these modifications are considered individually, in terms of the specific component of the system whose function may be altered.

Receptor Alterations

The avian erythrocyte has been an important model system for uncovering the nature of the receptor alterations that may contribute to heterologous desensitization. Several early lines of evidence suggested that the receptors were perturbed when these cells developed heterologous desensitization after prolonged exposure to a β-adrenergic agonist such as isoproterenol. First, the decrement in catecholamine-stimulated enzyme activity ($\sim$50%) was much greater than that in $(AlF_4)^-$ or guanine nucleotide–stimulated activity (10–20%) (Hoffman et al 1979). Second, polyacrylamide gel electrophoresis of photoaffinity-labeled β-adrenergic receptors from desensitized cells showed an altered electrophoretic mobility when compared to control cells, which suggests a covalent modification (Stadel et al 1982). Third, when such desensitized receptors were partially purified and reconstituted into phospholipid vesicles, their functionality was diminished when assessed by fusion with receptor-deficient cells (Strulovici et al 1984).

By incubating avian erythrocytes with [^{32}P]inorganic phosphate prior to desensitization and then isolating the receptors, it was found that phosphorylation of the β-adrenergic receptor, to a stoichiometry of 2–3 mol phosphate/mol receptor, occurred during the desensitization process (Stadel et al 1983a; Sibley et al 1984a). Under a number of circumstances the

phosphate/receptor stoichiometry was tightly correlated with the extent of desensitization (Stadel et al 1983a, Sibley et al 1984a).

Moreover, it has been demonstrated in vitro that the cAMP-dependent protein kinase (protein kinase A) can catalyze the addition of up to two moles of phosphate per mol of purified hamster lung β_2-adrenergic receptor (Benovic et al 1985). Agonist occupancy of the receptor enhances the rate of this phosphorylation reaction, but not the ultimate stoichiometry achieved. Further, it has been demonstrated that receptor phosphorylated by protein kinase A has a reduced ability to activate G_s in a reconstituted system. These data, then, suggest that one mechanism by which heterologous densitization can occur at the receptor level is a feedback regulatory effect mediated by protein kinase A such that agonist activation of the kinase leads to phosphorylation of the receptor (see Figure 2).

Protein kinase C is also able to phosphorylate and regulate the β-adrenergic receptor (Bouvier et al 1987; Kelleher et al 1984; Sibley et al 1984b). Tumor-promoting phorbol diesters, compounds that potently activate protein kinase C, stimulate β-adrenergic receptor phosphorylation and induce adenylyl cyclase desensitization in avian erythrocytes. Since a wide variety of hormones, drugs, and neurotransmitters activate protein kinase C, there is at least the potential for regulation of β-adrenergic receptor function via stimulation by these various agents.

It has been demonstrated in vitro that protein kinase C can phosphorylate the pure β_2-adrenergic receptor (Bouvier et al 1987). However, in contrast to the results obtained with cAMP-dependent protein kinase, the rate of receptor phosphorylation by protein kinase C is not influenced by agonist occupancy. To date, there are no reports assessing the functional consequences of in vitro β-receptor phosphorylation by protein kinase C. Consensus sites of phosphorylation for both protein kinase A and protein kinase C are found on pressumed cytoplasmic regions of the β-receptor (see Figure 1). These sequences reside on the large third cytoplasmic loop and the carboxyl terminal cytoplasmic tail.

Alterations in Guanine Nucleotide Regulatory Proteins

In addition to alterations in the receptors, there is evidence that guanine nucleotide regulatory proteins may be the locus of functionally significant changes in various forms of heterologous desensitization. A common approach for investigating this issue has been to induce heterologous desensitization in an in vitro cellular system and then to assay the functionality of the G_s component by solubilizing it from the plasma membrane and reconstituting it into cyc^- S_{49} lymphoma membranes. This cell type is genetically deficient in the α subunit of the G_s protein and hence lacks adenylyl cyclase activity. Addition of G_s protein to the membrane system

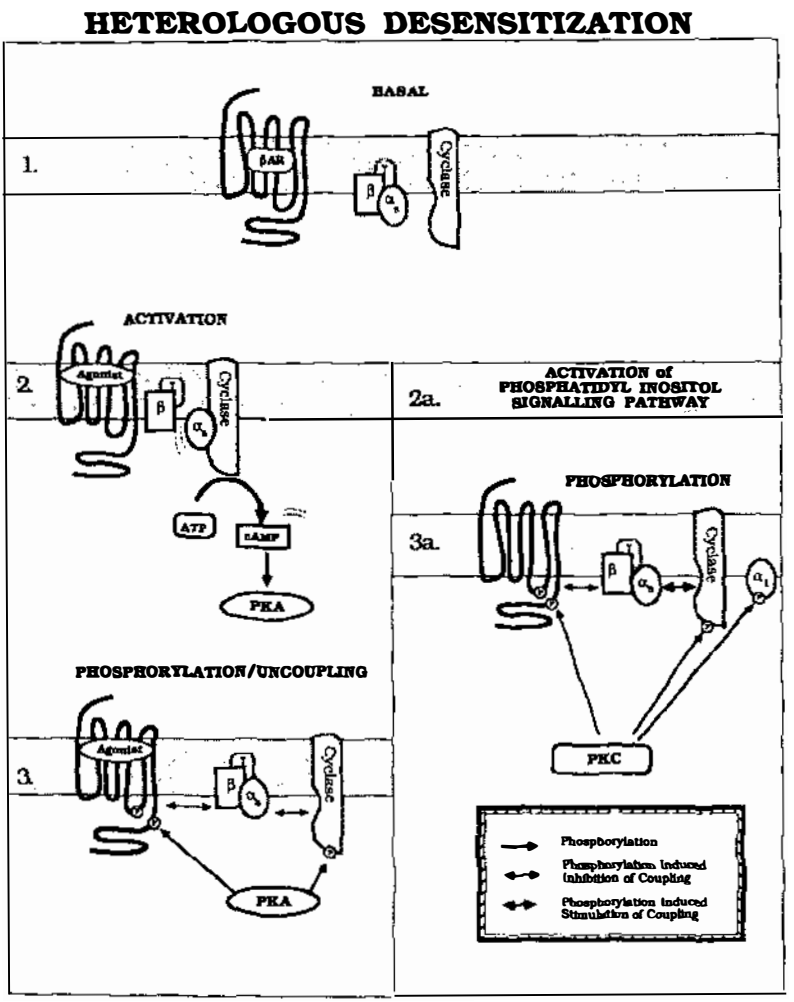

Figure 2 Heterologous desensitization of hormone-responsive adenylyl cyclase.

restores adenylyl cyclase activity and thus provides an assay for the functionality of the G_s protein added (Gilman 1987). Using this approach, Kassis & Fishman (1984) demonstrated that the G_s protein from fibroblasts, which displayed heterologous desensitization after exposure to prostaglandin E_1, was functionally impaired. Similar results have been obtained after heterologous desensitization of hepatocytes by prostaglandin E_1, and of ovarian cells desensitized by human chorionic go-

nadotropin. Conflicting results have been reported for avian erythrocytes desensitized with isoproterenol; one group (Briggs et al 1983) found decreased G_s function in turkey erythrocytes; another group failed to find an altered function in pigeon erythrocytes (Simpson & Pfeuffer 1980). Taken together, these findings suggest that at least some forms of heterologous desensitization involve functional alterations in the G_s protein. The nature of such alterations, however, remains completely unknown.

G_i may also be regulated. Rich et al (1984) reported that glucagon-induced heterologous desensitization in dog kidney epithelial cells (MDCK) was not associated with any alterations in G_s, but rather involved increases in the apparent levels of G_i. Thus, alterations in the G_s/G_i ratio may be another potential mechanism by which heterologous densitization is achieved, although again the mechanisms of any such regulation are unknown.

Finally, it has also been demonstrated in vitro that protein kinase C can phosphorylate the purified G_i protein. The phosphorylation occurs on the α_i subunit of G_i and is strikingly promoted by factors that dissociate α_i from the $\beta\gamma$ subunits (Katada et al 1985). This finding indicates that it is the free α_i subunit rather than the holoprotein that is the preferred substrate for the kinase, and that activation of the G_i protein in vivo might be required in order for this covalent modification to occur. However, it should be stressed that no evidence for the phosphorylation of G_i in intact cells has been reported. There is also no published information indicating whether phosphorylation of G_i in vitro leads to any changes in its function. Thus, the regulatory significance, if any, of this modification remains to be determined.

Adenylyl Cyclase Alterations

Since heterologous desensitization often involves broad patterns of refractoriness not only to hormones, but also to other classes of activators, such as $(AlF_4)^-$, forskolin, and guanine nucleotides, it seems reasonable to consider adenylyl cyclase itself as a possible locus of regulation. Nonetheless, very little evidence bearing on this hypothesis has been published. Yoshimasa et al (1988) documented that the cAMP-dependent protein kinase can phosphorylate the purified catalytic unit of adenylyl cyclase in vitro. When reconstituted in phospholipid vesicles with purified G_s, the phosphorylated cyclase preparations displayed an approximate 40% decrease in catalytic activity in the presence of the nonhydrolyzable GTP analog, GppNHp. Whether regulatory phosphorylation of the catalyst by the cAMP-dependent protein kinase occurs in vivo is presently unknown.

One other example of phosphorylation of adenylyl cyclase has been reported. In a variety of cells and tissues, exposure to phorbol esters, which

activate protein kinase C, leads to a general enhancement of adenylyl cyclase activity (Sibley et al 1986a). It has been shown that the catalytic unit of the cyclase (isolated by forskolin affinity chromatography is stoichiometrically phosphorylated in frog erythrocytes preloaded with [^{32}P]inorganic phosphate and then treated with active phorbol esters (Yoshimasa et al 1987). Moreover, the catalyst can be phosphorylated in vitro by protein kinase C (Yoshimasa et al 1988). Such in vitro phosphorylated enzymes show a modest ($\sim$20%) increase in activity when reconstituted in phospholipid vesicles with G_s. As with the other examples of modification of the cyclase described above, there is as yet no evidence documenting the physiological relevance of this phosphorylation. Figure 2 summarizes a variety of mechanisms that may contribute to heterologous desensitization.

HOMOLOGOUS DESENSITIZATION

Agonist-specific or homologous desensitization is defined as the loss of adenylyl cyclase responsiveness to a single hormone. At least three distinct phenomena may be involved in various aspects of this process: (*a*) an initial rapid uncoupling of the receptor from adenylyl cyclase activation, (*b*) a rapid sequestration or internalization of the receptor away from the cell surface, and (*c*) a slower down regulation or inactivation of the receptor.

Functional Uncoupling/Sequestration

There is abundant evidence linking cellular redistribution of the β-adrenergic receptor to homologous desensitization. This includes the early observations (Putman & Molinoff 1980; Toews et al 1983; Insel et al 1983) that, as assessed by radioligand binding studies on intact cells, preincubation of cells with an agonist converts a high proportion of the β-receptors to a form that has a lower apparent affinity for the agonist. This shift to lower affinity was interpreted as a slow equilibration of the hydrophilic agonist with a sequestered population of the receptor. More recently, several studies (Hertel et al 1983a,b) confirmed that agonist-induced desensitization is accompanied by a rapid decrease in the number of β-adrenergic receptors readily accessible to the hydrophilic ligand [^{3}H]CGP12177 with no change in the total number of cellular receptors recognized by the hydrophobic antagonist [^{3}H]dihydroalprenolol.

Although these data are consistent with a sequestration of the β-adrenergic receptor into a compartment that is less accessible to hydrophilic ligands, the first direct evidence that the receptor is indeed sequestered to a compartment distinct from the plasma membrane was provided by the

work of Chuang & Costa (1979) and Chuang et al (1980). They observed that exposure of frog erythrocytes to an agonist caused a decrease in the number of plasma membrane β-adrenergic receptors, which was paralleled by an increase in the "cytosolic" receptor number. Stadel et al (1983b) further documented that these cytosolic receptors are associated with a light membrane fraction. In mammalian cells, Harden et al (1980) demonstrated that pretreatment with isoproterenol promoted the sequestration of a significant portion of the β-adrenergic receptors from a heavy membrane fraction to a light membrane fraction (light vesicle) on sucrose gradients.

The data reviewed here strongly link the appearance of homologous desensitization to the sequestration of the β-adrenergic receptor away from the plasma membrane into a still poorly defined light vesicle fraction. However, is the loss of hormonal stimulation of adenylyl cyclase uniquely the consequence of the sequestration of the receptor? The notion that an early uncoupling of the receptor from adenylyl cyclase activation, which precedes the sequestration, contributes to the loss of hormonal responsiveness has emerged in the last few years. Harden et al (1980) demonstrated that in human astrocytoma cells the appearance of receptors in the light vesicle fraction (sequestration) was delayed $\sim$1 min relative to the rapid agonist-induced decrease in adenylyl cyclase hormonal responsiveness. Moreover, blockade of receptor sequestration by pretreatment of the cells with concanavalin A (Waldo et al 1983) or by incubation at 4°C (Homburger et al 1980) failed to prevent desensitization. Also consistent with an uncoupling event, distinct from the sequestration, is the observation of Kassis & Fishman (1984) that the β-adrenergic receptors from desensitized HeLa, cyc$^-$ S_{49} lymphoma, and C6 rat glioma cells have a decreased ability to stimulate a foreign adenylyl cyclase, as assayed by membrane fusion experiments. Taken together, these data support the notion that the β-adrenergic receptor itself is functionally altered in its ability to stimulate cyclase (uncoupled) following agonist-induced desensitization and that this uncoupling preceeds its sequestration.

Down-Regulation

Down-regulation may represent another specific mechanism for regulating hormone responsiveness. In contrast to receptor sequestration, down regulation of the β-adrenergic receptor occurs following more prolonged agonist stimulation and is characterized by a loss of total receptor binding sites from the cell (Homburger et al 1980; Shear et al 1976; Su et al 1980). Thus, the lost receptors are not associated with a membrane compartment accessible even to hydrophobic ligands. Although the precise fate of the down-regulated receptors has not been determined, two distinct mech-

anisms have emerged. In some systems, recovery to a normal level of cell surface receptors is slow and requires protein synthesis (Marshema et al 1980; Doss et al 1981; Frederich et al 1983), which suggests that the down-regulated receptors were proteolytically degraded. In other cases, recovery of β_2-adrenergic receptor binding sites appears to be independent of protein synthesis (Frederich et al 1983; Su et al 1976; Homburger et al 1984), which suggests that such down-regulated receptors can recycle back to the cell surface in a fully functional conformation.

Role of Receptor Phosphorylation in Homologous Desensitization

The first indirect evidence suggesting a role of phosphorylation in homologous desensitization came from cell-free studies, which demonstrated that desensitization of adenylyl cyclase activity stimulated by luteinizing hormone in ovarian cells can be reversed by phosphatase treatment (Hunzicker-Dunn et al 1979). More recently, using frog erythrocytes as a model system, Sibley et al (1985) showed directly that β-agonist-induced homologous desensitization is accompanied by a stoichiometric ($\sim$2 mol phosphate/mol receptor) phosphorylation of the β_2-adrenergic receptor. Moreover, the time courses of receptor phosphorylation and desensitization were very similar, reaching, for both phenomena, 50% of their maximum after a 30-min incubation with isoproterenol (Sibley et al 1985). Strasser et al (1986a) demonstrated that agonist-promoted phosphorylation of the β-adrenergic receptor is also observed in S_{49} lymphoma cells. Moreover, in the cyc^- and kin^- mutants of S_{49} lymphoma cells, which are devoid of G_s and cAMP-dependent kinase activity respectively, β-adrenergic agonists promote both desensitization and phosphorylation of the receptor. These findings strongly implicate cAMP-independent pathways for both phenomena and are in agreement with the notion that homologous desensitization does not appear to be cAMP mediated (Green & Clark 1981; Green et al 1981).

Involvement of a Receptor-Specific Kinase

As described above, β-adrenergic receptor phosphorylation accompanies homologous desensitization not only in frog erythrocytes (Sibley et al 1985) and wild-type S_{49} lymphoma cells, but also in mutant (kin^-, cyc^-) S_{49} cells (Strasser et al 1986a), which lack an agonist-promoted cAMP-dependent protein kinase response. It was initially speculated that the protein kinase(s) involved in receptor phosphorylation during homologous desensitization either were stimulated by a second messenger other than cAMP or preferentially phosphorylated the agonist-occupied form of the receptor. In fact, a kinase that is able to phosphorylate purified β-adren-

ergic receptor in an almost totally agonist-dependent fashion was identified and partially purified from the kin$^-$ mutant of S_{49} lymphoma cells (Benovic et al 1986b). This kinase, termed the β-adrenergic receptor kinase (βARK) is distinct from other kinases such as the cAMP-, cGMP-, Ca^{2+}/calmodulin-, and Ca^{2+}/phospholipid-dependent protein kinases. Furthermore, βARK does not phosphorylate such general kinase substrates as casein and histones. Thus, these studies suggested that a novel protein kinase, which preferentially phosphorylated the agonist-occupied form of the β-adrenergic receptor, might be involved in mediating the homologous form of adenylyl cyclase desensitization.

The β-adrenergic receptor kinase appears to be a ubiquitous protein present in a large number of tissues examined (Benovic et al 1987b). βARK has been purified from bovine brain to apparent homogeneity and consists of a single subunit of M_r 80,000. βARK phosphorylates the agonist-occupied receptor to a stoichiometry of $\sim$8 mol phosphate/mol receptor. The kinetics of the phosphorylation reaction ($K_m = 0.25$ μM for β-adrenergic receptor, $K_m = 35$ μM for ATP, $V_{max} = \sim 80$ nmol phosphate/min/mg protein) suggest a relatively high affinity interaction between the kinase and receptor.

The functional significance of β-adrenergic receptor phosphorylation by βARK has also been examined. When pure β-adrenergic receptor is phosphorylated by pure βARK, the resulting phosphorylated receptor has only a slightly reduced ability to interact with G_s (16% inactivation), as assessed by agonist-stimulated GTPase activity in a reconstituted system (Benovic et al 1987a). However, when pure β-adrenergic receptor is phosphorylated with a crude βARK preparation ($\sim$1% pure), the receptor interaction with G_s is significantly reduced ($\sim$80% inactivation). This suggests that the cruder βARK fraction contains a factor that enhances the uncoupling of phosphorylated β-adrenergic receptor and G_s. Moreover, the addition of pure retinal arrestin to β-adrenergic receptor phosphorylated by pure βARK enhanced the uncoupling of phosphorylated β-adrenergic receptor and G_s (41% inactivation) (Benovic et al 1987a). Arrestin (also called 48-kDa protein or S antigen) is involved in enhancing the inactivating effect of rhodopsin phosphorylation by rhodopsin kinase (see section on other G protein-coupled receptors below). These results suggest that a protein analogous to retinal arrestin may be involved in mediating the functional consequences of receptor phosphorylation by βARK.

While the β-adrenergic receptor coupled adenylyl cyclase system has proven to be a very useful model for studying homologous desensitization, many other adenylyl cyclase–coupled receptors are susceptible to the same type of desensitization. Thus, it is not unreasonable to speculate that the

mechanism by which these other receptors undergo homologous desensitization might also involve an agonist-induced receptor phosphorylation. The notion that each receptor has its own specific protein kinase, however, seems unlikely. A more plausible hypothesis is that a general receptor kinase exists that is able to phosphorylate different agonist-occupied receptors. Early in these studies it was found that stimulation of DDT_1 MF-2 hamster vas deferens smooth muscle cells or S_{49} lymphoma cells with a β-agonist leads to the sequestration of up to $\sim$80% of the βARK activity from the cytosol to the plasma membrane (Strasser et al 1986b). The sequestration was rapid and reversible and coincided temporally with the time courses of receptor phosphorylation and desensitization. This suggested that the agonist-induced sequestration of βARK may represent the first step in homologous desensitization. Moreover, this property provided a test for the hypothesis that βARK can phosphorylate different agonist occupied receptors. In S_{49} cells, prostaglandin E_1, which induces homologous desensitization to its own actions, also promotes βARK sequestration to the plasma membrane. This result suggests, albeit indirectly, that βARK may be a general adenylyl cyclase–coupled receptor kinase capable of phosphorylating and regulating the function of many receptors.

Similar results were obtained when S_{49} lymphoma cells were treated with somatostatin, a 14-amino acid peptide that inhibits adenylyl cyclase activity through a specific receptor. In these cells, somatostatin induced the sequestration of βARK from the cytosol to the plasma membrane to an extent comparable to β-agonists or prostaglandin E_1 (Mayor et al 1987). The sequestration process was rapid and reversible and was associated with desensitization of the somatostatin response, which suggests that βARK also phosphorylates and regulates receptors coupled to inhibition of adenylyl cyclase.

In order to determine more directly if such receptors serve as substrates for βARK, the effects of this kinase on the α_2-adrenergic receptor were studied. In this study, purified human platelet α_2-adrenergic receptor, reconstituted into phospholipid vesicles, was found to be a very good substrate for βARK (Benovic et al 1987c). The phosphorylation was dependent on the agonist occupancy of the receptor and was completely blocked by coincubation with α_2-antagonists. The time course of phosphorylation of the α_2-adrenergic receptor by βARK was virtually identical to that observed with the β-adrenergic receptor. In contrast, the α_1-adrenergic receptor, which is coupled to stimulation of phosphatidylinositol hydrolysis, did not serve as a substrate for βARK. These results strongly suggest that βARK is a general adenylyl cyclase–coupled receptor kinase.

Two studies attempted to determine whether agonist-induced desen-

sitization in vivo results in an alteration of the β-adrenergic receptor, which is stable to purification. Strasser et al (1985), using rats injected with isoproterenol to induce homologous desensitization, demonstrated that the β-receptor purified from the lungs of desensitized compared to control animals was fully functional in stimulating the GTPase activity of G_s and in stimulating the adenylyl cyclase activity in a hybrid cell system. In contrast, Sibley et al (1986b) showed that the phosphorylated β-adrenergic receptor, purified from isoproterenol-treated frog erythrocytes, is functionally impaired (35–40% inhibition) in its interaction with G_s in a reconstituted system.

As discussed above, during homologous desensitization the initial receptor uncoupling from adenylyl cyclase is followed by a rapid sequestration of the receptor from the cell surface. This sequestration event physically separates the receptor from the other components of the adenylyl cyclase system and therefore may contribute to the desensitization process. The sequestration also appears to act as a mechanism for dephosphorylation of the receptor, which then allows subsequent resensitization. Sibley et al (1986b) in fact demonstrated that, in isoproterenol-desensitized frog erythrocytes, the β-receptors remaining at the cell surface are highly phosphorylated, while receptors isolated from a sequestered membrane fraction are phosphorylated to a much lower extent. Moreover, they also demonstrated that this sequestered vesicular compartment contains a phosphatase activity that rapidly dephosphorylates [^{32}P]labeled β-adrenergic receptors. In vitro studies demonstrated that the types I, IIA, and IIB protein phosphatases do not dephosphorylate βARK-phosphorylated β-receptor (Yang et al 1988). However, a high molecular weight latent phosphatase 2 does dephosphorylate the receptor. Further studies are necessary to determine whether this latent phosphatase acts as a receptor phosphatase in vivo.

Identification of the Phosphorylation Sites and Their Contribution to the Desensitization Process

The recent deduction of the complete amino acid sequence of the mammalian β_2-adrenergic receptor from cDNA and genomic clones (Dixon et al 1986; Kobilka et al 1987a) now permits the identification of the different domains of the receptor potentially involved in its regulation. As mentioned above, several analogies including structural similarities have been drawn between the β_2-adrenergic receptor and other G protein–coupled receptors, more specifically rhodopsin (Dohlman et al 1987b). In both cases, specific receptor kinases, namely rhodopsin kinase and β-adrenergic receptor kinase, have been shown to phosphorylate their respective substrates in a stimulus-dependent fashion (Kühn & Dreyer 1972; Benovic et

al 1986a,b). Phosphopeptide analysis and sequencing of phosphorylated rhodopsin (Hargrave et al 1980; Thompson & Findley 1984; Miller & Dratz 1984) have demonstrated that multiple serine and threonine residues near the carboxyl terminus serve as the major sites of phosphorylation for rhodopsin kinase. Similarly, Dohlman et al (1987a) demonstrated that most, if not all, of the sites on the β-receptor phosphorylated by βARK were lost following carboxypeptidase treatment, which removes the cytoplasmic tail of the receptor. The serine- and threonine-rich carboxyl terminal segment of the β-adrenergic receptor therefore appears to be the major region of phosphorylation by βARK.

More recently, Bouvier et al (1988), using cells expressing a mutant β_2-adrenergic receptor that lacks the serine- and threonine-rich carboxyl terminus, showed that such a mutated receptor does not undergo the agonist-promoted phosphorylation observed in cells expressing the wild-type receptor. This observation strongly implicates βARK as the enzyme responsible for the agonist-induced phosphorylation of the β-receptor in whole cells. This study also implicates βARK in some aspect of agonist-specific (homologous) desensitization. Indeed, cells expressing the carboxyl terminal truncated receptor (which did not undergo agonist-induced phosphorylation) display a delayed onset of agonist-induced desensitization. Such mutated receptors, however, showed a normal desensitization pattern following longer agonist exposure (Strader et al 1987; Kobilka et al 1987b; Bouvier et al 1988). A normal (Strader et al 1987), as well as an increased (Bouvier et al 1988), sequestration of the receptor has been reported in cells expressing the truncated receptor. Moreover, in another form of the receptor in which carboxyl terminal serine and threonine residues have been mutated, desensitization is slowed even further. Agonist-promoted phosphorylation of the carboxyl terminus of the receptor, presumably by βARK, therefore appears to be a crucial event in the early stages of homologous desensitization. This agonist-induced phosphorylation may be a triggering event for the early uncoupling that precedes sequestration of the receptor.

Mechanism of Homologous Desensitization

Although a temporal sequence appears relatively clear in the process of homologous desensitization (functional uncoupling, sequestration, down regulation), no causal relationship has clearly been established between these events. Moreover, the molecular mechanism(s) that trigger these individual events remain poorly understood. However, covalent modification of the receptor itself, and more specifically phosphorylation of the β-adrenergic receptor, appears to play a crucial role in at least some aspect of homologous desensitization.

The current understanding of the biochemical mechanisms involved in homologous desensitization is depicted in Figure 3. One of the first steps that follows agonist occupancy and G_s activation is an agonist-induced phosphorylation of the receptor. This phosphorylation may initially involve the sequestration of a specific kinase, βARK, from the cytosol to the plasma membrane. βARK is then able to phosphorylate the agonist-occupied receptor at a number of sites predominately localized at the carboxyl terminus of the receptor. The phosphorylation leads to an

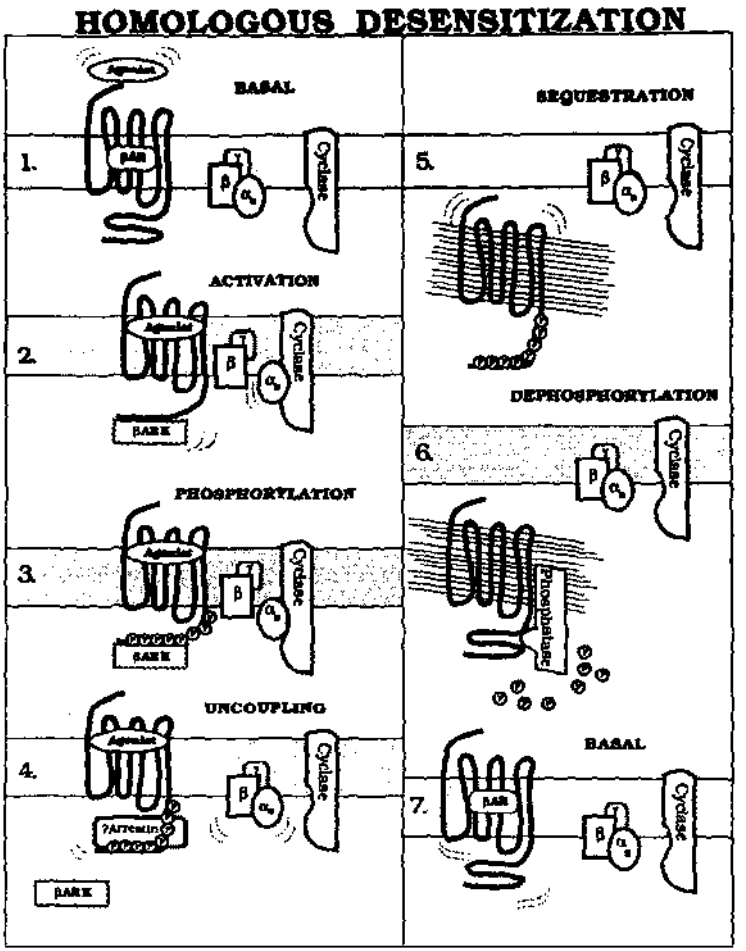

Figure 3 Homologous desensitization of hormone-responsive adenylyl cyclase.

uncoupling of the receptor from G_s by a mechanism that may involve the interaction of an arrestin like molecule with the phosphorylated receptor. The receptor is then sequestered from the cell surface by an unknown mechanism. The receptor appears to be dephosphorylated in this sequestered compartment and is recycled back to the plasma membrane where it is fully functional.

OTHER G PROTEIN–COUPLED RECEPTORS

Among other G protein–coupled receptors, rhodopsin, which is involved in the process of retinal light transduction, has been the most extensively studied with respect to mechanisms of coupling and regulation. This system is similar in structure and function to the hormone-responsive adenylyl cyclase system. The various components of the system—the receptor rhodopsin, the G protein transducin, and the effector cGMP phosphodiesterase—interact in a manner analogous to the components of hormone systems; moreover, some of these components, namely rhodopsin and transducin, have significant structural homology (similarity) with their counterparts from the hormone-responsive systems (Stryer 1986). Moreover, the mechanisms involved in regulating rhodopsin function are very similar to those described above for the regulation of the adenylyl cyclase activity stimulated by the β-adrenergic receptor (Applebury & Hargrave 1986; Lefkowitz & Caron 1988).

Absorption of a photon by rhodopsin in the retinal rod outer segment triggers at least two distinct events. First, activated rhodopsin interacts with and activates transducin, which in turn activates the effector enzyme cGMP phosphodiesterase, eventually leading to a nerve impulse. Second, photoactivated rhodopsin interacts with a protein kinase that phosphorylates it on multiple serine and threonine residues. This enzyme is specific for the bleached form of rhodopsin and has been designated rhodopsin (or opsin) kinase (Applebury & Hargrave 1986). Rhodopsin kinase is normally a soluble protein; however, when rhodopsin is activated by light, the enzyme becomes associated with the cytoplasmic face of the disc membranes (Kühn 1978). This suggests that a particular conformation of rhodopsin is responsible for interacting with rhodopsin kinase. The principal sites of phosphorylation on rhodopsin by rhodopsin kinase are on serine and threonine residues near the carboxyl terminal portion of the protein (Wilden & Kühn 1982; see Figure 1). However, additional evidence suggests that a region of loop V–VI of rhodopsin may also be phosphorylated by rhodopsin kinase (McDowell et al 1985).

Phosphorylation of rhodopsin by rhodopsin kinase is thought to serve as a stop signal for the phosphodiesterase-activating function of rhodopsin

(Kühn et al 1973; Liebman & Pugh 1980). The mechanism by which quenching of light transduction occurs is becoming better understood. While phosphorylation of rhodopsin alone decreases the interaction of rhodopsin with transducin (Shichi et al 1984; Wilden et al 1986), it is evident that another component is necessary to attain the full effect of phosphorylation on the deactivation process of rhodopsin (Wilden et al 1986). This protein, variously termed the S antigen, 48-kDa protein, or arrestin, is a major soluble component of rod outer segments that binds to phosphorylated rhodopsin, thereby inhibiting the ability of rhodopsin to activate transducin (Wilden et al 1986). Recently, the primary structure of arrestin was established by protein sequencing and cDNA cloning (Yamaki et al 1987; Shinohara et al 1987). While not significantly related to that of any other known proteins, the deduced sequence of the 48-kDa protein shows several amino acid stretches that are similar to functionally important regions of transducin (i.e. toxin ribosylation sites and phosphoryl binding sites). Thus, it is not surprising that arrestin is able to interact with phosphorylated rhodopsin and to inhibit its interaction with the G protein transducin (Shinohara et al 1987).

The data reviewed above suggest that the β-adrenergic receptor kinase and rhodopsin kinase are likely members of a family of kinases specific for G protein–coupled receptors. The ability of retinal arrestin to accentuate the inactivating effect of β-adrenergic receptor phosphorylation on the interaction of the β-receptor with G_s strongly suggests that a similar component exists in hormone receptor systems (Benovic et al 1987a). In analogy with retinal arrestin, this postulated protein would potentiate the uncoupling function of receptor phosphorylation.

Another class of receptors, those coupled to the activation of phospholipase C, also demonstrates desensitization phenomena (Leeb-Lundberg et al 1987). Members of this class, which also mediate their effects by interacting with G proteins, include the α_1-adrenergic, muscarinic, vasopressin, and angiotensin receptors. Agonist occupancy of these receptors results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate to inositol trisphosphate and diacylglycerol, which cause the mobilization of intracellular calcium and the activation of protein kinase C, respectively (Berridge & Irvine 1984). Phorbol esters mimic the protein kinase C activating function of these receptors by substituting for endogenously produced diacylglycerol (Castagna et al 1982).

Several studies have shown that phorbol esters can rapidly desensitize α_1-adrenergic receptor function (Cotecchia et al 1985; Cooper et al 1985; Corvera et al 1986; Lynch et al 1985; Colucci et al 1986). In the smooth muscle cell line DDT_1 MF-2, phorbol ester–mediated α_1-adrenergic desensitization is accompanied by phosphorylation of the α_1-adrenergic receptor

(Leeb-Lundberg et al 1985). A similar pattern of desensitization and receptor phosphorylation is observed with exposure of cells to the natural agonist norepinephrine (Leeb-Lundberg et al 1987). Comparison of peptide maps of the various phosphorylated α_1-receptors suggests that the same kinase (i.e. protein kinase C) is involved in the agonist as well as in phorbol ester–mediated phosphorylations (Bouvier et al 1987). As described above for the β_2-adrenergic receptor system, agonist-mediated uncoupling of the α_1-receptor response is accompanied by a rapid sequestration of the α_1-receptor binding site away from the cell surface (Leeb-Lundberg et al 1987). Unlike the agonist-promoted phenomenon, however, phorbol ester–mediated functional uncoupling of α_1-receptors does not promote a decrease in accessible cell surface receptors (Leeb-Lundberg et al 1987). Thus, in this system functional receptor uncoupling appears to require phosphorylation, whereas receptor sequestration is dependent on agonist occupancy and may or may not require receptor phosphorylation.

The biochemical events mediating muscarinic cholinergic responses in tissues have not been completely elucidated due to the fact that several receptor subtypes exist (Bonner et al 1987; Peralta et al 1987). Moreover, these receptors can couple to several different signal transduction systems (Ashkenazi et al 1987). Nonetheless, exposure of various tissues to muscarinic agonists leads to functional desensitization of their responses (Renaud et al 1980). Kwatra & Hosey (1986) recently demonstrated that muscarinic agonist exposure of chick cardiac tissue leads to a large increase in muscarinic receptor phosphorylation. This agonist-mediated phosphorylation correlates temporally and pharmacologically with a loss in agonist affinity of the receptor and with receptor desensitization (Kwatra et al 1987). Phosphorylation of the cardiac muscarinic receptor does not appear to be mediated by protein kinase C, calcium/calmodulin- or cyclic nucleotide–dependent protein kinases, which raises the possibility that a specific protein kinase, similar to the β-adrenergic receptor kinase, mediates this agonist-dependent phosphorylation of the receptor (Kwatra et al 1987).

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