β-Adrenergic Receptor-Coupled Adenylate Cyclase

Biochemical Mechanisms of Regulation

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Abstract

β-Adrenergic receptor-coupled adenylate cyclase is regulated by both amplification and desensitization processes. Desensitization of adenylate cyclase is divided into two major categories. *Homologous* desensitization is initiated by phosphorylation of the receptors by a β-adrenergic receptor kinase. This reaction serves to functionally uncouple the receptors and trigger their sequestration away from the cell surface. These sequestered receptors can rapidly recycle to the cell surface or, with time, become down regulated, being destroyed within the cell. Dephosphorylation of the receptors is accomplished in the sequestered compartment of the cell, which may functionally regenerate the receptors and allow their return to the cell surface. In *heterologous* desensitization, receptor function is also regulated by phosphorylation, but in the absence of receptor sequestration or down regulation. In this case, phosphorylation serves only to functionally uncouple the receptors, that is, to impair their interactions with the guanine nucleotide regulatory protein N_s. Several protein kinases are capable of promoting this phosphorylation, including the cAMP-dependent kinase and protein kinase C. In addition to the receptor phosphorylation, heterologous desensitization is associated with modifications at the level of the nucleotide regulatory protein N_s and perhaps N_i. Adenylate cyclase systems are also subject to amplification that involves a protein kinase C-mediated phosphorylation of the catalytic unit of the enzyme. Phosphorylation of the catalytic unit enhances its catalytic activity and results in amplified stimulation by the regulatory protein N_s. Other receptor/effector systems exhibit qualitatively similar regulatory phenomena, suggesting that covalent modification (phosphorylation) may represent a general mechanism for regulating receptor function.

Index Entries: β-Adrenergic receptor; adenylate cyclase; N proteins; desensitization; amplification; phosphorylation.

Introduction

Amplification and desensitization are well recognized phenomena in biological regulatory and sensory systems (Koshland et al., 1982). Considerably more is known about the molecular mechanisms of desensitization than those for amplification. Examples of systems in which desensitization is observed include chemotaxis of bacteria or mammalian polymorphonuclear leukocytes, neurotransmission by various neurotransmitters at synapses, stimulation of diverse physiological processes in eukaryotes by many drugs and hormones, and sensory perception. In the context of clinical therapeutics, desensitization significantly limits the efficacy of numerous pharmacological agents.

Common to most systems that display desensitization is the existence of receptors that mediate the effects evoked by the specific stimuli. Since such receptors constitute the first point of interaction of biologically active stimuli with cells, it is not unreasonable to suppose that regulation of receptor function might constitute the basis for some forms of desensitization.

Among the receptor–effector systems that mediate the effects of many hormones and drugs in humans and other animals few are more important than the adenylate cyclase system, which synthesizes the ubiquitous second messenger cyclic AMP. Stimulation of the enzyme by catecholamines, such as epinephrine and norepinephrine, is mediated by a specific receptor termed the β-adrenergic receptor. Persistent stimulation of this system by catecholamines or synthetic analogs leads to rapid desensitization of the cyclic AMP response, with consequent blunting of physiological responses, e.g., the therapeutic effects of adrenergic agents in the treatment of asthma and congestive heart failure.

Recent progress in elucidating the molecular properties and functions of these receptors has

shed new light on the mechanisms by which regulation of the receptors leads to desensitization. Moreover, the mechanisms uncovered may well be generally operative in mediating the desensitization response in diverse biological systems. Our goal here is to review advances in understanding the molecular basis of desensitization focusing on the β-adrenergic receptor-coupled adenylate cyclase effector system. In addition, we will review very recent information that indicates that this enzyme system is regulated by amplification processes as well as by desensitization.

Structure of β-Adrenergic Receptor-Coupled Adenylate Cyclase

Before considering regulation of adenylate cyclase, a brief review of the structural components of this system will be helpful. Hormone receptor-coupled adenylate cyclase appears to be comprised of at least three proteins: (1) the receptors, e.g., the β-adrenergic receptors for catecholamines; (2) the guanine nucleotide binding regulatory proteins (N_s and N_i); and (3) the enzyme catalytic unit (C) (Fig. 1). β-Adrenergic receptors have been purified from a variety of sources and reside on single polypeptides of M_r = 60,000–65,000 from mammalian tissues (Benovic et al., 1984; Cubero and Malbon, 1984). In amphibian and avian erythrocytes the receptors consist of peptides of M_r = 58,000 (Shorr et al., 1981) and $M_r \cong$ 40,000–50,000 (Shorr et al., 1982; Sibley et al., 1984), respectively. Guanine nucleotide-binding proteins are involved in both the stimulation (N_s) and inhibition (N_i) of adenylate cyclase activity. These proteins have also been purified and shown to exist as heterotrimers with subunit molecular weights of 42,000 or 41,000, respectively (α_s and α_i), and 35,000 (β_s and β_i) and ca. 5,000–10,000 (γ_s or γ_i) (Gilman, 1984; Spiegel, 1987). Pure preparations of these proteins have been reconstituted into phospholipid vesicles. In the case of N_s, coreconstitution with the pure β-adrenergic receptor establishes high-affinity agonist binding to the receptor and hormone-sensitive GTPase activity (Cerione et al., 1984). Fusion of pure β-adrenergic receptor preparations, which have been reconstituted into lipid vesicles, with cells that lack β-adrenergic receptors but contain adenylate cyclase leads to the establishment of a β-adrenergic receptor-responsive adenylate cyclase system (Cerione et al., 1983). Such studies document the functionality of the purified receptor proteins. Recently, the catalytic unit of adenylate cyclase has been purified to homogeneity (Pfeuffer et al., 1985; Smigel, 1986) and shown to be comprised of a single protein of $M_r \cong$ 150,000.

Recent information concerning the activation of adenylate cyclase suggests that agonist binding to the receptor first initiates the interaction of this protein with N_s to form a ternary complex. The coupling of these two proteins is readily detectable by assessing high-affinity, guanine nucleotide-sensitive agonist binding to the receptor. The binding of guanine nucleotides to N_s is thought to result in the dissociation of N_s from the receptor and the dissociation of the α (GTP binding) from the βγ subunits of N_s. The guanine nucleotide-liganded α_s combines with free or inactive catalytic units, which leads to stimulation of adenylate cyclase activity. Activity is terminated by a GTPase activity present on α_s. The interaction of N_s with the catalytic unit can be assessed by measuring guanine nucleotide or fluoride ion (which interacts with N_s) stimulation of adenylate cyclase activity. For a more detailed discussion of this topic, the reader is referred to several excellent reviews (Stadel et al., 1982a; Gilman, 1984; Schramm and Selinger, 1984).

Patterns of Adenylate Cyclase Desensitization

Hormone-induced desensitization has been extensively investigated in a variety of tissues and cells that contain β-adrenergic receptors

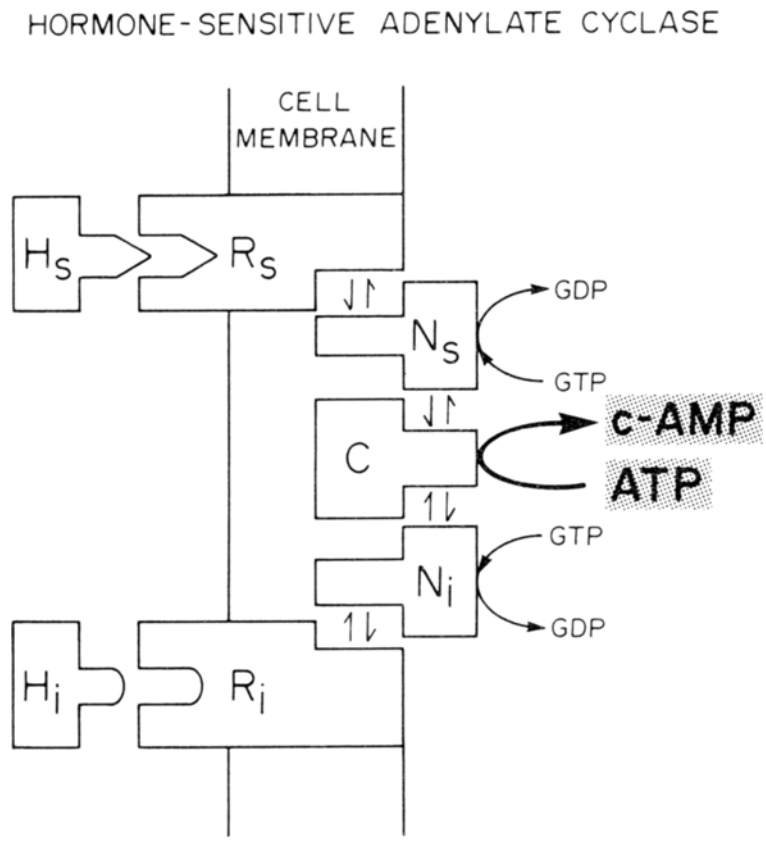

Fig. 1. Components of receptor-stimulated adenylate cyclase systems. H, hormone; R, receptor; N, guanine nucleotide regulatory protein; C, catalytic unit; s, stimulatory; i, inhibitory.

coupled to the stimulation of adenylate cyclase activity. Although the biochemical mechanisms for producing β-adrenergic receptor-coupled adenylate cyclase desensitization appear to be diverse, two major categories of refractoriness have been identified. These have been referred to as agonist-specific or *homologous desensitization* and as agonist-nonspecific or *heterologous desensitization.* The term homologous desensitization is used when the diminished response is observed *only* with the same receptor system that was activated during the agonist preincubation. Conversely, heterologous desensitization indicates that incubation with one agonist attenuates the response to multiple, different agonists operating through distinct receptors. Moreover, in some instances the pattern of unresponsiveness of adenylate cyclase in heterologous desensitization may be so broad as to include decreased sensitivity to activators that bypass the receptors, e.g., fluoride ion or guanine nucleotides. Obviously, the terms homologous and heterologous are simply operational or at best phenomenological and are not meant to be mechanistic in their usage. Nevertheless, these terms have been used in differing ways in the literature, leading to some confusion. As discussed below, there may well be multiple mechanisms of both homologous and heterologous desensitization, any of which may be operative in a given cell type under a certain set of conditions. Thus, it is an oversimplification to refer to either *the* mechanism of homologous or *the* mechanism of heterologous desen-

sitization. With these caveats in mind, we will retain the phenomenological distinction between homologous and heterologous desensitization in the following discussion of what is known about their various underlying biochemical mechanisms.

Heterologous Desensitization

Heterologous forms of desensitization of adenylate cyclase have been shown to occur in a wide variety of tissues and cell types. As discussed above, this form of desensitization represents a broad pattern of refractoriness in which the response to multiple hormones and sometimes nonhormonal effectors is impaired. In contrast to homologous desensitization, which may be unimechanistic, heterologous desensitization certainly occurs through more than a single mechanism. In many cell types, heterologous desensitization occurs in addition to the homologous form, thus making its analysis difficult. In general, however, the heterologous response occurs with a slower onset than the homologous one, suggesting that heterologous desensitization may represent an adaptive response to relatively prolonged stimulation.

Perkins and colleagues (Su et al., 1976; Johnson et al., 1978) were among the first to investigate heterologous desensitization using clonal astrocytoma cells. Prolonged incubation with either β-adrenergic agonists or prostaglandins diminished the subsequent capacity of both hormones to elevate intracellular cyclic AMP levels. This heterologous desensitization occurred more slowly than the homologous type of desensitization, which was also evident in these cells. Incubation of the cells with dibutyryl cyclic AMP produced refractoriness to both catecholamines and prostaglandins, suggesting that the heterologous desensitization might be cyclic AMP mediated. Production of the desensitization by any effector was not dependent on protein synthesis. Interestingly, when adenylate cyclase activity was examined in membranes from desensitized cells, the heterologous form of desensitization was lost, although the homologous form was retained. This latter property is not true of heterologous desensitization in a number of other systems. Brooker and coworkers have also shown that in C6-2B rat glioma cells a heterologous form of desensitization can be elicited with catecholamines, cyclic AMP analogs, cholera toxin, forskolin, and phosphodiesterase inhibitors, with no loss of β-adrenergic receptor binding (de Vellis and Brooker, 1974; Terasaki et al., 1978; Nickols and Brooker, 1979, 1980; Moylan et al., 1982; Barovsky et al., 1983). However, the heterologous form of desensitization in these cells can be largely attenuated or reversed by protein synthesis inhibitors. These findings lead to the proposal that incubation of C6-2B cells with agents that lead to increased intracellular cyclic AMP levels induces the synthesis of a "refractoriness protein," although the precise biochemical mechanism remains obscure.

Heterologous desensitization has also been shown to occur in a number of other mammalian tissues and cell types (Newcombe et al., 1975; Clark and Butcher, 1979; Koschel, 1980; Balkin and Sonnenberg; 1981, Harden, 1983; Attramadal et al., 1984; Noda et al., 1984). In most but not all systems this form of desensitization can be mimicked by incubation with cyclic AMP analogs, suggesting that heterologous desensitization is often cyclic AMP mediated.

Alterations in the Stimulatory Guanine Nucleotide Regulatory Protein (N_s)

Avian erythrocytes have been proven useful model systems with which to investigate the biochemical mechanisms of heterologous desensitization. This is related to the fact that these cells are available in large abundance and appear to exclusively exhibit the heterologous

form of adenylate cyclase desensitization. Hoffman et al. (1979) initially demonstrated that incubation of turkey erythrocytes with the β-adrenergic agonist isoproterenol produced an approximate 50% attenuation of subsequent catecholamine-stimulated adenylate cyclase activity. Since only β-adrenergic receptors are coupled to adenylate cyclase in these cells, stimulation by other hormonal effectors could not be assessed. There were also small but significant decrements in guanine nucleotide- and fluoride-stimulated enzyme activities observed as a result of desensitization. This suggests that the catecholamine-induced desensitization in the turkey erythrocyte is of the heterologous type. There was no reduction in the binding of the β-antagonist ligand [^{3}H] dihydroalprenolol in membranes derived from treated cells (Hoffman et al., 1979).

Stadel et al. (1981) subsequently showed that catecholamine-promoted desensitization in the turkey erythrocyte is associated with a functional uncoupling of the β-adrenergic receptor. This was evidenced by an impaired ability of the receptors to form a high-affinity, guanine-nucleotide-sensitive complex with agonists, as detected in radioligand binding studies. Importantly, the adenylate cyclase desensitization as well as the receptor uncoupling were partially mimicked by incubating the erythrocytes with cell-permeable analogs of cyclic AMP. This data indicated that there may be a cyclic AMP-mediated alteration of one or more of the adenylate cyclase components, which results in the desensitization response.

Similar findings have been reported for pigeon erythrocytes by Simpson and Pfeuffer (1980) and Hudson and Johnson (1981). Exposure of these cells to catecholamines or cyclic AMP analogs leads to a decrease in isoproterenol-, guanine nucleotide-, and fluoride-stimulated adenylate cyclase activities, with no change in receptor number. Hudson and Johnson (1981) additionally found that desensitization did not alter the ability of the solubilized N_s protein to reconstitute adenylate cyclase activity in S49 *cyc*$^-$ lymphoma cell membranes (which lack functional N_s activity). These workers did find, however, that desensitization resulted in a diminished ability of agonists and/or guanine nucleotides to induce a conformational change in the N_s protein, as assessed by peptide-mapping techniques.

Somewhat different results were obtained by Briggs et al. (1983) using turkey erythrocytes. These investigators found that when the solubilized N_s protein was quantitated by labeling with [^{32}P]NAD$^+$ and cholera toxin, desensitization resulted in a significant reduction of the ability of N_s to reconsititute enzyme activity in S49 *cyc*$^-$ membranes. These observations suggest that, at a minimum, heterologous desensitization of avian erythrocytes is associated with a functional modification of the guanine nucleotide regulatory protein N_s.

Investigations of heterologous desensitization in mammalian cells have also indicated that functional modifications of N_s occur. Kassis and Fishman (1982) demonstrated that treatment with prostaglandin E_1 (PGE_1) induced heterologous desensitization in human fibroblast cells and that N_s solubilized from these cells was less efficient in reconstituting adenylate cyclase activity in S49 *cyc*$^-$ membranes when compared to controls. Heterologous desensitization induced by prostaglandin E in liver (Garrity et al., 1983) and by human chorionic gonadotropin in ovaries (Kirchik et al., 1983) also results in impaired functionality of N_s, as determined with *cyc*$^-$ reconstitution. In contrast, Rich et al. (1984) have reported that glucagon-induced heterologous desensitization in MDCK cells was not associated with alterations in the reconstitutive ability of N_s, but instead involved increases in the apparent levels of the inhibitory guanine nucleotide regulatory protein, N_i. This novel finding suggests that alterations in the N_i/N_s stoichiometry may be one mechanism by which heterologous desensitization occurs.

Alterations in Receptor Function

Although heterologous desensitization results in impaired N_s functionality, this does not rule out other potential lesions in the adenylate cyclase system. In fact, the observation that catecholamine-stimulated adenylate cyclase activity in avian erythrocytes is desensitized by about 50%, whereas the fluoride ion- and guanine nucleotide-stimulated activities are reduced by only 10–20% after agonist-induced desensitization, is indicative of other processes that occur as well. Stadel et al. (1982b) first provided evidence for this possibility by demonstrating the β-adrenergic receptor peptides, as detected by photoaffinity labeling and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were of larger apparent molecular weight after desensitization of turkey erythrocytes. This indicated that the desensitization process coincided with a stable structural modification of the receptor.

Additional evidence for a covalent/functional modification of the receptor protein has been obtained by partially purifying the β-adrenergic receptors from desensitized turkey erythroctyes, reconstituting them into phospholipid vesicles, and fusing them with *Xenopus laevis* erythrocyte acceptor cells that possess N_s and C, but no β-adrenergic receptors. It was shown that the β-adrenergic receptors from desensitized cells were less efficient than those from control cells in reconstituting catecholamine responsiveness of the *X. laevis* adenylate cyclase activity (Strulovici et al., 1984).

β-Adrenergic Receptor Phosphorylation

Using [^{32}P]orthophosphate incorporation, it was subsequently shown that during the heterologous desensitization process in turkey erythrocytes, the β-adrenergic receptor undergoes phosphorylation (Stadel et al., 1983a). Sibley et al. (1984b) have investigated this phosphorylation process in detail. The β-adrenergic receptor in turkey erythrocytes is stoichiometrically phosphorylated under basal conditions containing 0.7–1.0 mol phosphate/mol receptor, with this stoichiometry increasing to 2–3 mol/mol upon maximal desensitization (Sibley et al., 1984b). Figure 2 shows an experiment comparing the phosphorylated receptor peptides from control and isoproterenol-desensitized cells. This phosphorylation occurs exclusively on serine residues in the receptor. We have also shown that the phosphate/receptor stoichiometry is tightly correlated with the degree of desensitization (Sibley et al., 1984b). For instance, Fig. 3 shows the dose–response relationship for isoproterenol-induced receptor phosphorylation and adenylate cyclase desensitization. As can be seen, between 10^{-9} and $10^{-6}M$ isoproterenol promotes desensitization of adenylate cyclase in parallel with receptor phosphorylation. In addition, the time courses for receptor phosphorylation and adenylate cyclase desensitization are identical, as are the rates of resensitization and the return of the phosphate/receptor stoichiometry to control levels (Sibley et al., 1984b). Moreover, incubation of the cells with cyclic AMP analogs causes *submaximal* phosphorylation of the β-adrenergic receptor, which is correlated with the *partial* desensitization of adenylate cyclase that these analogs evoke (Sibley et al., 1984b). These data thus indicate that in turkey erythrocytes, heterologous desensitization is tightly correlated with phosphorylation of the β-adrenergic receptor.

Additional evidence for a role of receptor phosphorylation in avian erythrocyte desensitization has come from experiments using a cell-free model system. Nambi et al. (1984, 1985) demonstrated that catecholamine-induced desensitization occurred in turkey erythrocyte lysates and that this process required added ATP, Mg^{2+}, and factor(s) present in the cytoplasmic fraction of the cell. If [γ-^{32}P]ATP was addi-

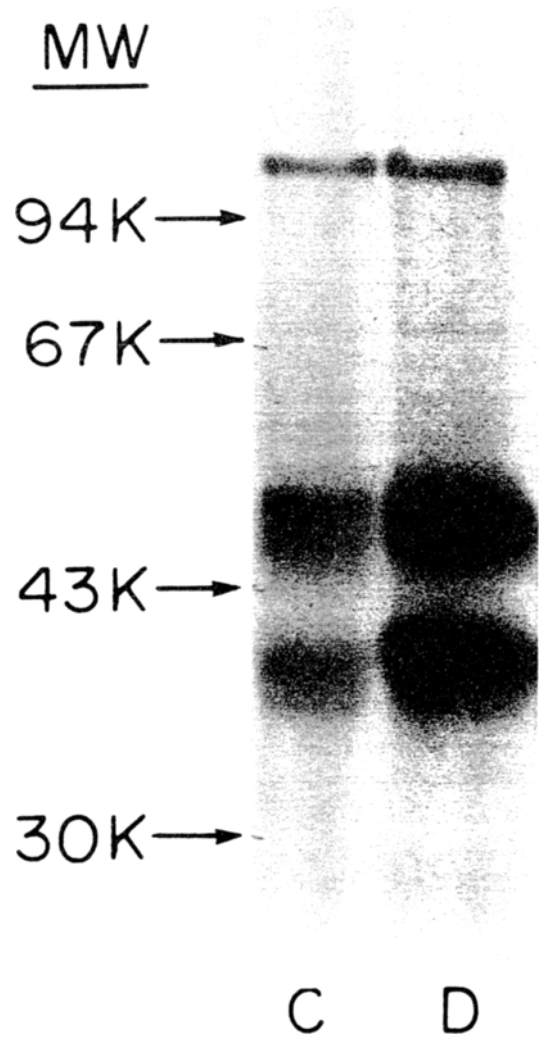

Fig. 2. SDS-PAGE of ^{32}P-labeled β-adrenergic receptor peptides from control and isoproterenol-desensitized turkey erythrocytes. The two major bands seen at 40 and 50 kdalton represent the turkey erythrocyte β-adrenergic receptor peptides. Lane C shows the receptor peptides from control cells, whereas Lane D represents those from desensitized cells.

tionally utilized, then isoproterenol could induce, in a stereospecific fashion, phosphorylation of the β-adrenergic receptor that was similar to that observed using intact cells.

Cyclic AMP-Dependent Protein Kinase

The observation that cyclic AMP analogs can partially reproduce the catecholamine-induced receptor phosphorylation and desensitization

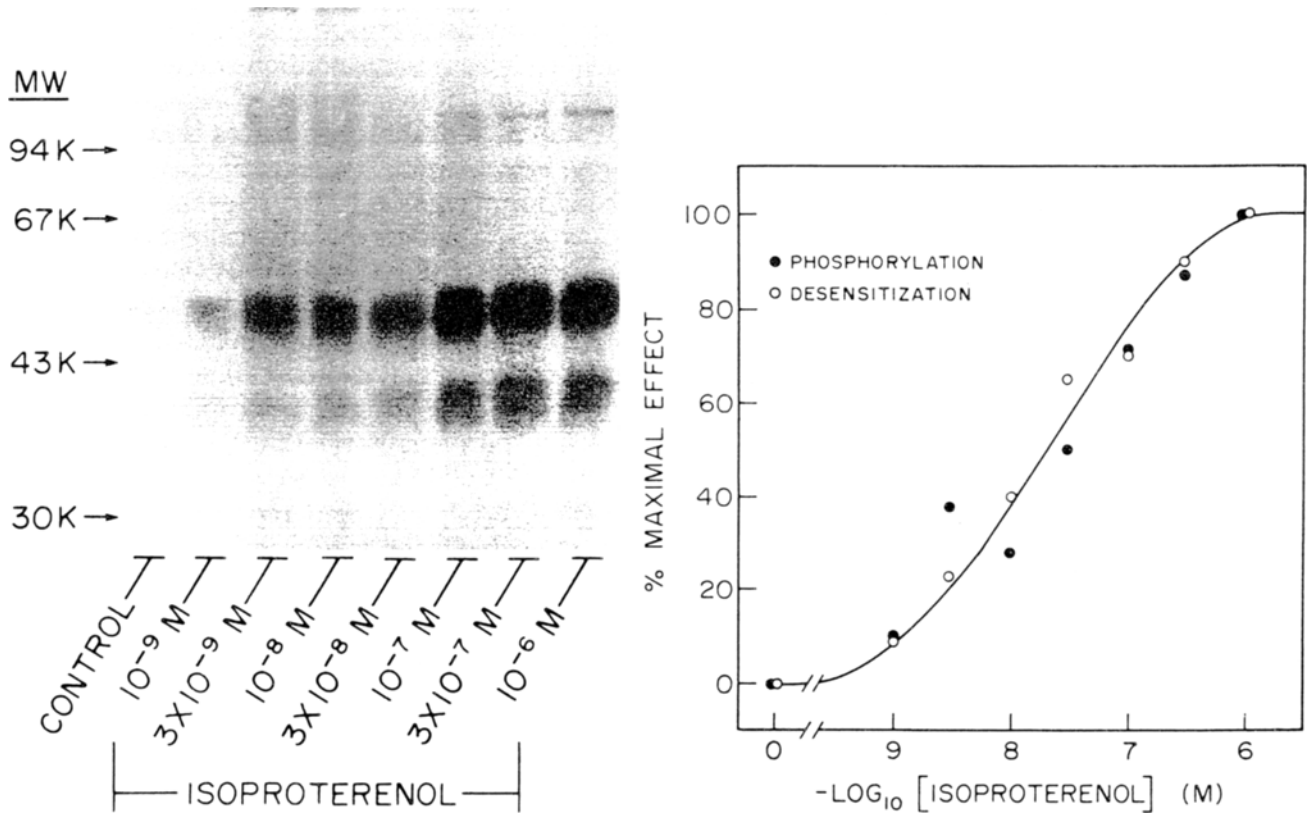

Fig. 3. Dose–response relationship for isoproterenol-induced receptor phosphorylation and adenylate cyclase desensitization. (A) Individual aliquots of ^{32}P-labeled cells were incubated with the indicated concentrations of isoproterenol prior to receptor purification and gel electrophoresis. (B) The phosphorylation data is expressed as an increase in the stoichiometric ratio of mol phosphate/mol receptor over the control sample. The adenylate cyclase data is expressed as a percentage of the maximal desensitization of the isoproterenol-stimulated enzyme activity.

in turkey erythrocytes suggests that cyclic AMP-dependent protein kinase is, at least, partially involved in the heterologous desensitization.

Benovic et al. (1985) have, in fact, recently demonstrated a cAMP-mediated phosphorylation of mammalian lung β-adrenergic receptor that is similar to the process exhibited by avian erythrocytes. Using pure lung receptor and pure cAMP-dependent protein kinase, it was shown that isoproterenol could enhance the rate of receptor phosphorylation by about twofold, which occurred on serine residues. Reconstitution of the phosphorylated receptor with the N_s protein demonstrated diminished agonist-promoted, receptor-mediated stimulation of GTPase activity compared with controls. Thus, the cAMP-dependent protein kinase-promoted phosphorylation is apparently functionally significant.

Protein Kinase C

An interesting question concerning heterologous desensitization is why incubation of cells with cAMP analogs produce only partial effects (Sibley et al., 1984b). One intriguing

hypothesis, suggested by the studies of Benovic et al. (1985), is that in order to obtain maximal phosphorylation of the β-adrenergic receptor, agonist occupancy must occur. Another possibility is that the receptor phosphorylation is not completely mediated by cyclic AMP and that other protein kinase systems may phosphorylate the β-adrenergic receptor as well. In this regard, we (Sibley et al., 1984a) and others (Kelleher et al., 1984) have recently shown that tumor-promoting phorbol esters, compounds that potently activate protein kinase C, are capable of stimulating β-adrenergic receptor phosphorylation concomitantly with adenylate cyclase desensitization. Interestingly, in duck erythrocytes (Sibley et al., 1984a) the phorbol ester-induced receptor phosphorylation is nonadditive with that produced by isoproterenol, suggesting a common mechanism or pathway of action (Fig. 4).

Recently, protein kinase C has been shown to directly phosphorylate the purified β-adrenergic receptor in vitro, albeit to a lower stoichiometry than that of the cAMP-dependent protein kinase (Bouvier et al., 1987). The phosphorylation by protein kinase C occurs on serine residues and is *not* enhanced by agonist occupancy of the receptor. Importantly, the sites on the receptor that are phosphorylated by protein kinase C and the cAMP-dependent protein kinase appear to be identical, as determined by peptide-mapping techniques (Bouvier et al., 1987).

Recent molecular cloning and sequence analysis of the β_2-adrenergic receptor (Dixon et al., 1986) has further elucidated the relationship of the protein kinase C and cAMP-dependent protein kinase phosphorylation sites. Figure 5 depicts the β_2-adrenergic receptor as it may be organized in the plasma membrane. The amino acid sequence was deduced from cDNA clones and direct protein sequencing, whereas putative transmembrane helices were assigned based on hydropathicity analysis (Dixon et al., 1986). As indicated in Fig. 5, there are two sites on the receptor consisting of the general amino acid sequence, Arg–Arg–X–Ser, which represent a concensus recognition site for the cAMP dependent protein kinase (Glass and Krebs, 1980). Both of these sequences contain a basic amino acid (lysine) either at or close to the carboxy-terminal side of the relevant serine residue, which renders this an excellent recognition site for protein kinase C as well (Kishimoto et al., 1985).

It thus appears as if protein kinase C and the cAMP-dependent protein kinase both phosphorylate the β-adrenergic receptor on identical sites. The phosphorylation by the cAMP-dependent protein kinase is enhanced by receptor agonist occupancy and represents a classical negative feedback regulatory loop. Phosphorylation of the β-adrenergic receptor by protein kinase C may represent a "cross-talk" pathway whereby receptor systems that stimulate phosphatidylinositol turnover and activate protein kinase C can negatively modulate adenylate cyclase-coupled receptors. In support of this hypothesis is the observation that muscarinic receptor agonists have been shown to promote β-adrenergic receptor desensitization in the heart, where muscarinic receptors are coupled to phosphatidylinositol hydrolysis (Limas and Limas, 1985).

Mechanisms of Heterologous Desensitization

Heterologous desensitization is thus associated with modifications in the adenylate system at both the level of the receptor and guanine nucleotide regulatory proteins (Fig. 6). It should be pointed out that structural and/or functional alterations in the enzyme catalytic unit cannot be excluded, although there is no evidence for this at present.

It seems reasonable to propose that the mechanisms of heterologous desensitization elucidated in erythrocytes are not unique to this

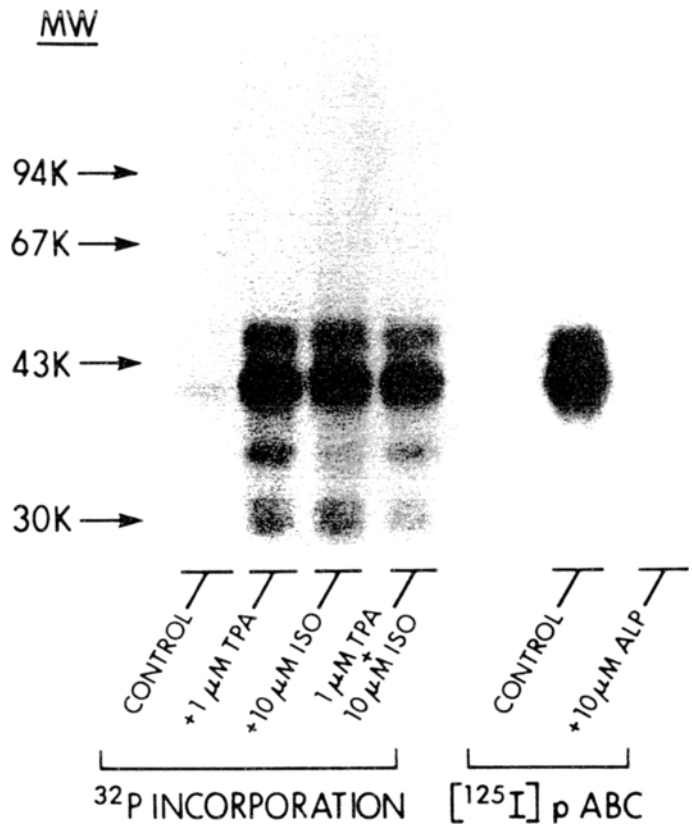

Fig. 4. SDS-PAGE of ^{32}P-labeled and [^{125}I]para-azidobenzylcarazolol ([^{125}I]pABC)-labeled β-adrenergic receptor peptides from duck erythrocytes. The two major peptides of molecular mass 40 and 48 kdalton represent the β-adrenergic receptor peptides in duck erythrocyte membranes, as indicated in the right-hand panel visualized through covalent incorporation of the photoaffinity probe [^{125}I]pABC. For ^{32}P labeling, the cells were preincubated with ^{32}Pi prior to desensitization with either the phorbol diester, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), isoproterenol (ISO), or both TPA and ISO.

cell type. Thus, one major means of achieving heterologous desensitization would be modification(s) in the N_s or N_i proteins, as discussed above. The nature of these modifications is not yet known, although an attractive hypothesis is that a phosphorylation event is somehow involved. Interestingly, Zick et al. (1986) have recently shown that the α subunit of transducin, a retinal, guanine nucleotide-binding regulatory protein, is capable of undergoing multisite phosphorylation. Although in some heterologous desensitization systems, cAMP seems to induce the N_s/N_i modification (Simpson and Pfeuffer, 1980; Stadel et al., 1981), in others it seems not to be involved (Rich et al., 1984; Noda et al., 1984).

Another major pathway of heterologous desensitization is modification of the receptor proteins. In this case the effect is not receptor sequestration or down-regulation, as in homologous desensitization (*see below*), but a functional uncoupling of the receptor from adenyl-

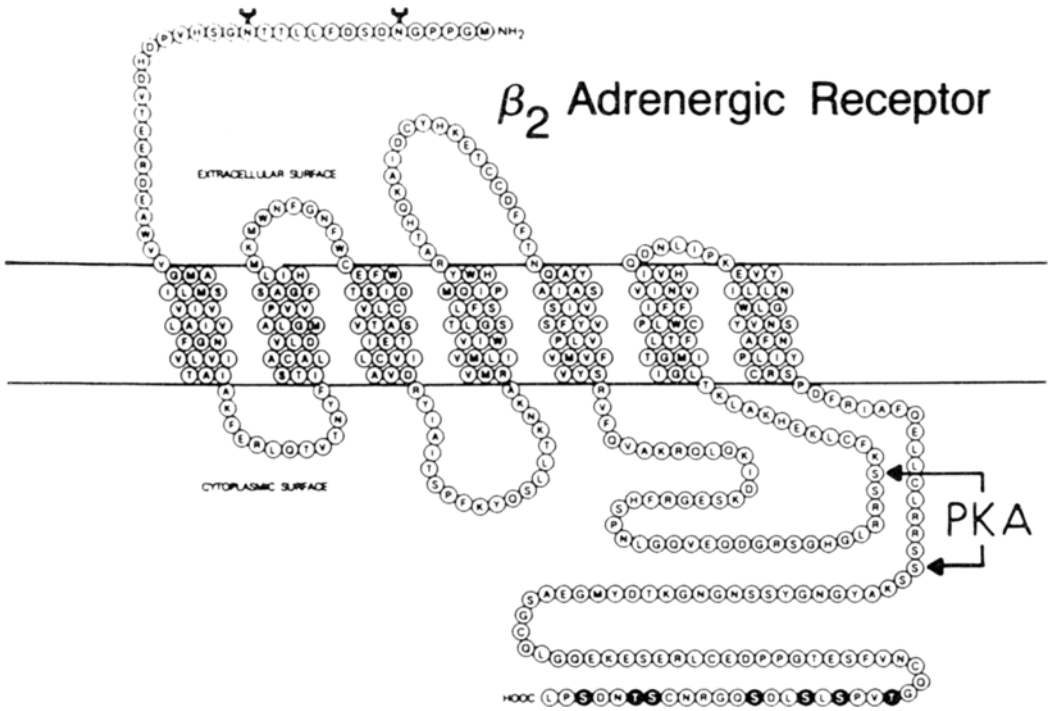

Fig. 5. Structure of the β-adrenergic receptor as it may be organized in the plasma membrane. The sites of phosphorylation of the β-adrenergic receptor by the cAMP-dependent protein kinase (and probably protein kinase C) are indicated. The solid circles represent the serine and threonine rich region in the carboxy terminus, which may serve as sites of βARK phosphorylation.

ate cyclase. Evidence from avian erythrocytes indicates that phosphorylation is the probable mechanism for producing the functional modification. Moreover, because of the heterologous nature of the desensitization it is reasonable to suppose that all the receptors coupled to adenylate cyclase would probably be modified in this way, that is, phosphorylated.

It is likely that differing extents of receptor and N_s/N_i modifications occur in different cell types and under different conditions. In some cells that exhibit heterologous desensitization the modification in N_s/N_i might predominate. In other cells, including the erythrocytes, modification of the receptor protein may be the most relevant mechanism. Evidence for the latter possibility has come from several systems that exhibit heterologous desensitization in the absence of any alterations in guanine nucleotide- or fluoride ion-stimulated enzyme activities (Rich et al., 1984; Koschel, 1980; Attramadal et al., 1984).

Although this hypothesis is attractive, it must be reconciled with the observation that in some cell types, heterologous desensitization is not observed upon cell lysis, arguing against any stable covalent modifications in the adenylate cyclase system. However, one potential explanation is that any modification is rapidly reversed upon cell lysis, e.g., by phosphatase action. The observation that protein synthesis is required in some cell types for heterologous desensitization is also compatible with our hypothesis. It is possible that in some cells, a mediator of the modification(s) is a rapidly turning-over enzyme.

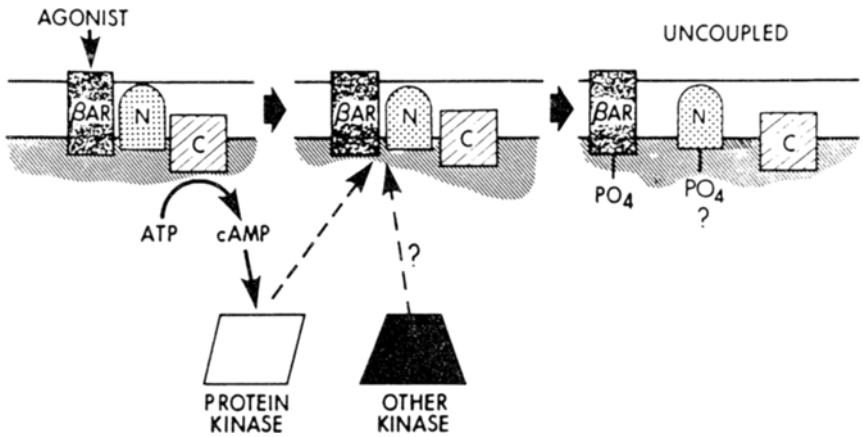

Fig. 6. Heterologous mechanisms of β-adrenergic receptor-coupled adenylate cyclase desensitization.

Homologous Desensitization

Homologous patterns of β-adrenergic receptor desensitization occur widely and have been extensively investigated. The status of the β-adrenergic receptor during homologous desensitization was first investigated in frog erythrocyte membranes after the cells were incubated with isoproterenol under conditions leading to a homologous form of desensitization. There was a decrease in the number of assayable β-adrenergic receptors directly comparable to the decrease in hormone responsiveness (Mukherjee et al., 1975,1976; Mickey et al., 1975, 1976; Wessels et al., 1978,1979). Moreover, the time course of loss of β-adrenergic receptors paralleled the time course of loss of isoproterenol-stimulated adenylate cyclase activity. In contrast, homologous desensitization in cultured mammalian cells exhibits more complex phenomena, consisting of multiple sequential events (Shear et al., 1976; Su et al., 1979, 1980; Harden et al., 1979; Homburger et al., 1980; Fishman et al., 1981). An initial step involves a rapid "uncoupling" of the receptors from the other components of the adenylate cyclase system, which involves sequestration of receptors from the cell surface (*see below*). This can be measured as a loss in hormone-sensitive adenylate cyclase activity and a loss in high-affinity, nucleotide-sensitive agonist binding. A subsequent event involves a down-regulation of receptor number exhibited by a decrease in the ability to detect β-adrenergic receptors with radiolabeled antagonists. These processes seem to occur in all normal cells that exhibit homologous desensitization, although their kinetics can vary considerably. Homologous desensitization is thus associated with alterations occurring at the receptor level of the adenylate cyclase system.

The availability of a variant line (*cyc*$^-$) of the S49 murine lymphoma cell, which lacks the guanine nucleotide regulatory protein N_s and, hence, hormone-sensitive adenylate cyclase activity, has provided an important tool for probing many aspects of receptor-adenylate cyclase function. Using this cell line, Clark and colleagues (Green and Clark, 1981; Green et al., 1981) have shown that agonist occupation of the β-adrenergic receptor in the absence of receptor–N_s coupling or cyclic AMP generation is sufficient to promote desensitization in these cells. Since *cyc*$^-$ cells lack a functional N_s protein, hormone responsiveness can only be tested af-

ter reconstituting native N_s from wild-type cells with *cyc*$^-$ membranes. Catecholamine-stimulated adenylate cyclase activity was decreased when the *cyc*$^-$ membranes were derived from cells that had been preincubated with agonist. Since agonist desensitization of *cyc*$^-$ occurred in the absence of a functional N_s protein or activation of adenylate cyclase, these data suggest the defect to be localized to the β-adrenergic receptor. This makes intuitive sense, since any defect distal to the receptor protein (that is, nucleotide regulatory proteins or catalytic unit) would probably lead to a heterologous form of desensitization. Moreover, these data indicate that homologous desensitization is not cAMP-mediated.

Other results have further suggested that the N_s protein is unaltered during homologous forms of desensitization (Green and Clark, 1981; Iyengar et al., 1981). Such studies have utilized reconstitution of N_s from desensitized cells into acceptor β-adrenergic receptor systems and have demonstrated no impairment of function. Moreover, as noted above, guanine nucleotide- and fluoride ion-stimulated adenylate cyclase activities are unchanged during homologous desensitization, indicating that N_s–C coupling and the catalytic unit are both normal.

Receptor Sequestration

The fact that the locus of the defect in homologous desensitization seems to be the β-adrenergic receptor and the observation that the receptors appear to decrease in number during homologous desensitization suggest that the receptors are perhaps sequestered or internalized away from the cell surface during desensitzation. Chuang and Costa, using frog erythrocytes, initially demonstrated that associated with desensitization and loss of β-adrenergic receptor sites from the plasma membranes was the appearance of receptors in what they defined as the soluble fraction of the cell (Chuang and Costa, 1979; Chuang et al., 1980). This soluble fraction represented a 30,000*g* supernatant that likely contained small membrane particles perhaps containing the receptors. The agonist-promoted appearance of the receptors in this supernatant fraction was blocked by antagonists, as was the desensitization.

Harden and collaborators (Harden et al., 1980; Waldo et al., 1983; Toews et al., 1984,1986) have presented further evidence for a redistribution of β-adrenergic receptors from the plasma membrane fraction into smaller membrane particles during the desensitization process. Using 1321N1 astrocytoma cells, it was shown that after the initial, rapid uncoupling and desensitization of the β-adrenergic receptor and adenylate cyclase, all the receptors were still accessible to ligand binding and could be pelleted at 40,000*g* after lysing the cells. However, in the presence of concanavalin A, a sequestered, light-membrane receptor fraction could be separated from the plasma membranes and the remainder of the receptors and adenylate cyclase on sucrose gradients, indicating a shift in the membrane localization of the receptors. Similar findings have been reported by these authors and others using other cells (Frederich et al., 1983; Hertel et al., 1983a,b; Clark et al., 1985; Kassis and Sullivan, 1986; Kassis et al., 1986).

Using the frog erythrocyte model system, our laboratory has shown that a portion of the desensitized, sequestered receptors can be recovered in a light-membrane fraction obtained by centrifuging the cell cytosol at 158,000*g* for 1 h (Stadel et al., 1983b). This membrane fraction shows markedly diminished activities of typical plasma-membrane marker enzymes, including adenylate cyclase. The receptors in these light-membrane particles appear to be totally uncoupled from the nucleotide regulatory protein and show only low-affinity agonist binding. Reconstitution of cholate-solubilized N_s activity from the membrane particles into S49 *cyc*$^-$ cell

membranes (which lack N_s) shows, in fact, that the sequestered membrane fraction is nearly devoid of this protein component. Further characterization of the sequestered receptors by photoaffinity labeling and SDS-PAGE has indicated that the sequestered receptors are not grossly altered or degraded in any fashion. These findings thus suggest that desensitization in frog erythrocytes is associated with a sequestration of the receptors into a membrane domain or compartment that is devoid of the other components of the adenylate cyclase system.

In frog erythrocytes, as well as other cells, the sequestered receptors can recycle back to the plasma membrane without undergoing degradation, provided that the desensitizing agonist is removed after a short period of time (Strulovici and Lefkowitz, 1984).

Recently, additional evidence for a sequestration event has come from studies with the newly developed radioligand [^{3}H]CGP-12177 (Staehelin et al., 1983; Staehelin and Hertel, 1983). CGP-12177 is a relatively hydrophilic antagonist as compared with the more hydrophobic antagonist radioligands that have been previously used to study β-adrenergic receptors. Because of its relative hydrophilicity [^{3}H]CGP-12177 would be expected to label only the β-adrenergic receptors present on the cell surface when binding assays are performed on intact cells. Hertel and colleagues (Hertel et al., 1983a,b) have shown, using C6 glioma cells, that isoproterenol treatment rapidly induces a loss of β-adrenergic receptors as assessed by [^{3}H]CGP-12177 binding, which parallels the decrease in hormone-stimulated adenylate cyclase activity. However, the total number of receptors as determined by binding of the hydrophobic antagonist radioligand [^{3}H]dihydroalprenolol did not change. Separation of the β-adrenergic receptors on a sucrose gradient showed that the loss of [^{3}H]CGP-12177 binding was related to a movement of the receptors from the plasma membrane to a vesicular cell compartment that was accessible to [^{3}H]dihydroalprenolol, but not to [^{3}H]CGP-12177.

An additional finding that may be related to the agonist-induced sequestration of β-adrenergic receptors is the observation that, as assessed by radioligand-binding studies on intact cells, the affinity of the receptors for agonists declines with time during agonist exposure to intact cells. Pittman and Molinoff initially demonstrated that with L6 muscle cells a low concentration of isoproterenol inhibited [^{125}I]hydroxybenzylpindolol binding during the initial minutes of the binding assay, but did not affect the binding of the radiolabeled antagonist once equilibrium was achieved (Pittman and Molinoff, 1980, 1983). This suggested that the receptor's affinity for the agonist decreased during the incubation. Toews and colleagues (Toews et al., 1983,1984; Toews and Perkins, 1984) have additionally performed agonist competition curves of [^{125}I]pindolol binding on intact astrocytoma cells and demonstrated that, initially, agonists competed with high affinity, but that with time the apparent agonist-binding affinities were decreased.

Preincubation of the cells with agonists converts about half of the β-adrenergic receptors from a form exhibiting high affinity for the agonists to a form with lower apparent affinity. Antagonists do not have this effect. These results have been interpreted to indicate that the low-affinity form of the β-adrenergic receptor observed in such whole cell experiments is related to slow equilibration of the hydrophilic agonists with a population of β-adrenergic receptors that have been internalized because of agonist exposure. Interestingly, this effect occurs in the *cyc*$^-$ variant cell, which lacks a functional N_s protein (Insel et al., 1983; Hoyer et al., 1984). Apparently, the rapid change in affinity of the β-adrenergic receptor for agonists does not require the interaction of the receptor with N_s nor the activation of

adenylate cyclase and generation of cyclic AMP. This is consistent with the data mentioned earlier, that agonist-induced homologous desensitization does not require β-adrenergic receptor–N_s coupling in S49 cells.

The physical mechanism of the receptor sequestration is unknown, although a role for the cellular cytoskeleton has been hypothesized. In this regard, microtuble inhibitors, such as colchicine and vinblastine, and the microfilament disrupter cytochalasin B have been shown to attenuate catecholamine-induced desensitization in several systems (Kurokawa et al., 1979; Simantov et al., 1980; Insel and Koachman, 1982; Limas and Limas, 1983).

A major unanswered question about β-adrenergic receptor sequestration is the precise cellular location of the sequestered receptors. One hypothesis is that the receptors are internalized into cytosolic vesicles via a coated-pit mechanism, as has been shown for peptide hormone receptors (Pastan and Willingham, 1981). The observation that upon lysis of desensitized cells the sequestered β-adrenergic receptors appear to have been translocated into light-membranous particles is consistent with this possibility. However, Strader et al. (1984) have shown, using frog erythrocytes, that the production of these vesicular particles is very much dependent upon the method of cell lysis. When very "gentle" methods of cell lysis were used, the sequestered β-adrenergic receptors were shown to be localized to the plasma membrane fraction. This suggests that in the intact frog erythrocyte the receptors are never present in free-floating cytoplasmic vesicles, but rather are contained within structures that remain contiguous with the inner surface of the plasma membrane. Insel and coworkers have recently reached similar conclusions using the S49 lymphoma cell system (Mahan et al., 1985).

Receptor Down Regulation

In addition to an early desensitization/sequestration event, homologous desensitization in some cells appears to be associated with an actual disappearance of receptors such that they cannot be detected with any radioligand in any cell fraction (Shear et al., 1976; Su et al., 1979,1980; Homburger et al., 1980). This phenomenon has come to be referred to as "down regulation" of the receptors (in the jargon of the field) as opposed to the sequestration of receptors described above. Down regulation appears to occur more slowly than the initial sequestration process in most cell types. The exact fate of these β-adrenergic receptors is not known with certainty. In some cases it seems clear that the receptors are proteolytically degraded and new receptor synthesis is required to regenerate them (Frederich et al., 1983; Morishima et al., 1980; Doss et al., 1981; Hughes and Insel, 1986; Neve and Molinoff, 1986). However, in other cases, β-adrenergic receptor number can return to control levels even in the absence of new protein synthesis (Doss et al., 1981; Su et al., 1976). This suggests that with time these down-regulated receptors can regain their binding activity. Interestingly, in contrast with the sequestration event, β-adrenergic receptor–N_s coupling appears to be required in order for down regulation to occur to a maximal extent. Thus, in S49 *cyc*⁻ and UNC variant cells, which possess lesions in N_s, agonist-induced down regulation of β-adrenergic receptors does not occur or is blunted despite the fact that these cells apparently exhibit the desensitization/sequestration process (Shear et al., 1976; Su et al., 1980; Mahan et al., 1984). This suggests that the down regulation involves an N_s-mediated targeting of the receptors through an, as yet, undefined mechanism.

Although β-adrenergic receptor–N_s coupling appears to be necessary for down regulation to occur, activation of adenylate cyclase and the generation of cyclic AMP may not be required. Experiments with HC-1 hepatoma cells that possess β-adrenergic receptors and N_s, but not a functional catalytic unit, demonstrated that isoproterenol pretreatment could produce a loss of high-affinity agonist binding and a down regulation of hydrophobic antagonist ([^{125}I]hydroxybenzylpindolol) binding (Su et al., 1980). Additionally, the S49 variant cell *kin*$^-$, which lacks cyclic AMP-dependent protein kinase activity, exhibits desensitization and down regulation of β-adrenergic receptors in response to catecholamine exposure, indicating that this kinase is not involved in these processes (Shear et al., 1976; Mahan et al., 1984).

Although in most cell types cyclic AMP does not appear to be involved in homologous desensitization, it should be noted that prolonged (12–24 h), nonhormonally induced elevation of cyclic AMP levels in some cells has been observed to produce a partial down regulation of the β-adrenergic receptors (Moylan et al., 1982; Bobik and Little, 1984; Zaremba and Fishman, 1984; Fishman et al., 1985). The physiological significance of this finding is unclear, however, since catecholamines could not maintain prolonged elevated cyclic AMP levels because of the relatively rapid desensitization process in most cells.

Alterations in Receptor Function

Homologous desensitization thus seems to be composed of multiple, related events serving to sequester or remove the β-adrenergic receptor from its normal location in the plasma membrane. Although the receptor is eventually sequestered from N_s and C during the desensitization process, a central question relates to whether the receptor is functionally modified as well. Perkins and colleagues (Waldo et al., 1983; Toews et al., 1984; Hertel et al., 1986) have presented evidence that very short-term exposure of cells to isoproterenol leads to uncoupling of the β-adrenergic receptor and desensitization of adenylate cyclase stimulation *prior* to receptor sequestration. Moreover, pretreatment of astrocytoma cells with concanavalin A blocks the sequestration process in these cells, yet does not block the desensitization and uncoupling events that occur maximally (Waldo et al., 1983; Toews et al., 1984). These data suggest that at least early in the desensitization process there is a functional change that occurs in the receptor.

In addition, Fishman and colleagues, using reconstitution and membrane-fusion techniques, have demonstrated that catecholamine-induced homologous desensitization in various cultured cells results in a functional alteration of the β-adrenergic receptor (Kassis and Fishman, 1984; Kassis et al., 1986). In contrast, Strulovici et al. (1983), using similar fusion techniques, have tested the activity of the *sequestered* receptors from desensitized frog erythrocytes and showed that they were functionally active. Similar conclusions were reached by Clark et al. (1985) in characterizing the sequestered receptors found in S49 cells. In order to resolve this apparent discrepancy, we purified β-adrenergic receptors that were derived largely from the cell surface of control and desensitized frog erythrocytes and tested their functional activity using a reconstitution assay (Sibley et al., 1986a). Figure 7 shows that the β-adrenergic receptors purified from the desensitized cells are functionally impaired in their ability to couple with the N_s-regulatory protein and stimulate its GTPase activity. It thus appears that, at least in frog erythrocytes, homologous desensitization does indeed involve a functional alteration of the β-adrener-

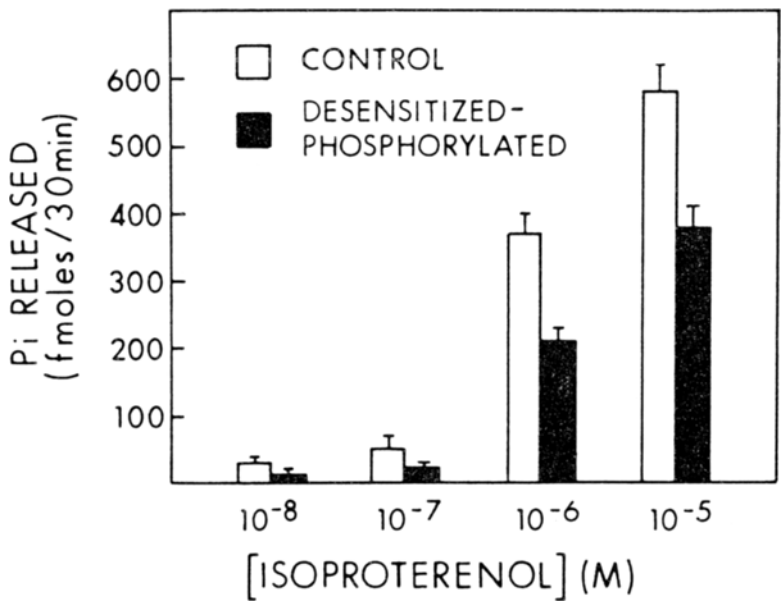

Fig. 7. GTPase activity of phospholipid vesicles containing N_s and β-adrenergic receptors purified from control or desensitized cells. Frog erythrocytes were incubated in the absence (control) or presence of isoproterenol (desensitized) prior to receptor purfication and reconstitution with N_s. Equal amounts of control and desensitized receptor were reconstituted. GTPase activity, as a function of the isoproterenol concentration, was then assessed.

gic receptor on the cell surface, but that this modification may be reversed upon sequstration of the receptor within the cell (*see below*).

β-Adrenergic Receptor Phosphorylation

Intact Cell Studies

The mechanisms by which agonists promote homologous desensitization of the β-adrenergic receptor also appear to involve receptor phosphorylation. Evidence for this was first obtained by Sibley et al. (1985) using frog erythrocytes, in which β-adrenergic agonists were shown to promote receptor phosphorylation in conjunction with homologous desensitization (Fig. 8). The agonist-induced receptor phosphorylation is pharmacologically specific and stoichiometric, occurring to about 2 mol phosphate/mol receptor. The phosphorylation reaction temporally coincides with the receptor sequestration and desensitization processes (Sibley et al., 1986a). Prostaglandin E_1 does not promote β-adrenergic receptor phosphorylation, although this hormone elevates cAMP levels in these cells. This suggests that the observed receptor phosphorylation is not mediated by the cAMP-dependent protein kinase. Additional evidence that this kinase is not involved in homologous-induced receptor phosphorylation has come from studies using S49 lymphoma cells (Strasser et al., 1986b). It was observed that in the S49 mutant cell lines *cyc*$^-$ and *kin*$^-$, which are deficient in the N_s protein and cAMP-dependent protein kinase, respectively, agonists promoted β-adrenergic receptor phosphorylation to the same degree as in

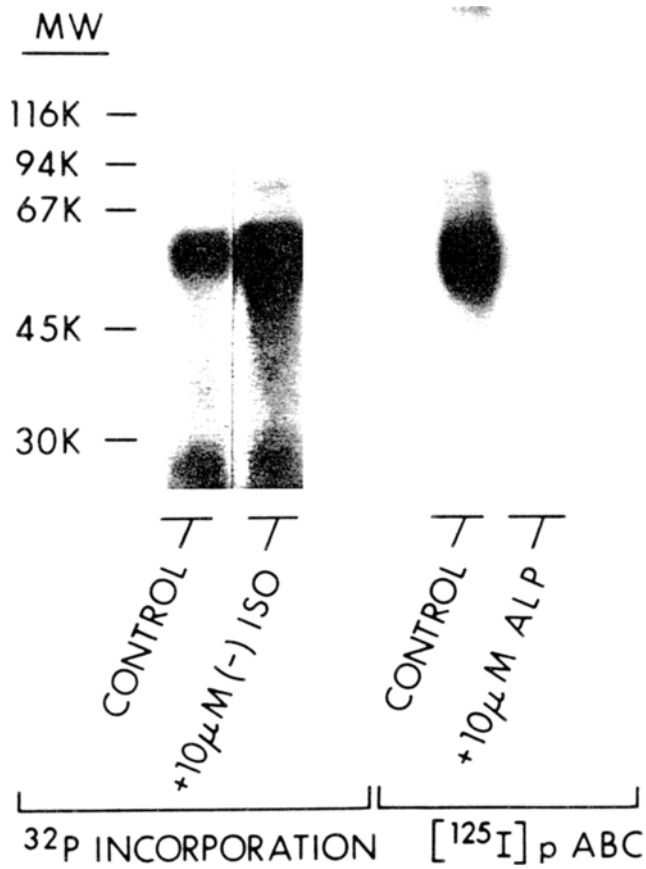

Fig. 8. SDS-PAGE of ^{32}P-labeled and [^{125}I]pABC-labeled β-adrenergic receptors from frog erythrocytes. For ^{32}P labeling, the cells were incubated with ^{32}Pi prior to homologous desensitization with isoproterenol. For [^{125}I]pABC labeling, frog erythrocyte membranes were incubated with [^{125}I]pABC in the absence (control) or presence of alprenolol (+10 μ*M* ALP) to block incorporation.

wild-type cells. These results indicate that: (1) Receptor–N_s coupling is not necessary for receptor phosphorylation to occur—agonist occupancy is sufficient, and (2) the receptor phosphorylation is not mediated by the cAMP-dependent protein kinase.

β–Adrenergic Receptor Kinase

Recently, Benovic et al. (1986a,b) have identified a novel cAMP-independent kinase that seems to be involved in homologous desensitization and phosphorylation of the β-adrenergic receptor. Termed the β-adrenergic receptor kinase (or βARK), it is ubiquitous in mammalian tissues thus far examined and appears to be cytosolic. It has the unique property that it only phosphorylates the agonist-occupied form of the receptor. The unoccupied or antagonist-occupied receptor is not a substrate for the enzyme. β-Adrenergic receptor kinase is distinct from other kinases, such as the cAMP-, Ca^{2+}/calmodulin-, and Ca^{2+}/phospholipid-dependent protein kinases. Moreover, βARK does not phosphorylate such general kinase substrates as casein or histones.

When a cell is stimulated by a β-adrenergic agonist, βARK is translocated from the cytosol to the plasma membrane, where it phosphorylates the agonist-occupied receptor (Strasser et al., 1986a).

When a cell containing both prostaglandin E_1 (PGE_1) receptors and β-adrenergic receptors, both coupled to adenylate cyclase, is subjected to homologous desensitization with PGE_1, βARK can be shown to be translocated from the cytosol to plasma membrane (Strasser et al., 1986a). Conversely, occupancy of α_1-adrenergic receptors, which are coupled to the phosphatidylinositol cycle, does not lead to βARK translocation. These findings suggest that, in reality, βARK may be a general adenylate cyclase-coupled receptor kinase. The current unavailability of purified preparations of other adenylate-cyclase-coupled receptors, however, precludes direct testing of this hypothesis at present.

The sites of phosphorylation on the β-adrenergic receptor by βARK are currently not known. It can be seen in Fig. 5, however, that the carboxy terminal cytoplasmic tail of the receptor is rich in serine and threonine residues, which may serve as recognition sites by βARK. This hypothesis is currently being investigated.

The suggestion that multiple adenylate cyclase-coupled receptors might be able to serve as substrates for a single receptor kinase (βARK) raises a provocative question, since receptor phosphorylation catalyzed by this enzyme is postulated to lead to *homologous* desensitization. How could covalent modification of many receptors by the same enzyme lead to the agonist-specific pattern of refractoriness observed in homlogous desensitization? The key regulatory feature of this system is that only the agonist-occupied receptor is a substrate for the enzyme. Thus, assuming that all adenylate cyclase-coupled receptors share a common domain bearing the sites phosphorylated by βARK, then this domain must be exposed only after agonist occupancy of the receptors induces crucial conformational changes. As a result, only those receptors that are agonist occupied, i.e., activated, will be substrates for the enzyme. Unoccupied receptors will not be modified. This elegant mechanism then seems to intimately couple the processes of receptor activation and desensitization.

Dephosphorylation and Resensitization

The observation that homologous desensitization results in a functional modification of the cell-surface β-adrenergic receptors yet the sequestered receptors appear functionally normal, suggests that dephosphorylation of the sequestered receptors might occur. We directly tested this hypothesis by purifying the sequestered receptors from ^{32}P-labeled, desensitized frog erythrocytes (Sibley et al., 1986a). Figure 9 shows that the phosphate content of the desensitized sequestered receptors (Lane 3) is much less than that of the desensitized receptors derived from the cell surface (Lane 2). In fact, the sequestered receptors exhibit a phosphate content similar to that of the receptors purified from control cells (Fig. 9, Lane 1). We have further documented that high levels of a β-adrenergic receptor phosphatase activity appears to be associated with the sequestered vesicle membrane fraction (Sibley et al., 1986a). This phosphatase might represent a specific receptor phosphatase or a previously unrecognized activity of one of the known protein phosphatases. The dephosphorylation reaction occurring within the sequestered membrane compartment may functionally regenerate the receptor and allow it to recycle back to the plasma membrane.

In summary, the molecular events proposed to be involved in homologous desensitization of the β-adrenergic receptor are depicted in Fig.

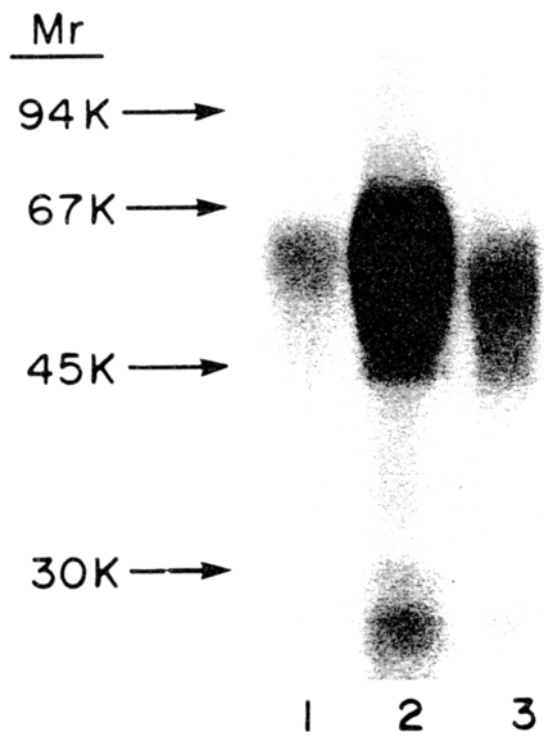

Fig. 9. SDS-PAGE of ^{32}P-labeled β-adrenergic receptors from control and desensitized frog erythrocytes. Lane 1 shows a receptor preparation from control cells. Lane 2 shows a receptor preparation that contains the plasma membrane receptors from desensitized cells. Lane 3 shows the receptors that were purified from the sequestered vesicle membranes in desensitized cells. Equal amounts of receptors were loaded on each gel lane.

10. Agonist occupancy results in the translocation of βARK from the cytosol to the plasma membrane through an as yet to be defined mechanism. Subsequently, the receptors become phosphorylated and functionally uncoupled from adenylate cyclase activation. Shortly thereafter, the receptors become sequestered or internalized within the cells into an undefined intracellular vesicular compartment. Within this sequestered compartment the receptors become dephosphorylated and functionally regenerated. High levels of a β-adrenergic receptor phosphatase activity appear to be associated with the sequestered vesicle compartment. When the agonist is removed or its concentration decreased sufficiently, the receptors can redistribute back to their basal levels in the plasma membrane.

Homologies with Other Receptor Systems

It has become increasingly apparent that diverse biological phenomena are characterized by adaptive processes analogous to the

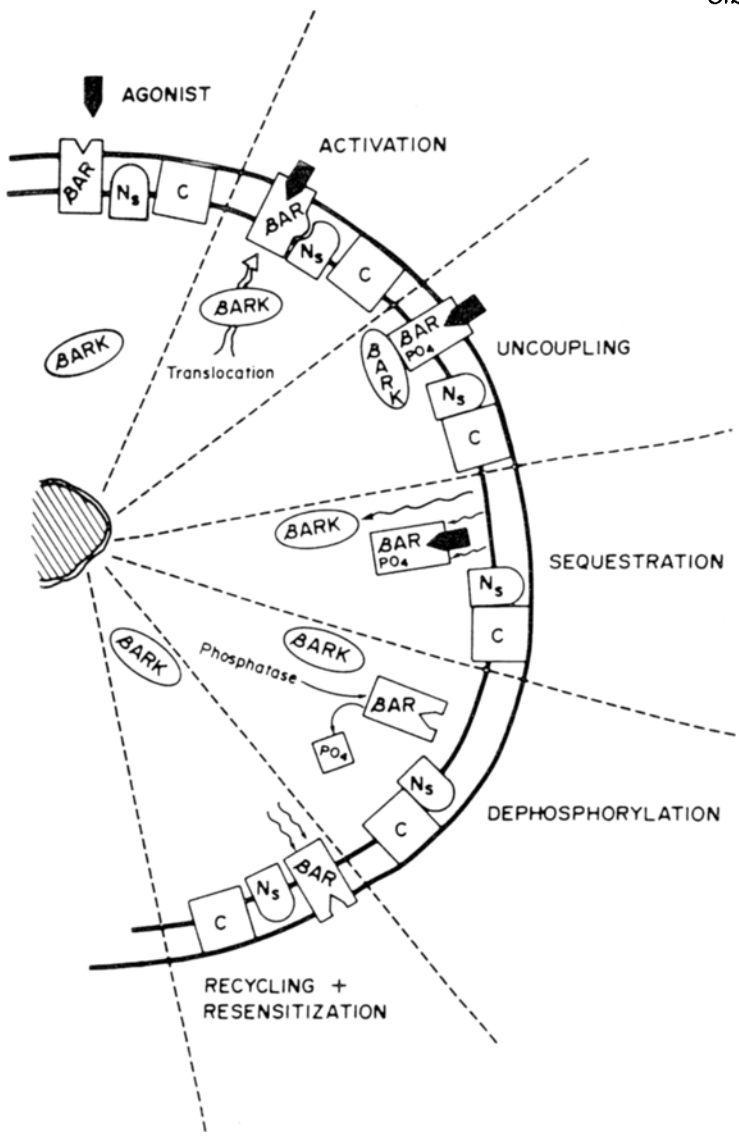

Fig. 10. Homologous desensitization of β-adrenergic receptor-coupled adenylate cyclase.

desensitization observed in β-adrenergic receptor-coupled adenylate cyclase systems. Not surprisingly, many of the other adenylate cyclase-coupled receptors display desensitization phenomena qualitatively similar to those described above. Thus far, little information is available concerning the molecular mechanisms involved. It will be of interest to see if these are identical to those uncovered for the β-adrenergic receptors.

A variety of receptors coupled via guanine nucleotide regulatory proteins to phosphatidylinositol (PI) turnover also demonstrate agonist-induced desensitization and in some cases down regulation. These include receptors for catecholamines (α_1-adrenergic), acetylcholine (muscarinic cholinergic), vasopressin, and angiotensin, among others. Phorbol esters mimic the actions of these agents by activating protein kinase C, as does diacylglycerol, ordinarily generated by the agonist stimulation of phospholipase C. It has been recently demonstrated that treatment of cultured, smooth-muscle cells with phorbol es-

ters leads to desensitization of the α_1-adrenergic receptor-promoted PI turnover response (Cotecchia et al., 1985; Leeb-Lundberg et al., 1985). More recently it has been shown that α_1-adrenergic agonists also promote α_1-adrenergic receptor desensitization, sequestration, and phosphorylation, all of which are temporally correlated (Leeb-Lundberg et al., 1987). Unlike the situation for the adenylate cyclase-coupled β-adrenergic receptors, there is as yet no evidence for specific receptor kinases involved in these phosphorylation reactions. In fact, based on peptide mapping and in vitro phosphorylation experiments, it appears that protein kinase C is itself responsible for phosphorylating the α_1-adrenergic receptors (Bouvier et al., 1987). α_1-Adrenergic agonists increase the rate, but not the extent of this phosphorylation, by analogy with the situation for cAMP-dependent protein kinase phosphorylation of the β-adrenergic receptor. Thus, a classical feedback loop may operate whereby diacylglycerol, generated by agonist stimulation of PI turnover, activates protein kinase C, which phosphorylates and desensitizes the α_1-adrenergic receptor, thus dampening the response. The cAMP-dependent kinase will also phosphorylate the α_1-adrenergic receptor, although agonists do not promote this reaction (Bouvier et al., 1987). The functional significance, if any, of this phosphorylation is presently unknown.

The situation for the muscarinic cholinergic receptor seems quite analogous to that of the α_1-adrenergic receptor. Stimulation of several cell types with either cholinergic agonists or phorbol esters leads to desensitization of muscarinic receptor-mediated stimulation of phosphatidylinositol hydrolysis and sequestration of muscarinic receptors away from the cell surface (Masters et al., 1985; Orellana et al., 1985; Harden et al., 1985; Liles et al., 1986). Specific muscarinic cholinergic desensitization of muscarinic receptors coupled to inhibition of adenylate cyclase has also been observed (Green and Clark, 1982). Moreover, in cardiac tissue it has recently been demonstrated that a cholinergic agonist causes a 10-fold increase in phosphorylation of the muscarinic receptor (Kwatra and Hosey, 1986). The nature of the kinase(s) involved in these regulatory events remains to be determined.

An additional analogy may be drawn with a mechanism of light adaptation recently elucidated for the rhodopsin-coupled effector system in the rod outer segments of the retina. As reviewed in detail elsewhere (Stryer, 1986) this system is structurally and functionally analogous to hormone-responsive adenylate cyclase (Dixon et al., 1986; Dohlman et al., 1987). The analogous components are a photon of light instead of hormone, rhodopsin instead of receptor, a GTP-binding and hydrolyzing protein termed transducin instead of N_s, and a cGMP phosphodiesterase, which controls retinal concentrations of cGMP, instead of the adenylate cyclase enzyme. Rhodopsin undergoes a phosphorylation reaction that is catalyzed by a highly specific kinase termed rhodopsin kinase (Kuhn et al., 1973; Bownds et al., 1972). Phosphorylation by rhodopsin kinase requires the bleached form of rhodopsin as its substrate, that is, the form that has interacted with the "agonist" light. Phosphorylation of rhodopsin occurs on multiple serine and threonine residues clustered within the carboxy terminus of rhodopsin (Hargrave et al., 1980) and results in an impaired ability of rhodopsin to interact with transducin. Thus, this system is desensitized by an agonist-promoted phosphorylation reaction that uncouples the receptor from its GTP-binding regulatory protein. Although phosphorylation of rhodopsin will reduce its interaction with transducin, an additional protein is required for complete uncoupling of this system. This protein is referred to as "48K" protein, S antigen, or arrestin, which binds to phosphorylated rhodopsin (Wilden et al., 1986) and physically prevents the interaction of rhodopsin with

transducin. Given the striking analogies between the rhodopsin and β-adrenergic receptor systems, a fascinating hypothesis is that there is an analog of the "48K" protein in the adenylate cyclase system whose action is necessary to express the functional effects of βARK phosphorylation on the receptor.

The picture that emerges from these findings is of a variety of pathways by which receptors might be regulated. There are classical feedback regulatory loops, such as the cAMP-dependent protein kinase phosphorylation of the β-adrenergic receptor or protein kinase C phosphorylation of the α_1-adrenergic receptor. The rates of these reactions are enhanced by agonist occupancy of these receptors. "Cross-talk" pathways also exist by which, for example, protein kinase C can phosphorylate the adenylate cyclase-coupled β-adrenergic receptor or cAMP-dependent protein kinase the α_1-adrenergic receptor. These reactions are not promoted by agonist occupancy of the substrate receptor, and their physiological regulatory significance is less clear. Finally, there exist specific receptor kinases, such as rhodopsin kinase and βARK, which phosphorylate and "desensitize" particular receptors in a completely agonist-dependent fashion. Such kinases potentially lead only to agonist-specific or homologous desensitization since crucial agonist-promoted conformational changes in the receptor are required to transform them into substrates for the regulatory enzyme.

Amplification of Adenylate Cyclase Activity By Protein Kinase C

It has recently become apparent that β-adrenergic receptor-coupled adenylate cyclase is regulated by amplification as well as by desensitization events. Evidence for this was first obtained from experiments that involved incubating cells with phorbol esters, compounds that potentialy activate protein kinase C (Nishizuka, 1984), and subsequently examining the effects on adenylate cyclase activity. Activation of protein kinase C with phorbol esters leads to enhanced basal adenylate cyclase activity as well as that stimulated by various hormonal and nonhormonal effectors (Sibley et al., 1986b; Bell et al., 1985; Cronin and Canonico, 1985; Sulakhe et al., 1985; Hollingsworth et al., 1986; Johnson et al., 1986). This amplification effect has also been observed by directly adding purified protein kinase C to adipocyte membranes (Naghshineh et al., 1986).

Of all the cell systems thus far examined, the most dramatic effects have been demonstrated in the frog erythrocyte (Sibley et al., 1986b). Figure 11A shows that incubation of these cells with a phorbol ester results in a 100–300% amplification of β-adrenergic agonist-, prostaglandin-, guanine nucleotide-, as well as forskolin- and Mn^{2+}-stimulated enzyme activities. The fact that the forskolin- and Mn^{2+}-stimulated activities are increased is especially provocative since these agents are known to directly stimulate the catalytic unit of adenylate cyclase. This might suggest that the catalytic unit undergoes a stable protein kinase C-induced modification that results in the increased activity. In fact, we have recently demonstrated that in frog erythrocytes, phorbol ester activation results in stoichiometric phosphorylation of the catalytic unit of adenylate cyclase (Yoshimasa et al., 1987). Figure 11B shows that under basal conditions there is virtually no phosphate incorporated into the catalytic unit of adenylate cyclase purified from untreated ^{32}P-labeled cells. However, after treatment with TPA the catalytic unit is phosphorylated to about 3 mol phosphate/mol enzyme. We have also demonstrated that purified protein kinase C will directly phosphorylate the adenylate cyclase

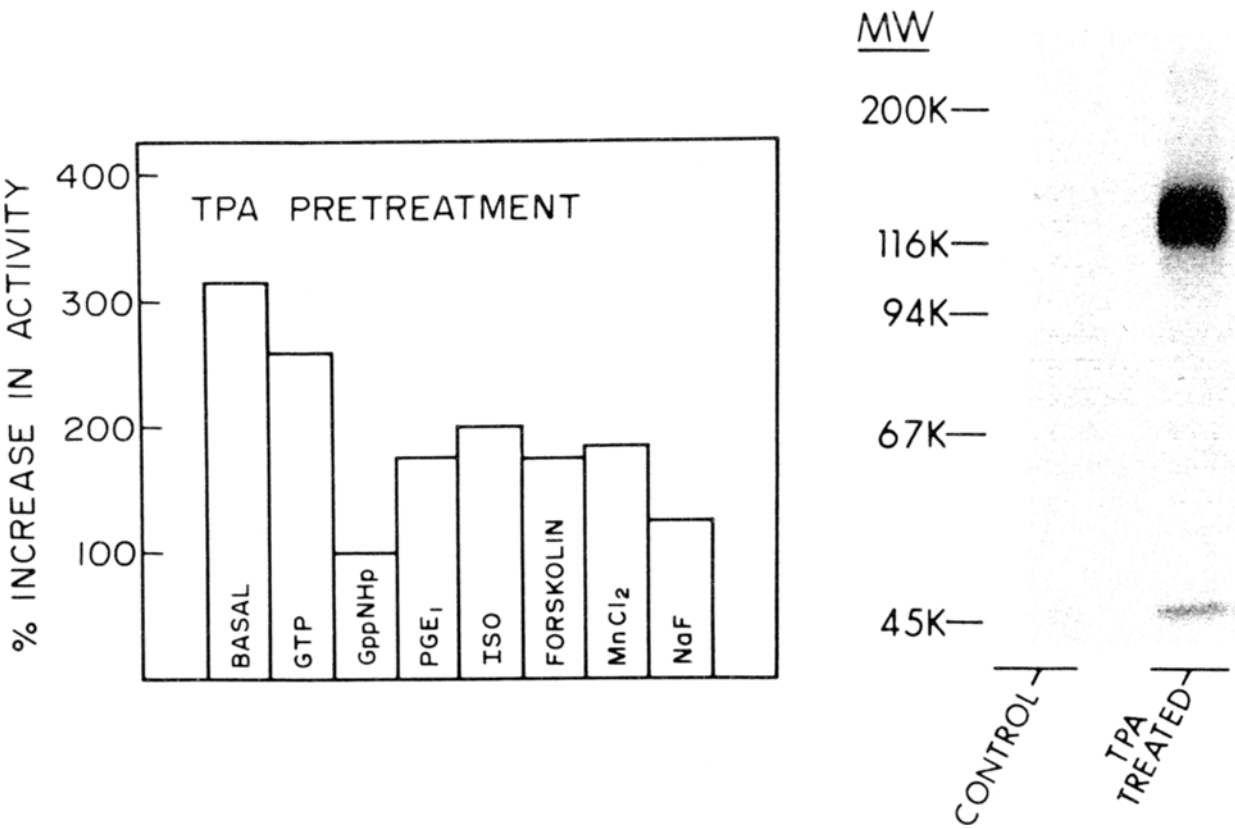

Fig. 11. Phorbol ester treatment promotes enhanced adenylate cyclase activity and phosphorylation of the catalytic unit of adenylate cyclase in frog erythrocytes. (A) Cells were pretreated with the phorbol ester, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), and then adenylate cyclase activities were assessed with the indicated effectors. Data is plotted as the % increase in activity relative to control activities. (B) Cells were prelabeled with ^{32}Pi and then incubated in the absence (control) or presence TPA. The catalytic unit was subsequently purified as described in Yoshimasa et al. (1987).

catalytic unit purified from bovine brain (Yoshimasa et al., 1987).

It is not clear why in some cells activation of protein kinase C results in receptor phosphorylation and desensitization (*see above*), whereas in others the results are catalytic unit phosphorylation and amplification. Clearly, more work is necessary in order to resolve this important issue.

It has recently been speculated that the observed amplification of adenylate cyclase activity in phorbol ester-treated cells may be related to an impairrment in function of N_i—the inhibitory guanine nucleotide regulatory protein (Jakobs et al., 1985; Katada et al., 1985; Olianis and Onali, 1986; Bell and Brunton, 1986). The α subunit of N_i has, in fact, been demonstrated to be a substrate for protein

kinase C in vitro (Katada et al., 1985). However, the protein kinase C-induced phosphorylation of the N_i α subunit was not observed to be stoichiometric, and the functional activity of the phosphorylated protein was not directly tested. Moreover, there has been no report of N_i phosphorylation in any intact cell system. Although our present data (Sibley et al., 1986b; Yoshimasa et al., 1987) do not rule out an additional effect of protein kinase C on the N_i-inhibitory pathway, phosphorylation of the adenylate cyclase catalytic unit could be sufficient to explain the observed phorbol ester-induced amplification effect.

The protein kinase C-catalyzed phosphorylation of the adenylate cyclase catalytic unit may provide a physiological mechanism by which receptor systems that promote PI turnover and protein kinase C activation can modulate receptor systems coupled to adenylate cyclase. That this may be the case is suggested by recent reports that activation of receptors that stimulate PI turnover, such a α_1-adrenergic, histamine, and angiotensin II (Hollingsworth et al., 1985; Nabika et al., 1985; Sugden et al., 1985), can lead to adenylate cyclase amplification.

Summary

Multiple mechanisms appear to be involved in regulating the responsiveness of β-adrenergic receptor-coupled adenylate cyclase systems. These mechanisms include both amplification and desensitization and seem to be achieved through covalent modification of one or more of the receptor/ enzyme components. With respect to desensitization, at least two major mechanisms have been elucidated. Homologous desensitization is initiated by phosphorylation of the receptor by a β-adrenergic receptor kinase. This reaction serves to functionally uncouple the receptors and perhaps trigger their translocation out of their normal plasma membrane environment. The site of receptor sequestration is unclear and might lie within the plasma membrane or within the cell. The sequestered receptors can rapidly recycle to the cell surface or, with time, become down regulated, perhaps being destroyed within the cell. Dephosphorylation of the receptors appears to be accomplished in the sequestered compartment of the cell, which may functionally regenerate the receptors and allow their return to the cell surface. In heterologous desensitization, receptor function is also regulated by phosphorylation, but in the absence of receptor sequestration or down-regulation. In this case, phosphorylation serves only to functionally uncouple the receptors, that is, to impair their interactions with the guanine nucleotide regulatory protein N_s. Several protein kinases appear to be capable of promoting phosphorylation of the receptors, including the cAMP-dependent protein kinase and protein kinase C. In addition to the receptor phosphorylation, heterolgous desensitization seems to be associated with modifications (phosphorylation?) at the level of the nucleotide regulatory proteins N_s, and perhaps N_i, which impairs activation of the enzyme.

Recent evidence indicates that adenylate cyclase systems are subject to amplification as well as desensitization. This appears to involve a protein kinase C-mediated phosphorylation of the catalytic unit of the enzyme. Phosphorylation of the catalytic unit seems to enhance catalytic activity and to result in amplified stimulation by the α subunit of N_s. Further studies of the mechanisms of regulation of adenylate cyclase-coupled receptors are likely to help elucidate modes of regulation of a wide variety of receptor-coupled functions in diverse types of cells.

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