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From Epinephrine to Cyclic AMP

ALEXANDER LEVITZKI

Binding of catecholamines to the β-adrenergic receptor results in the activation of adenylate cyclase and the intracellular formation of adenosine 3′,5′-monophosphate (cAMP). In the past 20 years the events that lead from hormone binding at the cell surface receptor site to the synthesis of cAMP at the inner layer of the membrane have been intensively studied. Signal transduction in this system involves the sequential interaction of the β-adrenergic receptor with the guanine nucleotide–binding protein (G_s) and the adenylate cyclase catalyst (C). The mechanism of signal transduction from the receptor through G_s to C, as well as the role of the adenylate cyclase inhibitory G protein G_i, is discussed.

ADENOSINE 3′,5′-MONOPHOSPHATE (cAMP) AND THE ENzyme that synthesizes the molecule, adenylate cyclase, exist in almost every form of life and in every tissue of higher organisms. The ubiquitous role of cAMP and its involvement in the regulation of a multitude of biochemical pathways is now well established. Adenylate cyclase is usually activated as a response to external stimuli—hormones and neurotransmitters in mammalian tissues and glucose in yeast. An experimental system that has become a focus in the study of transmembrane signaling is the β-adrenergic receptor–dependent adenylate cyclase. This system was originally studied in avian erythrocytes by the discoverers of cAMP, who were also the first to show that epinephrine (adrenaline) activates adenylate cyclase and that all the activity resides in the cell membrane (*1*). Pharmacological and physiological experiments (*2*) substantiated by biochemical data (*3*) have defined three types of adrenergic receptors: the β_1- and β_2-adrenergic receptors, which activate adenylate cyclase; the α_2-adrenergic receptor, which inhibits adenylate cyclase; and the α_1-adrenergic receptor, which activates phospholipase C.

The clinical importance of all the receptor subtypes has induced pharmaceutical chemists to develop a wide spectrum of selective drugs that show high affinity for these receptors. These drugs became the basis for the biochemical identification and characterization of these receptors. In 1974 the biochemical criteria for the identification of β-adrenergic receptors were established and, by the use of [^{3}H]propranolol (*4*), [^{3}H]dihydroalprenolol (*5*), and ^{125}I-labeled hydroxybenzylpindolol (*6*), it was possible to identify and quantitate β-adrenergic receptors in a radioreceptor assay. Since 1981 the ligand of choice has been ^{125}I-labeled (−)-cyanopindolol (*7*) because it exhibits 40 times as high affinity and 45 times as high specific radioactivity as either (−)-propranolol or (−)-dihydroalprenolol and is more selective than (−)-hydroxybenzylpindolol. When accurate binding experiments were possible, then substantiation of the pharmacological evidence (*8*) for the existence of two closely related classes of β-adrenergic receptors, β_1 and β_2 (*9*), could be achieved.

(−)-Propranolol was the basis for the design of the first radioactively labeled β-adrenergic affinity label. A bromoacetyl analog of

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(−)-propranolol inhibits irreversibly the response of adenylate cyclase to catecholamines but does not impair its activation by NaF (*10*), which acts directly through the activating G protein G_s (or N_s) (*11*). By using the tritiated affinity label the protein subunit of the β-adrenergic receptor was identified (*12*). At a later stage, other affinity labels based on (−)-carazolol and (−)-cyanopindolol (*13*) were shown to label the β-adrenergic receptor protein from different sources.

The involvement of guanosine triphosphate (GTP) in signal transduction was first demonstrated by Rodbell and his colleagues. The original findings (*14*) revealed that GTP enhances and may even be essential for signal transduction from the receptor to the catalytic unit. These findings were quickly bolstered by the use of the nonhydrolyzable GTP analog guanosine-5′-[β,γ-imino] triphosphate (GPPNHP), which magnified the stimulatory effects of GTP (*15*). When GPPNHP was used, β-adrenergic agonists facilitated the rate of adenylate cyclase activation by the nucleotide (*15*). Because GPPNHP and not GTP was used in these experiments, the enzyme became permanently active. These findings (*15*) supported the idea that under physiological conditions GTP is hydrolyzed at the regulatory site to the inactive ligand guanosine diphosphate (GDP), thereby terminating the hormonal signal. The role of the hormone-bound receptor, therefore, must be to facilitate the replacement of GDP by GTP to reactivate the enzyme. The direct demonstration, in the turkey erythrocyte system, of a β-adrenergic receptor–dependent slow guanosine triphosphatase (GTPase) [catalytic rate (k_{cat}) = ~12 min^{-1}] provided definite proof for the involvement of GTP in the regulation of adenylate cyclase (*16*).

The existence of a specific G protein was clearly established when it became possible to resolve adenylate cyclase into its GTP-binding protein and the catalytic moiety. The resolved G protein was able to restore β-adrenergic receptor–sensitive adenylate cyclase activity in membranes of S49 cyc^- cells, which have the β-adrenergic receptor and the catalytic moiety of adenylate cyclase (C) but lack the transducer G_s protein (*17*). Other experiments demonstrated the reconstitution of GPPNHP-dependent cyclase activity after separation of the G protein from C on a GTP affinity matrix and adding it back to the resolved catalyst C (*18*). These reconstitution experiments were complemented by the identification of the GTP-binding subunit with a [^{32}P]GTP-derived photoaffinity label (*18*) and by cholera toxin–catalyzed [^{32}P]adenosine diphosphate (ADP)–ribosylation of the GTP-binding subunit (*19*). It took another few years to purify the β-adrenergic receptor and the adenylate cyclase catalytic unit (C). These purified components were then reconstituted in synthetic phospholipid bilayers to generate the β-adrenergic receptor–sensitive enzyme. These reconstitution experiments (see below) proved that the three components are sufficient to generate the transmembrane signaling in the β-adrenergic receptor–dependent adenylate cyclase. The β-adrenergic receptor–dependent cyclase was the first transmembrane signaling system to be resolved into its components and fully reconstituted (*20*, for review).

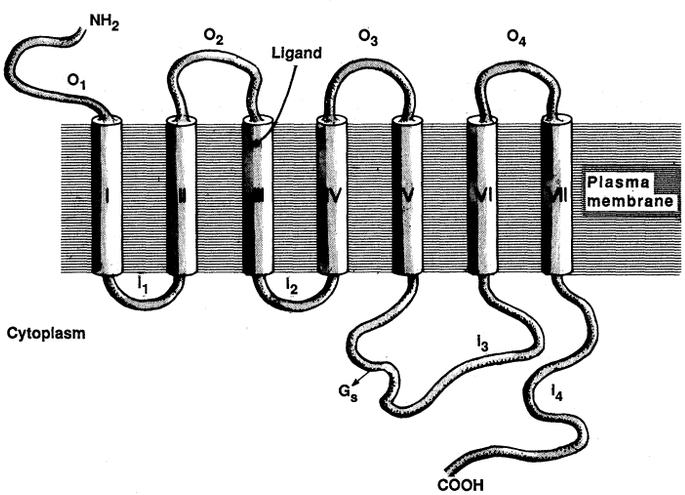

Fig. 1. Schematic structure of the β-adrenergic receptor and its homologs. The receptor has a molecular size of ~46 kD and is highly hydrophobic. The hydrophobicity results from the presence of seven sequences of hydrophobic amino acids, which can be arranged in seven putative transmembrane domains. The NH_2-terminus is extracellular and the COOH-terminus intracellular. Transmembrane domain III probably participates in ligand binding, suggesting a hydrophobic environment for the ligand. The intracellular domain i_3 seems to be crucial for the interaction between the receptor and the stimulatory G protein G_s. The mechanism of signal transduction from the agonist binding site to the domain of interaction between the β-adrenergic receptor and G_s (domain i_3) is not known. It is also possible that oligomerization of the receptor or G_s is involved, although there is no evidence to suggest this.

The β-Adrenergic Receptor—An Archetype

The observation that the β-adrenergic receptor can be solubilized in digitonin without losing its ligand-binding properties (*21*) allowed investigators to purify completely the β_2-adrenergic receptor as well as the β_1-adrenergic receptor (*22*) by using an (−)-alprenolol–based affinity column. Partial sequence data enabled the cloning and sequencing of the β_2- (*23, 24*) and later of the β_1-adrenergic receptors (*25*). The most striking feature of the receptor structure that was deduced from the sequence data was the seven putative transmembrane spanning domains (Fig. 1). This feature immediately focused attention on rhodopsin, to which the β-adrenergic receptor was found to be homologous, especially in the putative transmembrane domains (*26*). Soon afterward the cloning and sequencing of muscarinic receptors, of the α_2-adrenergic receptor (*27*), and of the *Saccharomyces cerevisiae* pheromone receptors STE2 and STE3 (*28*) revealed similar structural organization. All these receptors interact with heterotrimeric G proteins (*28*, for review). These observations classify the β-adrenergic receptor as a member of a growing family of receptors, all of which activate an effector system through a G protein.

Another significant finding is that the ligand binding domain of the β-adrenergic receptor is probably located within a protein domain that is embedded in the phospholipid bilayer (Fig. 1). The amino acid Asp^{113} within the third putative transmembrane domain is essential for ligand binding (*29*). This negatively charged residue is presumed to bind the protonated form of the catecholamine. [By analogy, it is likely that an Asp residue (Asp^{105}) within the third putative transmembrane domain of the muscarinic receptor is involved in the binding of positively charged acetylcholine.] The involvement of the third transmembrane sequence in ligand binding does not preclude the involvement of other transmembrane sequences. Indeed, a recent analysis of the peptide sequence that becomes covalently labeled by the β-adrenergic receptor affinity label *p*-(bromoacetamido)benzyl-1-[^{125}I]iodocarazolol localizes it to the second membrane-spanning domain (*30*). Deletions of cytoplasmic domains had little or no effect on the ligand-binding properties of the mutated β-adrenergic receptor (*31*).

These findings corroborate affinity-labeling experiments that demonstrate that the β-adrenergic receptor binding site is submerged within the lipid bilayer (*32*). The irreversible β-adrenergic blocker ^{125}I-labeled *N*-bromoacetylamino-cyanopindolol ([^{125}I]BAM-CYP) labels a glycolipid that is associated with the receptor binding site. Binding activity could be restored only after solubilization of the labeled membranes in deoxycholate and then molecular sieve

chromatography to separate the β-adrenergic receptor from the [^{125}I]BAM-CYP glycolipid (*32*). The presence of the receptor ligand binding site within a hydrophobic domain offers an explanation as to why hydrophobic β-adrenergic blockers bind more tightly than the physiological hydrophilic catecholamines (up to 10^4 times as tightly). The receptor ligand dissociation constant for (−)-epinephrine and (−)-norepinephrine is in the range of 2 to 3 μ*M*, whereas the dissociation constant for (−)-propranolol is 0.0012 μ*M* (1.2 n*M*) (*4*) and for (−)-cyanopindolol is 0.00003 μ*M* (0.03 n*M*) (*7*). A similar relation exists between the dissociation constants of agonists and antagonists of muscarinic receptors, α_2-adrenergic receptors, and α_1-adrenergic receptors. It is likely therefore that the hydrophobic blockers characteristic for these receptors interact with hydrophobic areas of the protein or with the lipid bilayer near the receptor ligand binding site.

The localization of the ligand binding site to the third putative transmembrane domain poses a dilemma as to the type of conformational transition that translates β-adrenergic agonist binding to the activation of G_s. Deletion analysis of the cytoplasmic loops tentatively identifies the COOH-terminal domain (residues 239 to 272) of the third intracellular loop i_3 (Fig. 1) as the domain that interacts with the G protein G_s. Deletion of this domain (*31*) eliminates the ability of the receptor to activate adenylate cyclase (through G_s). The β-adrenergic receptor with this particular deletion exhibits extraordinarily high affinity toward the agonist (−)-isoproterenol (*31*) and is probably "locked" into a high-affinity form.

Figure 1 does not immediately suggest how the putative transmembrane domain III transmits a conformational change to domain i_3, which presumably interacts with G_s. One possibility is that domain i_2, which immediately follows domain III, is moved as a consequence of ligand binding and in turn pushes the i_3 domain, which most likely interacts with G_s protein. The COOH-terminal domain of the G_s protein is believed to interact with the β-adrenergic receptor because in the *unc* mutation of G_s, coupling of β-adrenergic receptor to G_s is attenuated, whereas G_s to C coupling is intact. This mutation involves a Pro to Arg replacement in the COOH-terminal portion of G_s (*33*).

β-Adrenergic Receptor to Cyclase Coupling

The coupling of β-adrenergic receptors to adenylate cyclase has been studied both in the native system as well as in reconstituted material generated from the isolated components: β-adrenergic receptor, G_s, and the catalyst C. It had been established that the β-adrenergic receptor and GTP act in synergy and that the process of cyclase activation is first-order with a characteristic rate constant of ~0.7 min^{-1} at 37°C (*15*). This finding was interpreted to mean that during activation the agonist-bound receptor catalyzes the exchange of GDP with GPPNHP to yield a permanently active enzyme, since GPPNHP cannot be hydrolyzed. Direct measurements demonstrated that [^{3}H]GDP release from turkey erythrocyte membranes is facilitated by β-adrenergic agonists and is therefore β-adrenergic receptor–specific (*34*). It was therefore suggested (*34*) that the interaction of GDP-bound G_s protein with the agonist-bound β-adrenergic receptor results in the "opening" of the GTP binding site, allowing the GDP to GTP (or GPPNHP) exchange reaction to occur. Furthermore, a detailed kinetic analysis of the turkey erythrocyte system suggests that the G_s protein can attain an open and a closed state. In the presence of both hormone and GTP, the G_s protein shuttles between these two forms continuously at relatively high frequency (*35*). Experiments with purified G_s reconstituted with both resolved and purified β-adrenergic receptor demonstrate that the β-adrenergic receptor to G_s coupling in the reconstituted

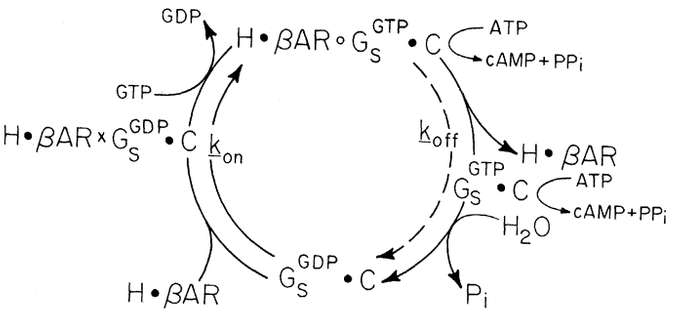

Fig. 2. Activation of adenylate cyclase by β-adrenergic receptors. The agonist (H)-bound β-adrenergic receptor (βAR), during its transient encounter with the G_s-cyclase complex ($G_s^{GDP} \cdot C$), induces the "opening" of the guanine nucleotide binding site. During this brief period, GDP dissociates from the open site and is replaced by incoming GTP; the agonist-bound receptor then dissociates from the GTP-loaded complex. The receptor-catalyzed nucleotide exchange results in the formation of an active, GTP-bound enzyme that produces cAMP. The association of the β-adrenergic receptor with G_s is less tight (○) than the association with G_s^{GDP} (×). Both interactions are weaker than the interaction between G_s and C (●).

system retains the basic kinetic features seen in the membranes. Thus, the role of the agonist-bound receptor is to facilitate GDP to GTP exchange on G_s concomitant with its activation (*36*). In these experiments C was absent so the parameter measured was the facilitation of [^{35}S]guanosine-5′-[γ-thio]triphosphate ([^{35}S]GTPγS) binding and conversion of inactive G_s (GDP) to active G_s (GTPγS) (*36*), which, because of its stability, can be assayed by its ability to activate cyclase in the G_s-deficient S49 cyc^- system. These experiments also demonstrated that the basic features of G_s activation by the β-adrenergic receptor do not require the presence of the catalyst. Other experiments performed on native membranes in which C was inactivated with *N*-ethylmaleimide also showed that the β-adrenergic receptor to G_s coupling retains its kinetic features (*37*). This aspect of the interaction between receptor and G protein is probably common to many G protein transduction systems.

The activation of the G_s–adenylate cyclase by the agonist-bound β-adrenergic receptor is a catalytic event, and the receptor is not permanently associated with the G_s-cyclase complex. Rather, the receptor forms a transient complex (Fig. 2). During that transient encounter between the agonist-bound β-adrenergic receptor and G_s-C, GDP is exchanged with GTP at the α subunit of G_s, with the subsequent dissociation of the agonist-bound β-adrenergic receptor from the complex. This unique kinetic feature of the system termed "collision coupling" (*38*) accounts for the finding that the rate constant of activation of the G_s-C complex (k_{on}) linearly depends on the concentration of the agonist-bound β-adrenergic receptor in the system:

$$k_{on(observed)} = k_{on(intrinsic)} \times [R]_{total} \times [H]/(K_H + [H])$$

where $[R]_{total}$ is the total receptor concentration in the system, [H] is the agonist concentration, and K_H is the dissociation constant of agonist (H) to the receptor. This behavior, originally found in the native turkey erythrocyte membrane (*38*), was confirmed in reconstituted β-adrenergic receptor–G_s mixtures generated from resolved β-adrenergic receptor and G_s and later from the purified components (*39*).

This kinetic feature is the origin of a tenfold amplification in the system since one receptor can activate numerous G_s-cyclase molecules (Fig. 2). This approximately tenfold amplification is in addition to the ~100-fold amplification due to the slow GTPase activity associated with the enzyme. This feature reveals another similarity with the rhodopsin-transducin system in which up to 300 molecules

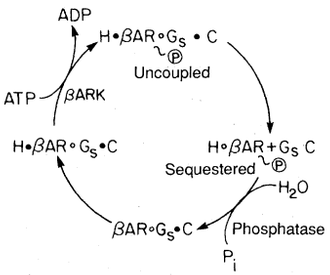

Fig. 3. The desensitization of the β-adrenergic receptor. Agonist (H)-bound adrenergic receptor (βAR) activates adenylate cyclase ($G_s \cdot C$) within seconds by forming a transient complex. The agonist-bound receptor is a substrate for the β-adrenergic receptor kinase, βARK, which catalyzes the phosphorylation of the receptor. The phosphorylated receptor is functionally uncoupled from adenylate cyclase. The phosphorylated receptor also becomes sequestered into a specialized membrane compartment, probably coated pits. The delocalized and sequestered receptor is found in light-density vesicles, which may be either recycled back to the membrane or translocated to the lysosomal compartment for degradation. Upon recycling, the receptor is probably dephosphorylated by a specific phosphatase, which allows functional resensitization.

of guanosine 3′,5′-monophosphate (cGMP) phosphodiesterase become activated by photobleached (activated) rhodopsin within the first 0.5 second after a flash (*40*). Biochemical experiments confirm the transient nature of the β-adrenergic receptor to G_s interaction. These studies reveal that the agonist-bound β-adrenergic receptor forms a complex with G_s in its GDP-bound form, whereas the two proteins readily dissociate when GDP is exchanged with GTP or its nonhydrolyzable analogs (*41*).

Binding experiments reveal (*42*) the existence of two classes of β-adrenergic receptors—receptors that bind β-adrenergic agonists with high affinity and receptors that bind these agonists with low affinity. Apparently, the two populations of receptors represent the receptor associated with G_s (GDP)-C, which exhibits high affinity, and the free receptor, which exhibits low affinity, toward β-adrenergic agonists. The existence of heterogeneous populations of β-adrenergic receptor also results in complex ligand displacement curves when β-adrenergic agonists are tested as inhibitors of β-adrenergic antagonist binding. The addition of GTP or its nonhydrolyzable analogs shifts the displacement curve to higher agonist concentrations and converts it to a simple noncooperative displacement curve. This phenomenon is due to the conversion of a mixed population of receptors to a homogeneous, low-affinity population of receptors not complexed with G_s (*42*). The complex H–β-adrenergic receptor–G_s–C can be stabilized by treatment of the agonist-bound state with *N*-ethylmaleimide, in the absence of GTP. Most likely the aklylation of a sulfhydryl group on G_s "locks" the complex in the agonist-bound associated state (*43*).

Measurements of the decay of the β-adrenergic receptor–induced active state of bound G_s-C to its inactive GDP-bound state (*44*) revealed a first-order process (*38*) typified by the first-order constant k_{off} (*38, 44*) of 12 to 15 min^{-1} for a full agonist at 37°C. Comparison of the values of k_{on} to those of k_{off} established that k_{off} is 8 to 12 times as high as k_{on} (*45*). This relation accounts for two characteristic features of the β-adrenergic receptor–dependent adenylate cyclase: (i) only a small portion of the enzyme is in the active form at any given time, and (ii) a large fraction of the amplification in the system is accounted for by the slow GTPase step (k_{cat} = ~12 to 15 min^{-1}) as compared to the turnover number of activated C (k_{cat} = ~1100 min^{-1} to 1400 min^{-1}) (*46*). Namely, the active G_s^{GTP}-C species can produce ~100 cAMP molecules before it decays by the GTPase off-step. Since one receptor can activate approximately ten adenylate cyclase molecules, an overall amplification of $\sim 10^3$ is obtained in the β-adrenergic–dependent adenylate cyclase.

The molecular understanding of the events that occur during adenylate cyclase activation by β-adrenergic receptors also allows a better insight into the meaning of "partial agonism." A partial agonist, such as phenylephrine or dopamine, elicits less than maximal response as compared to a full agonist such as (−)-epinephrine, (−)-norepinephrine, or (−)-isoproterenol. Kinetic analysis reveals that partial agonists exhibit smaller k_{on} values, whereas the k_{off} values remain unaltered (*45*). Furthermore, a linear correlation was found between the extent of adenylate cyclase activation in the presence of GTP and the rate constant k_{on}, which is measured in the presence of GPPNHP (*45*). These results suggest that the better the agonist the higher the fraction of receptors that can be converted to their active state and couple to G_s. Indeed, binding studies corroborated that the extent to which β-adrenergic agonist can stimulate adenylate cyclase is proportional to the fraction of β-adrenergic receptors that can be converted to the high-affinity state (*47*).

Role of Different Protein Components in Signal Transduction

Reconstitution experiments reveal that in order to generate β-adrenergic receptor–dependent cyclase one needs to reconstitute three purified components: β-adrenergic receptor, G_s, and C (*39*). Although hormone-dependent cyclase activity can be clearly demonstrated, the "basal" adenylate cyclase activity in the reconstituted system is high compared to the basal activity in the native membrane. It has been suggested that G_i tonically inhibits the basal activity and that this effect can be mimicked by the βγ subunits (*48*), which are common to G_s and G_i (*49*). The possibility that βγ subunits directly inhibit C is still the subject of intensive investigations and discussion. The argument (*49, 50*) is twofold: whether all four components (β-adrenergic receptors, G_s, G_i, and C) are separate but transiently interacting and whether G_s and G_i undergo subunit dissociation during the process of adenylate cyclase activation and inhibition by receptors.

According to one view, G_s ($\alpha_s\beta\gamma$) and C are separate units and α_s-GTP binds to C once it is generated by the activation of G_s (*49*). The released βγ subunits actually compete with C for α_s. The species α_s-GDP is assumed to exhibit higher affinity toward βγ subunits than α_s-GTP, whereas the relation is reversed with respect to C. This sequence of events suggests that, subsequent to GTP hydrolysis, α_s-GDP dissociates from α_s-GDP-C and rebinds to βγ subunits.

This mechanism, however, cannot account for all the findings. For example, (i) the process of adenylate cyclase activation is first-order, which argues for a permanent G_s to C association (*51*), and (ii) both turkey erythrocyte adenylate cyclase (*52*) and the bovine brain enzyme (*53*) can be partially purified as a 1:1 G_s to C complex in both the basal GDP state and the GPPNHP-activated form. It is, however, possible to accommodate a modified version (*50, 54*) of the dissociation model (*49*) with the kinetic and biochemical findings, if it is assumed that α_s remains associated with C at all times, while the βγ subunits are allowed to dissociate and play an inhibitory role on the subunit α_s. In this modified mechanism it is still assumed (*54*) that G_i, upon activation by an inhibitory receptor, releases βγ subunits thus favoring the G_s-associated ($\alpha_s\beta\gamma$) inhibited state. According to both dissociation models (*49, 54*), no physical interaction of α_i with the adenylate cyclase system is required for G_i to inhibit the enzyme. Attempts to demonstrate inhibition of C by G_i when the two pure components were reconstituted together failed (*55*), but these experiments were conducted in the absence of G_s. The attenuating effects of G_i on adenylate cyclase were significant in the presence of G_s (*48*), so it is possible that G_i associates with G_s-C but not with the resolved catalytic unit C.

Recent biochemical findings on highly purified GPPNHP-activated adenylate cyclase from turkey erythrocytes reveal that βγ subunits remain associated with the α_s-C complex (*56*). These findings argue against the dissociation of βγ subunits from the G

protein upon its activation. Because it is not yet clear whether βγ subunits mediate adenylate cyclase inhibition, we explored alternative mechanisms (*54*) for G_i-mediated inhibition that do not involve βγ subunit dissociation. Treatment of S49 cell membranes with NAD^+ and pertussis toxin, which catalyzes the ADP-ribosylation of G_i, induces a threefold reduction in the affinity of β-adrenergic receptors toward (−)-isoproterenol (*57*). Thus, G_i associates directly with β-adrenergic receptor–G_s-C complex. Had G_i been unable to react directly with the complex, such G_i to β-adrenergic receptor "cross talk" would not have been observed. This finding supports the observation (*48*) that G_i attenuates adenylate cyclase activity in the presence of G_s.

Role of Lipids

Coupling between β-adrenergic receptors and G_s or G_s-C takes place only when both components are present in a phospholipid bilayer. The coupling between the receptor and cyclase is sensitive to detergent and is inhibited at detergent concentrations that are one-tenth of those required to inhibit the G_s to C interaction (*58*). A number of combinations of phospholipids were effective in bringing about β-adrenergic receptor to G_s coupling as well as β-adrenergic receptor to G_s to C coupling in reconstitution experiments (*20, 39, 48*). However, a combination of phosphatidylethanolamine:phosphatidylserine (3:2, w/w) was the most effective in bringing about stimulation by GTP and isoproterenol (*39*). Studies on the reconstitution of crude but resolved β-adrenergic receptors with crude G_s demonstrated that the most effective coupling was obtained when the phospholipid was combined with α-tocopherol or a free fatty acid (*59*). It is likely that these additives substitute for the glycolipid associated with the β-adrenergic receptor in turkey erythrocytes (*32*).

Desensitization and Down Regulation

The binding of catecholamines to β-adrenergic receptors triggers the activation of adenylate cyclase within seconds (see above). Prolonged exposure of the receptor to agonists results in the progressive loss of response to the bound ligand. This phenomenon of time-dependent attenuation of responsiveness or refractoriness is common to many receptor systems. This phenomenon, also known as desensitization, is important physiologically as well as in certain pathophysiological conditions (*60*) such as bronchial asthma. Insight into the biochemical basis of this phenomenon in the β-adrenergic receptor system has recently been obtained from studies on intact cells as well as from in vitro studies with biochemical and molecular biological techniques.

Two types of desensitization have been defined (*61*): homologous desensitization and heterologous desensitization. Homologous desensitization refers to the loss of responsiveness to the stimulating catecholamine where the response to other hormones or neurotransmitters affecting the same tissue via adenylate cyclase remains intact. Biochemically, homologous desensitization results in the loss of adenylate cyclase responsiveness to the stimulating catecholamine, but not to ligands acting by other receptors linked to the same pool of enzyme. Also, stimulatory effects of AlF_4^-, GPPNHP, or forskolin, which stimulate adenylate cyclase by receptor-independent mechanisms, remain unperturbed. Homologous desensitization occurs within 1 to 3 minutes of exposure to the catecholamine.

Heterologous desensitization refers to the decline of adenylate cyclase response to other stimulating agents such as AlF_4^-, guanyl nucleotides, or nonadrenergic agonists that couple to the enzyme through other receptors. It is generally accepted that the onset of heterologous desensitization begins after the fast homologous desensitization step. Desensitized β-adrenergic receptors become sequestered and move to vesicles that can be separated from the plasma membrane by ultracentrifugation. These vesicles are devoid of G_s, G_i, or adenylate cyclase (*62*). The hydrophobic ligand ^{125}I-labeled pindolol penetrates the cell membranes and therefore can be used to determine the total number of β-adrenergic receptors in both compartments. The hydrophilic β-adrenergic antagonist [^3H]GCP-12177 can monitor the loss of surface receptors (*63*) because it cannot penetrate the cell membrane. In the presence of pore-forming antibiotics [^3H]CGP-12177 measures both populations of receptors—the surface ones as well as the internalized vesicular receptors. The uncoupling reaction, which is induced by exposure to β-adrenergic agonists and results in the formation of delocalized low-affinity β-adrenergic receptors, can be inhibited by treating the cell with the lectin concanavalin A (*63*). It is likely that concanavalin A, because of its binding to numerous glycoproteins on the membrane surface, prevents the movement of the receptors away from the adenylate cyclase complex to a unique membrane compartment and then to light endocytotic vesicles.

The desensitized, uncoupled, and free β-adrenergic receptors exhibit lower affinity to β-adrenergic agonists than do coupled receptors (*64*). The β-adrenergic receptor–containing vesicles from desensitized frog erythrocytes could functionally reconstitute β-adrenergic agonist–sensitive adenylate cyclase when they were fused with *Xenopus laevis* erythrocytes, which are devoid of β-adrenergic receptor (*65*). This finding demonstrates that no irreversible lesion has occurred during the process of desensitization.

Circumstantial evidence pointed to the involvement of ATP in β-adrenergic receptor desensitization (*66*). The hypothesis that some modification of the receptor occurs was strengthened by the finding that reduction in β-adrenergic agonist affinity as a result of prolonged exposure of cells to β-adrenergic agonists occurs also in S49 cyc^- cells, which lack G_s (*67*). Furthermore, exposure of S49 cyc^- cells to catecholamines, followed by reconstitution with externally added G_s, resulted in diminished β-adrenergic agonist–dependent adenylate cyclase activity as compared to untreated cells (*68*). These experiments suggest that β-adrenergic receptor is desensitized and probably covalently modified when challenged with an agonist, even in the absence of G_s. A recent finding, however, seems to contradict these results; a deletion of a β_2-adrenergic receptor sequence that is involved in receptor to G_s coupling [portion of domain i_3 (Fig. 1)] also results in the elimination of receptor desensitization and sequestration in cells transfected with the mutated receptor (*69*). However, in the deletion study, the authors did not measure the agonist affinity as a result of exposure of the mutated β-adrenergic receptor to β-adrenergic agonists; this was performed on the S49 cyc^- cells (*67*). It is feasible that the agonist-induced change in the β-adrenergic receptor occurs in the absence of the receptor-to-G_s coupling but that the subsequent steps are altered because of the aberrant structure of the mutated β-adrenergic receptor.

Removal of the β-adrenergic agonist from desensitized cells results in the fast reappearance of receptors on the cell surface, concomitant with the recoupling to adenylate cyclase (*64*). Some experiments strongly suggest that very prolonged exposure of cells to catecholamine results in irreversible loss and degradation of β-adrenergic receptors, possibly in a lysosomal compartment. Reappearance of receptors after very prolonged exposure seems to involve de novo protein synthesis (*70*).

Early experiments aimed at revealing molecular mechanisms of desensitization were conducted on cell-free homogenates prepared from cells that exhibit fast ($t_{1/2}$ = ~1 min) homologous desensitization to β-adrenergic agonists. Although these experiments indicated that adenosine triphosphate (ATP) and, therefore, a phosphoryl-

ation event is involved (*66*), only recently was phosphorylation of the agonist-bound β-adrenergic receptor by a specific β-adrenergic receptor kinase (βARK) demonstrated (*71*). This phosphorylation was also correlated with the loss of the coupling between the phosphorylated receptor and G_s (*71*) (Fig. 3). Because βARK may not be entirely specific for the β-adrenergic agonist–bound receptor, it may also be involved in the agonist-dependent phosphorylation of the adenylate cyclase–stimulating prostaglandin E_1 (PGE_1) receptor and of the α_2-adrenergic receptor, which inhibits adenylate cyclase (*72*). It is therefore thought (*72*) that βARK is specific for receptors that either stimulate or inhibit adenylate cyclase.

The βARK system is analogous to the rhodopsin system in which rhodopsin kinase is specific for the bleached (activated) rhodopsin (*73*). In the rhodopsin-transducin system a 48-kD protein (arrestin) specifically interacts with the phosphorylated form of rhodopsin, preventing it from interacting with transducin (*74*). Tentative evidence for the existence of a protein that may be analogous or homologous has recently been published (*75*). It will be of great interest to examine whether this protein binds to the desensitized, phosphorylated receptor and prevents it from interacting with G_s. The homology between a domain in arrestin and the COOH-terminus of the α_t subunit of transducin suggests (*33*) that the two proteins compete for the same domain on the rhodopsin molecule. Because the COOH-terminal region of the α_s subunit of G_s is most likely the domain that interacts with the β-adrenergic receptor (*33*), a homolog of the 48-kD protein (arrestin) may interact preferentially with the βARK phosphorylated β-adrenergic receptor and prevent it from interacting with G_s. It is also likely that a specific phosphatase is responsible for the removal of the phosphate group or groups from the desensitized β-adrenergic receptor (Fig. 3).

Deletion analysis (*69*) has demonstrated that a β_2-adrenergic receptor lacking the COOH-terminal portion desensitizes normally and becomes sequestered like the β_2-adrenergic receptor. This region, which is rich in serine residues, had been suggested to be a potential target for βARK (*23*). In view of the deletion studies, further work is needed to precisely assign the β-adrenergic receptor sequence targeted by βARK and the role of phosphorylation in the overall process. Interestingly, a deletion of the region (amino acids 239 to 272), which is essential for the β-adrenergic receptor to G_s coupling but is not essential for ligand binding, results in the complete loss of the ability of the receptor to be desensitized and sequestered (*69*). The authors (*69*) suggest that β-adrenergic receptor to G_s coupling is therefore essential for desensitization. This interpretation is not compatible with other data that show that in intact S49 cyc^- cells, which lack G_s altogether, the β-adrenergic receptor undergoes desensitization [see above and (*67, 68*)]. The behavior of the mutated receptor may be altered, as compared to the native receptor. The deletion of amino acids 239 to 272 may also encompass potential phosphorylation sites for βARK, such as the amino acids 259 to 262 (*23*).

In S49 cells (*76*) heterologous desensitization occurs at low concentrations (50 n*M*) of (−)-epinephrine and is mediated by cAMP. Homologous desensitization, according to this study, occurs at higher concentrations of (−)-epinephrine (10 μ*M*) and is probably mediated by βARK (*76*). If this recent finding is corroborated in other systems, many of the apparent discrepancies described above will be clarified.

Glucocorticoids stimulate the synthesis of β-adrenergic receptors and reverse the β-adrenergic agonist–induced down regulation of the receptors (*77*). This may be the reason for the effectiveness of steroids in the treatment of bronchial asthma. The discovery of glucocorticoid-responsive elements in the human β_2-adrenergic receptor (*24*) gene supports the notion that receptor expression is regulated by glucocorticoids.

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Research Articles

Phase Determination by Multiple-Wavelength X-ray Diffraction: Crystal Structure of a Basic "Blue" Copper Protein from Cucumbers

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A novel x-ray diffraction technique, multiple-wavelength anomalous dispersion (MAD) phasing, has been applied to the de novo determination of an unknown protein structure, that of the "blue" copper protein isolated from cucumber seedlings. This method makes use of crystallographic phases determined from measurements made at several wavelengths and has recently been made technically feasible through the use of intense, polychromatic synchrotron radiation together with accurate data collection from multiwire electronic area detectors. In contrast with all of the conventional methods of solving protein structures, which require either multiple isomorphous derivatives or coordinates of a similar structure for molecular replacement, this technique allows direct solution of the classical "phase problem" in x-ray crystallography. MAD phase assignment should be particularly useful for determining structures of small to medium-sized metalloproteins for which isomorphous derivatives are difficult or impossible to make. The structure of this particular protein provides new insights into the spectroscopic and redox properties of blue copper proteins, an important class of metalloproteins widely distributed in nature.

THE CLASSIC PHASE PROBLEM IN X-RAY CRYSTALLOGRAPHY can be solved with the use of anomalous scattering effects. As the energy of an incident x-ray beam is varied across the absorption edge of an element, there may be substantial changes in the real and imaginary components (f' and f'') of the x-ray scattering. In crystal structures that contain atoms with large "anomalous scattering" effects, the net observed intensity of each Bragg reflection will then be energy dependent. In such cases, the differences between the Bragg intensities measured from a single crystal at several x-ray energies may be used to directly derive crystallographic phases and hence to determine the crystal structure. Multiple-wavelength anomalous dispersion (MAD) phase assignment is potentially applicable to any macromolecular crystal structure that contains one or more anomalous scatterers (*1*). Metallopro-

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