Future Directions

A combination of molecular biological and electrophysiological methodology offers new opportunities for investigating the molecular basis of the brain's electrical activity. Electrophysiological analysis can provide detailed functional descriptions (like that in Fig. 1), but the exact nature of the underlying molecular mechanisms cannot be revealed. Structural techniques, including molecular biological methods, can elucidate the structure of channels, but are not appropriate for investigating the extremely rapid events characteristic of channel function. A combined approach should, however, offer the key for linking structure and function. The basic idea behind the combined approach is to make specific changes in structure and then evaluate the functional sequelae. This approach is now possible through the use of site-directed mutagenesis to substitute specific amino acids, expression systems to manufacture the modified proteins, and single-channel recording and other modern electrophysiological methods for analyzing altered function (*32*).

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Message Transmission: Receptor Controlled Adenylate Cyclase System

Michael Schramm and Zvi Selinger

The adenylate cyclase system is located in the cell membrane and is apparently present in every cell type of higher organisms; it is controlled by specific receptors for neurotransmitters and hormones which turn the enzyme on, while other receptors cause its inhibition. Cyclic adenosine monophosphate (cyclic AMP), which the enzyme produces from adenosine triphosphate (ATP), has been implicated in many processes, from activation of glycogenolysis and lipolysis (*1*) to actions more specific for the nervous system, such as the function of opiates (*2, 3*), the control of electrical activity (*3, 4*) and the initiation of simple behavior patterns (*5*). Thus, it is accepted by now that the receptor activated adenylate cyclase serves as a major transmembrane signaling system: A neurotransmitter released presynaptically binds to the postsynaptic receptor facing outward in the cell membrane, and the receptor communicates this information inward, through the membrane, by activating the adenylate cyclase (*6, 7*). The cyclic AMP formed in turn activates the specific protein kinase originally discovered by Krebs and his collaborators (*8*). The kinase then phosphorylates certain specific proteins that cause the final biological response. This protein kinase is the only molecule known at present to interact specifically with cyclic AMP in eukaryotes.

The early studies of Sutherland and co-workers already revealed that adenylate cyclase activity is extremely high in brain (*1*). Later, it was shown by Bloom *et al.* (*4*) that noradrenaline acting on beta-adrenergic receptors stimulates cyclic AMP synthesis in the Purkinje cells of the cerebellum and that the cyclic AMP formed inhibits specific electrical activities of these cells.

The functions of two other neurotransmitters, adenosine and dopamine, have been studied in the nervous system in connection with the adenylate cyclase. Adenosine raised special interest when it was shown to cause a dramatic increase in cyclic AMP in brain slices (*1*). Various electrophysiological effects of adenosine have been studied (*3*), and the location and characteristics of the receptors in brain have been elucidated by Snyder and his collaborators (*9*). The role of cyclic AMP in an adenosine effect must, however, be ascertained in each case since it has been shown that there are two classes of adenosine receptors at the cell membrane, facing out: Ra, stimulating adenylate cyclase, and Ri, inhibiting the enzyme (*10*). Because of the great variety of biological responses elicited by adenosine and because of the medical implications, a considerable number of adenosine analogs with receptor selectivity are being studied (*11*).

When the dopamine-stimulated adenylate cyclase was discovered (*12, 13*), it was hoped that it might explain all the major actions of dopamine. However, like epinephrine, adenosine, and several other neurotransmitters, the action of dopamine is not limited to one type of receptor. Kebabian and Calne proposed a D_1 receptor which activates the adenyl-

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ate cyclase and a D_2 receptor which inhibits the enzyme (*13*). Additional classes of dopamine receptors have been recognized, which might turn out to be different conformations of D_1 and D_2 (*14*).

Cyclic AMP formation apparently plays an important role also in developmental aspects of the nervous system. Nirenberg *et al.* have reviewed their work showing that cyclic AMP regulates the formation of functional synapses between neuroblastoma hybrid cells and muscle cells in culture (*15*). The role that cyclic AMP plays in some neuronal systems is further emphasized by studies (*5*) on the marine snail *Aplysia*. Sensitization of the animal resulted in an enhanced reaction of gill mantle contraction when a stimulus was applied later on. This behavior was traced to a sustained increase in adenylate cyclase activity in a neuron.

Interest in the adenylate system can also be attributed to its intricate mechanism. The system is without precedent in membrane biochemistry in that a neurotransmitter or hormone (H) binds to a receptor on the outer side of the cell membrane, controlling the activity of an enzyme on the inner side of the membrane; several distinct membrane proteins, present only in minute amounts, must interact; several small molecules from inside the cell are involved and reactions which deactivate or inhibit the system are operative at a number of steps.

Research on the action mechanism of the adenylate cyclase system is largely done on material from which highly purified cell membranes are readily obtained. The relatively simple biochemical composition of the avian erythrocyte membrane permitted several findings to be made which were confirmed only later in other systems. Also, many of the studies on the molecular mechanism of action of the receptor were performed on the beta-adrenergic receptor because of the availability of an almost inexhaustible arsenal of highly specific agonists and antagonists.

Summary. The adenylate cyclase system is composed of an activating hormone or neurotransmitter (H), its receptor (R), the guanosine triphosphate (GTP) binding protein (G_s), and the catalytic unit (C). The activation of the receptor R involves a transient change in conformation, from a loose binding of the neurotransmitter H to an extremely tight interaction, termed locking. The system is regulated in the activation steps and also by three deactivation processes. A guanosine triphosphatase activity is built into the G_s protein so that the active G_{sGTP} has only a limited lifetime during which it is able to activate C. In addition, the continued occupation of R by H causes desensitization of R. Finally, there are inhibitory receptors, such as α-adrenergic and opiate receptors, which inhibit the adenylate cyclase by way of a specific GTP binding protein (Gi). Yet to be determined are the conformational transformations of pure R on binding of an agonist or a partial agonist; the genes that code for the many different receptors that activate the adenylate cyclase, and the possibility that the G components interact with systems in the cell other than the adenylate cyclase.

Activation of the Adenylate Cyclase by Neurotransmitter

Three protein components are known to participate in the process: the neurotransmitter or hormone receptor (R), the guanyl nucleotide binding protein (G), and the catalytic unit of the adenylate cyclase (C) (*16*). A schematic description of the interaction of the components in activation and deactivation of the adenylate cyclase system is given in Fig. 1. The scheme emphasizes several points. The receptor R interacts with G and not with C. Thus, the catalytic unit of the adenylate cyclase system is activated by R indirectly. Once R has activated G by facilitating the binding of guanosine triphosphate (GTP) (*17*), R will not be required again to maintain adenylate cyclase activity until the GTP bound to G is hydrolyzed (*18, 19*). Thus G is visualized as shuttling between R and C (*20, 21*). The findings discussed below show that activation by a shuttling mechanism can indeed be experimentally set up. A different mechanism has, however, been suggested on the basis of kinetic analyses in the cell membrane (*22*). Accordingly, HR activates a stable GC complex by a "collision coupling" mechanism.

With the introduction of the hydrolysis resistant analogs of GTP, GPP(NH)P (*23*), and GTPγS (*24, 25*), it was shown that HR was indeed only required to facilitate the formation of the activated form of G, $G_{GPP(NH)P}$ (*19*) (Fig. 1). The adenylate cyclase remained fully activated even when a receptor blocking agent was later added which displaced the hormone from R. The specific receptors for the many different hormones and neurotransmitters which activate adenylate cyclase systems were shown to be interchangeable (*26, 27*). A theory suggesting that several receptors floating in the same cell membrane might all interact with a single adenylate cyclase had been proposed earlier (*28*). The existence of G as a separate entity became apparent when it was found that cyc^- cells (see *16*), which lack adenylate cyclase activity, were defective specifically in this protein (*29, 30*). Since C could still be activated by Mn^{2+} (*29*), it appeared that C, too, was a separate component.

Components and Their Interaction

R and the HRG complex. The first adenylate cyclase activating receptor, the β-R, was successfully purified to homogeneity about 2 years ago (*31, 32*). The molecule was shown to consist of a single chain of about 60,000 daltons, which may exist in the native cell membrane as a dimer (*33*). The purified protein is not only capable of specific ligand binding but also functions as a β-R. The β-R was implanted (*34*) in the cell membrane of *Xenopus* erythrocytes which possess an adenylate cyclase but no β-R. After implantation, the adenylate cyclase could be activated by β-R specific catecholamines (*35*). Recently, a reconstitution of a functional HRG system, consisting of the highly purified β-R and G, was constructed (*36*), indicating that no essential component is missing.

The key question in our trying to understand the role of R in the adenylate cyclase system is what R actually does when a neurotransmitter binds to it and how this is different from the interaction of R with an antagonist which also binds specifically to the neurotransmitter binding site (*7*). An early finding on the binding of labeled glucagon to its receptor in liver cell membranes gave the first clue (*37*). The affinity of R for the hormone was considerably higher in the absence of guanyl nucleotides than in their presence.

This finding was subsequently confirmed for most agonist-R systems which activate the adenylate cyclase. In the case of the β-R, for which a large number of specific blocking agents is available, it was demonstrated that the guanyl nucleotides only decrease the binding affinity of R for agonists but not for antagonists. These findings suggested that in the absence of guanyl nucleotides the activated HRG complex predominates and that this complex shows a relatively high

affinity for H (Eq. 1). When GTP is added, HRG dissociates (Eq. 2) and HR, which has a lower affinity for H then predominates (Eq. 3) (*21*).

$$H + RG \rightleftarrows HRG \quad (1)$$

$$HRG + GTP \rightarrow HR + G_{GTP} \quad (2)$$

$$HR \rightleftarrows H + R \quad (3)$$

Since antagonists do not produce an activated RG complex, their binding to R is not affected by guanyl nucleotides. Binding measurements are performed under steady-state conditions, giving the average affinity for all the R complexes and conformations in the system and not the actual characteristics of any particular molecular species.

Further insight into the activation process resulted from studies based on some earlier observations by Vauquelin *et al.* (*38*). The HRG complex was trapped, by alkylation of an -SH group, which was apparently located in the G component (Fig. 2) (*39*). When GTP at very high concentration was subsequently added, dissociation of the complex still took place, but in slow motion, requiring many minutes instead of a second or less in the native system. Fully functional R was released; H, which had been locked in R, was also released; and the G component was found to be inactive because of the alkylation. Thus in the normal process of interaction between the H-activated R and G, both proteins undergo a conformational change so that R transiently locks H while G exposes a previously masked -SH group as well as the site for GTP binding. GTP then enters and the HRG complex dissociates as in Eqs. 1 to 3. If, instead, an alkylating agent is added, the HRG complex is trapped. Somewhat similar observations were made by Heidenreich *et al.* (*39*) although they had no indication that functional R can be recovered from the trapped complex and therefore thought that alkylation takes place on R. The study by Korner *et al.* (*40*) also revealed that the site on G which interacts with R is different from the site which interacts with C. After alkylation of SH groups in absence of an agonist, G essentially lost its ability to activate a C component when assayed by fusion to cyc$^-$ cells (*20*). However, G still showed an undiminished interaction with R. When an agonist was added, the HRG complex was still formed, the masked -SH group became exposed, and the complex could be trapped by alkylation.

The specific conformational change of R, in its interaction with an agonist and the lack of such a conformational change in the interaction with antagonists was also studied in a simple system. The turkey erythrocyte β-R specifically locked previously bound agonist when deoxycholate was added (*41*). The response occurred even in absence of a functional G component. When deoxycholate was removed, the β-R returned to the low affinity interaction with the agonist; β-R also retained its full capacity to activate an adenylate cyclase system when implanted in a cell membrane after the detergent was removed. Since the G component was apparently not required for locking induced by deoxycholate, it was concluded that the detergent nonspecifically mimics the action of G in disrupting certain hydrophobic interactions that restrain the association of H with R (Fig. 3). Thus, the specific response of β-R to the binding of an agonist can be demonstrated and some of the properties studied at the level of β-R itself. It is thought that this locking of the agonist in the binding site represents the information which propagates along the R molecule, through the cell membrane, in its interaction with G (*7*).

Reconstitution of R function. A hormone responsive system was constructed from separate solubilized preparations of R and of G (*42*). After supplementation with phospholipids and removal of detergent, addition of hormone plus GPP(NH)P resulted in accumulation of the active $G_{GPP(NH)P}$ (termed G*). The

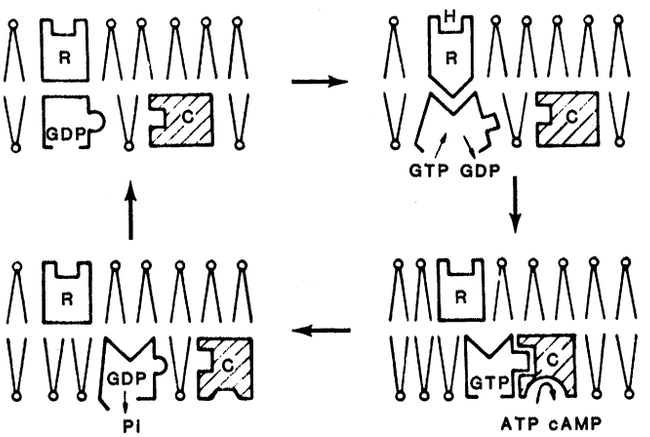

Fig. 1. Scheme of component interactions in the activation and inactivation of the adenylate cyclase system. A section of the cell membrane shows the three components (upper left). Phospholipid is represented by the polar heads with a pair of fatty acid tails interspersed between the components. When the neurotransmitter or hormone, H, binds to R, HR interacts with the binding protein G to release GDP and to facilitate the tight binding of GTP (upper right). The G binding protein thus activated by GTP associates with the catalytic unit C to form the active enzyme complex GC (lower right). Hydrolysis of the GTP at the G protein site, with release of inorganic phophorus (P_i), results in dissociation of G from C and cessation of enzyme activity (lower and upper left areas). R is arbitrarily drawn as limited to the outer layer of the cell membrane. There appears to be no evidence as yet that R transverses the entire membrane.

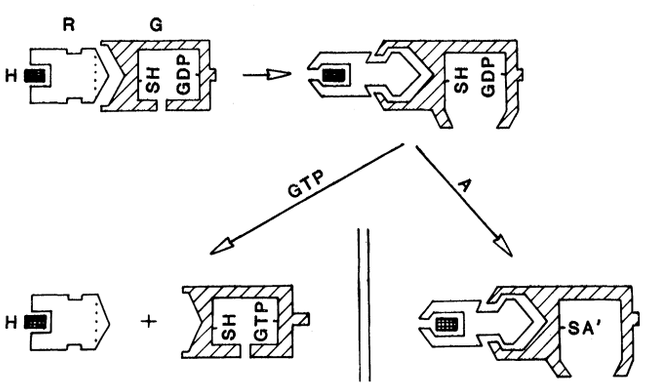

Fig. 2. Transient locking of H in R, the putative activated state of the HRG complex. (Upper left) The low affinity HR complex demonstrates a pointed recognition site for binding to the G protein. (Upper right) HR interacts with G in a mutual induced fit which locks H in R and exposes a specific -SH as well as the GTP binding site in G. (Lower right) Alkylating agent, A, reacts with the -SH group to form SA', thus trapping HRG in the conformation as above. (Lower left) The normal reaction sequence; GTP binds to its specific site in G, causing dissociation of the HRG complex. The activated G can now combine with C to produce the active adenylate cyclase (not shown).

amount of G* formed was subsequently determined by implantation in cyc^- membranes and measurement of the adenylate cyclase activity it produced by forming the G*C complex. The relative amounts of R and of G in the reconstituted HRG system could be varied, and it was thus possible to show that R indeed functioned catalytically, that the rate depended on the amount of R, as also indicated by studies in cell membranes (*43*), that functional C was indeed not required for R activity and that the rate limiting step was apparently not the encounter of R with G in the membrane but the activation of G in the HRG complex.

Further studies with the reconstituted system showed that $G_{GPP(NH)P}$ and G_{GDP} apparently compete for the same site on HR (*44*). However, $G_{GTP\gamma S}$ did not recognize that site on R. In line with these observations and in confirmation of earlier observations in the native membrane, HR in presence of GTP deactivated $G_{GPP(NH)P}$ but not $G_{GTP\gamma S}$ (*44*).

$$HRG_{GPP(NH)P} + GTP \rightarrow HR + G_{GTP} + GPP(NH)P \quad (4)$$

$$G_{GTP} \rightarrow G_{GDP} + P_i \quad (5)$$

These observations led to the conclusion that the two synthetic nucleotide triphosphates induce in G different conformations with respect to the interaction with R although both activate G with respect to C. $G_{GTP\gamma S}$ is probably closer to the natural GTP in its chemical structure and properties. The different properties of the two synthetic nucleotide triphosphates should be considered when they are injected into cells to probe the function and role of adenylate cyclase systems.

Recently, a hormone responsive system was reconstituted in phospholipid vesicles with the use of highly purified G from rabbit liver and β-R from turkey erythrocytes (*45*). The rate of H-stimulated G activation was followed by measurement of the binding of $GTP\gamma^{35}S$ (Eq. 6). The demonstration of the H-enhanced guanosine triphosphatase reaction in this system pointed to the importance of detergent removal and incorporation in phospholipid vesicles for reconstitution of the native properties of G (*45*).

$$H_\beta\text{-}RG + GTP\gamma^{35}S \rightarrow H_\beta\text{-}R + G_{GTP\gamma^{35}S} \quad (6)$$

Up until now the reconstitution of a complete R-G-C system has met with limited success (*46*). However, recent developments in the purification of C improve the chances for reconstitution.

Role of Mg^{2+} in the HRG interaction. Study of the adenylate cyclase from a number of cellular sources indicated that the neurotransmitters and hormones stimulate the system by increasing its affinity for Mg^{2+} as well as its maximal activity. The effect of Mg^{2+} affinity apparently involves the G component, which requires Mg^{2+} for its activation by GTP. At high Mg^{2+} concentration, G becomes activated even without the action of HR (*3, 21, 47*). However, Mg^{2+} has effects, presumably on G, also in the absence of guanyl nucleotides; it causes an increase in the binding affinity of β-R for the agonist, and it enhances the locked conformation of the HRG complex (*39, 40*). Even after inactivation of the G component, the locking of H in R, which is facilitated by deoxycholate, was shown to require Mg^{2+} (*41*), an indication that R itself may require Mg^{2+} for action.

Molecular properties of the Gs and Gi components. A new era in this field started when the purification of the Gs protein to homogeneity was achieved (*48*). This result proved that, despite the availability of only minute amounts of the adenylate cyclase components, their study and characterization as molecular entities is feasible. Gs is composed of two subunits, α, 45,000 daltons and β, 35,000 daltons (*49*). A third subunit, γ, of 10,000 daltons may also be part of the G component (*50*). The activation process is visualized in Eq. 7.

$$G_{\alpha\beta} + GTP\gamma S \xrightarrow{Mg^{2+}} G_{\alpha GTP\gamma S} + \beta \quad (7)$$

As the α subunit tightly binds the GTPγS, the β subunit dissociates. In absence of HR, this activation reaction requires a high Mg^{2+} concentration. Fluoride, in the presence of Al^{3+}, activates G to produce G-AlF_4^-, and this reaction appears to account for the well known, but hitherto enigmatic activation of adenylate cyclase by fluoride (*49*). The activated G_α subunit suffices to activate C when implanted in cyc^- membranes. The β subunit plays a role in inhibition of adenylate cyclase. These studies were done in the presence of detergent to keep the somewhat hydrophobic G component soluble. There is therefore little doubt about the interpretation of the findings with regard to subunit dissociation during activation. It remains, however, uncertain whether dissociation takes place in the native membrane. There are examples of subunit dissociation caused by small amounts of detergent when allosteric effectors bind to a protein (*51*).

The molecular properties of the Gi protein, which function in inhibition of adenylate cyclase, are similar to those of Gs. It has been obtained in homogenous form (*49, 52*) and like Gs, it is a heterotrimer, consisting of α, β, and γ subunits of 41,000, 35,000, and 10,000 daltons, respectively. The β subunits of Gi and Gs are identical (*49*), the activating process for Gi is also identical to that described in Eq. 7 for Gs. In the membrane, Gi activation is produced by certain neurotransmitters which interact with specific inhibitory receptors (Ri), as discussed below.

The catalytic unit of the system, C, is the component we know least about, mainly because of its lability. Bender and Neer (*53*) prepared from brain a fraction containing C but no G (*24, 25*). The C activity was stimulated and stabilized by the calcium binding protein calmodulin (*53*). Recently, the plant diterpene forskolin has been used in an affinity column (*54*) to partially purify C after it had been shown (*55*) that this agent strongly stimulates the adenylate cyclase. Forskolin appears to interact with C directly and still permits the interaction of G* with C.

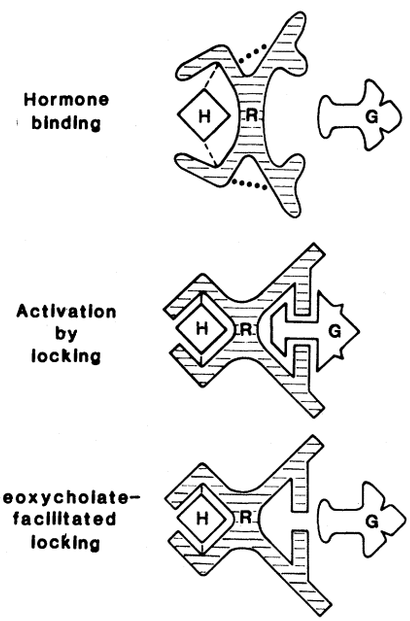

Fig. 3. A model of the conformational transition involved in R activation. (Top) The HR low affinity binding; R is restrained in its interaction with H by hydrophobic interactions within R, symbolized by the dotted lines. Such hydrophobic interactions might, however, operate equally well between R and the lipids in the cell membrane in which the entire system is embedded. (Center) G has interacted with HR to form the HRG activated complex. The forces involved in the formation of this complex have overcome the restraining hydrophobic interactions within R so that a close fit between the binding site and H is now possible. (Bottom) The detergent deoxycholate disrupts the hydrophobic interactions and thus facilitates locking of H in R even in the absence of G.

Lateral mobility of the components in the cell membrane. The shuttle mechanism as depicted in Fig. 1, as well as the collision coupling mechanism (*22*) for activation of the adenylate cyclase system, both suggest some lateral mobility of the components. The evidence previously discussed, showing that R can function catalytically (*42, 43*) also requires either R or G to be mobile. The functional implantation of an R preparation free of G in a foreign adenylate cyclase system (*41, 42*) proves that lateral mobility can be used for R-G coupling. However, in a study of cultured liver cells, Henis *et al.* (*56*) did not find evidence for lateral mobility of β-R, suggesting that, either mobility was occurring below the macroscopic level of the measurements or that G was perhaps the mobile component. After studying β-R in turkey erythrocyte membranes, Rimon *et al.* (*57*) concluded that membrane fluidity was limiting β-R function, apparently because of the nature of the lipids in these membranes (*58*).

Lipid requirements. The critical conformational changes that take place in the individual components suggest that interaction with certain membrane lipids plays an important role (*42, 45, 47*). A nonpurified β-R preparation, after extensive delipidation, had some rather specific requirements for reconstituting its agonist and antagonist binding properties (*59*). Phosphatidylethanolamine by itself was sufficient to restore specific binding to R, while acidic phospholipids were ineffective. Surprisingly, R could be reconstituted even without phospholipids; a mixture of cholesterol hemi-succinate and mono-oleylglycerol, at the proper molar ratio, effectively reconstituted specific binding to R. The specific role of the phospholipids in the HRG interaction remains to be determined.

Deactivation Processes in the Adenylate Cyclase System

To make a signal transmission system highly efficient it is required that there be not only a mechanism to turn it on, but also a procedure to turn it off. There are at least three different deactivation processes operating in the adenylate cyclase system, and these are considered in turn.

The Rs activated guanosine triphophatase. This reaction (*18*) deactivates G_{GTP} as a result of a slow hydrolysis of the tightly bound GTP (Figs. 1 and 4). Recent studies indicate that the hydrolytic activity is inherent in the purified Gs protein (*45*). The term R- or H- activated guanosine triphosphatase is somewhat misleading. As a result of the action of HR, more G_{GTP} is produced (Eqs. 1 to 3) and thus more substrate becomes available for guanosine triphosphatase action. This reaction ensures that, if neurotransmitter is removed or inactivated, cyclic AMP production will rapidly come to a standstill because all G_{GTP} will be converted to the inactive G_{GDP} (GDP, guanosine diphosphate). It was indeed calculated for the turkey erythrocyte system that because of the continuous action of the guanosine triphophatase only one-fifth of the adenylate cyclase is in the activated form at any given time, even in the presence of saturating amounts of H (*60*). The guanosine triphosphatase is suppressed when Gs undergoes adenosine diphosphate ribosylation by cholera toxin and this explains rather well the enhancement of adenylate cyclase activity by the toxin. The mechanism of action of the toxin and the role of cyclic AMP in cholera have been the subject of many studies (*61*).

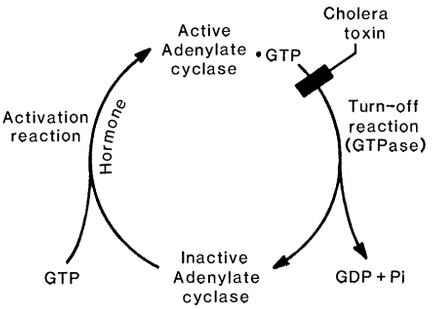

Fig. 4. The on-off cycle of the adenylate cyclase system. The figure illustrates the simultaneous activation and deactivation of the system, even in presence of H and GTP. The fraction of the total enzyme which is in the activated form at any time will depend on the ratio between the rate of activation and the rate of deactivation by the guanosine triphosphatase (GTPase). Cholera toxin increases the fraction of enzyme that is in the activated form, in proportion to its blocking effect on the guanosine triphosphatase. Other deactivation processes (discussed in the text) are disregarded in the drawing. [Courtesy of Raven Press, from (*50*)]

Desensitization and down regulation of R. Much of this work has been done on β-R of astrocytoma cultures by Perkins *et al.* (*62*), on C6 glioma by Fishman and co-workers (*63*), on erythrocytes by Lefkowitz and collaborators (*31, 64*), and on other receptors by several additional groups (32, 65). Occupation of R by an agonist for any length of time leads to a decrease in the activation of the adenylate cyclase. At least in one instance, desensitization seemed to be associated with phosphorylation of β-R (*31, 32*). Homologous desensitization refers to loss of stimulation of adenylate cyclase, limited to the specific R occupied by its agonist; other receptors, coupled to the same adenylate cyclase, retain the ability to activate the system when the respective agonists are added. Heterologous desensitization signifies that all receptors coupled to a single adenylate cyclase show a diminished ability to activate the system. In that case desensitization probably occurs at the level of Gs (*63*). In several instances, desensitization is followed by down regulation, the disappearance of receptors from the cell membrane as measured by neurotransmitter or antagonist binding. It has been shown for β-R in astrocytoma cells that the missing receptors, after a short incubation, can still be located in a light vesicle fraction when the cells are homogenized. It is likely that the vesicles were formed by a process of endocytosis. In frog erythrocytes the β-R in the vesicles apparently did not undergo any irreversible changes in the molecular structure; the receptors were found to be functional when coupled to the adenylate cyclase in *Xenopus* erythrocytes by the fusion-implantation procedure (*64*).

Desensitization and even down regulation may occur to an appreciable extent in 1 minute or less, whereas agonist binding to receptors on the cell is routinely assessed after 30 minutes of incubation intended to ensure steady-state conditions. Thus Pittman and Molinoff (*65*) showed that β-R on intact L6 cells demonstrate high affinity binding in the first minute, which rapidly changes to a low affinity during the standard incubation period of the binding assay. It has been suggested by Perkins *et al.* (*62*) that the lower affinity may actually reflect the diminished permeation of the hydrophilic agonist through the membrane of the vesicles in which the receptors are being segregated as discussed above.

Deactivation through the action of inhibitory neurotransmitters. In the early studies of Sutherland and colleagues, it was already noted that acetylcholine causes some inhibition of adenylate cyclase in heart membrane preparations (*1*). Sharma *et al.*, studying the effect of opiates on neuroblastoma cells, showed inhibition of adenylate cyclase by these drugs (*2*). They suggested that application of opiates to the cells in culture for an extended time led to a gradual increase in the amount of adenylate cyclase, so that when the opiate is removed, the concentration of cyclic AMP rises above the normal and that such a rise might trigger the well known withdrawal symptoms. The action of noradrenaline on the α-adrenergic receptors in platelets and adipocytes was also

characterized by strong inhibition of adenylate cyclase activity. In addition to the agonist, Na^+ ions were required for the action of the inhibitory receptors (*66*). The effect of Na^+ on binding of agonists to certain receptors in brain was found by Snyder and his collaborators (*9*). The first indication that GTP is also required for the inhibition of adenylate cyclase caused by certain neurotransmitters and hormones came from the work of Rodbell and co-workers (*67, 68*). Final proof for the separate existence of Gs and Gi came when both were obtained as highly purified proteins (*49, 52*). Just as cholera toxin had been most helpful in identifying and characterizing Gs, pertusis toxin now proved invaluable in the study of Gi. Katada and Ui first showed that the ADP ribosylation of a 41,000-dalton protein, catalyzed by the toxin, blocks the inhibitory action (*69*). In presence of the toxin, labeled NAD served to tag Giα specifically in order to monitor this component in electrophoretic separations and during purification (*49, 52*).

We now come to the question of what is the molecular mechanism by which the inhibitory receptors act. At present several possibilities are under consideration. The studies of Klee and colleagues (*70*) on the action of the opiate Ri demonstrated agonist specific stimulation of a guanosine triphosphatase. It was therefore thought that the inhibitory receptors might bring about a stimulation of this enzyme's activity in Gs, thus enhancing the deactivation of G_{GTP} (Eq. 5). Jakobs and Schultz (*71*), however, found action of Gi on C in cyc^- cells, which have no Gs; and they therefore suggested that the guanosine triphosphatase activity observed is inherent in Gi_{GTP} itself and represents the turn-off reaction of the inhibitory response, analogous to that of the stimulatory response already discussed in Fig. 4. The findings of Gilman and co-workers (*48, 49*) with highly purified Gs and Gi, suggest that the inhibition is due, at least in part, to the sequence of reactions in Eqs. 8 and 9.

$$\mathrm{HriGi}\alpha\beta + \mathrm{GTP} \rightarrow \mathrm{Hri} + \mathrm{Gi}\alpha_{\mathrm{GTP}} + \beta \quad (8)$$

$$\mathrm{Gs}\alpha_{\mathrm{GTP}} + \beta \rightarrow \mathrm{Gs}\alpha\beta + \mathrm{GTP} \quad (9)$$

Since the β subunits of Gi and Gs are identical, an excess of this subunit would accumulate in the steps shown in Eqs. 7 and 8 relative to the amount of the adenylate cyclase activating Gs. By mass-action the concentration of $Gs\alpha_{GTP}$ would decline as the rising concentration of the β subunit leads to association and thus deactivation (Eq. 9). Although there is experimental support for this formulation, there are also observations that are difficult to explain by this mechanism (*49, 52*). Thus inhibition can be demonstrated in subjects that lack the Gsα subunit. The probable interactions are summarized in Fig. 5.

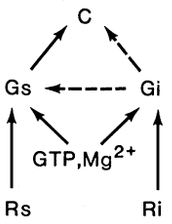

Fig. 5. Interactions of Gs and Gi in the adenylate cyclase system. Dotted arrows show presumed actions.

The cyclic AMP diesterase (*72*) is outside the scope of our article. It is mentioned here, however, because it represents the major pathway for actively decreasing cyclic AMP concentrations, whereas the above-discussed adenylate cyclase deactivation processes prevent an increase in cyclic AMP in the cell.

Implications for the Present and Future

Investigators have sometimes thought that cyclic AMP is not an intermediate in a given neurotransmitter or hormone response based on the following findings. The concentration of H producing half-maximal cyclic AMP concentrations in the cell (or half maximal adenylate cyclase stimulation in a membrane) was much higher than the concentration of H required to produce the half-maximal biological response being studied. The reason for this is that under ordinary conditions activation of only a small percentage of the receptors suffices to produce a maximal biological response. At such a low concentration of H it is not possible to obtain reliable measurements of cyclic AMP production. It is, however, often possible to measure the extent of activation of the cyclic AMP–dependent protein kinase (*73*) and thus to obtain an answer to the question whether adenylate cyclase stimulation is involved.

In contrast to other tissues where there are many identical cells, individual neurons or small groups often have specific properties and functions. In order to study the adenylate cyclase, the type of R coupled to the enzyme, and the effect on it by various stimuli, it is therefore necessary to assay adenylate cyclase activity in single neurons. Indeed, this task has been achieved (*5, 74*). The individual cell may also be treated with agents known to affect cyclic AMP production; GPP(NH)P or GTPγS can be injected; agonists and antagonists specific for receptors which stimulate or inhibit cyclic AMP production can be applied to the cell to observe the effect on induced or spontaneous electrical activity, transmitter release, phosphorylation of particular proteins, metabolism, axonal transport, or on other responses of that particular nerve cell.

When it was found that the different receptors are interchangeable, it was proposed that all the receptors that couple to adenylate cyclase have a similar structure, differing only in the specific site which binds the neurotransmitter or hormone (*26*). Now that the β-R has been purified to homogeneity, it might be possible to obtain antibodies to that part of R which couples to G. Such an antibody could also be useful for the purification of all other receptors that couple to adenylate cyclase because it would be expected to interact with any one of these. Further purification and molecular biological analyses may also provide information about the smaller differences, for example, between β_1 and β_2 receptors (*31*) and thus give us some idea of their structure-function relationships.

A number of investigators have already called attention to the fact that Gs and Gi seem to be part of a family of GTP binding proteins (*49, 75*). Thus, transducin, the light activated guanosine triphosphatase in the rod outer segments of the retina, has a subunit structure and properties strikingly similar to those of Gi and Gs (*76*). The *ras* gene products which bind GTP and are located in the cell membrane might also be part of this family (*49*). Since partial sequences of those proteins are being elucidated it will also become clear whether they are related to the well-known GTP binding factors that participate in protein synthesis, Tu and Ts in prokaryotes and EF in eukaryotes. It should also be emphasized that signal transmission from HR to G may not be exclusively channeled to C; it is possible that G, or a subunit thereof, might interact with other systems in the cell membrane (*49*).

The factors controlling the fundamental activity of the adenylate cyclase system (in the absence of H) have so far eluded the investigator. Now that β-R, Gs, and Gi are highly purified, it should be possible to assess the contribution of each when reconstituted into phospholipid vesicles. With the successful delipidation and relipidation (*59*) the role of specific lipids in the above-discussed interactions should also become clearer.

A quarter of a century after the discovery of cyclic AMP (*1*) a thorough understanding of this transmembrane signaling system now seems within reach.

References and Notes

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16. G (or G/F), the guanyl nucleotide binding protein that activates the catalytic unit of the adenylate cyclase. F signifies that this protein component is the one that interacts with fluoride (AlF_4), resulting in activation of adenylate cyclase. Activation by fluoride thus bypasses the R component. Gs (or Ns), refers to the same G as above, emphasizing its stimulating function in the adenylate cyclase system and distinguishing it from Gi (or Ni), the guanyl nucleotide binding protein that functions in the inhibition of adenylate cyclase. GPP(NH)P, a synthetic analog of GTP in which the oxygen between the β and γ phosphates has been replaced by an imino group. Since it is not hydrolyzed by phosphatases it produces persistent activation of Gs and Gi. GTPγS, an analog of GTP in which an oxygen on the gamma phosphate is replaced by sulfur. It is relatively stable to guanosine triphosphatases and therefore acts like GPP(NH)P. G_{GTP}, $G_{GTP\gamma S}$, $G_{GPP(NH)P}$, G*, and G_{GDP}, refer to the G protein to which the respective guanyl nucleotide is tightly bound. The first three represent activated forms of G which in turn activate C. The guanosine diphosphate–containing component is the inactive form of G. S49-cyc$^-$ or Ac$^-$, a variant of an S49 lymphoma cell line which lacks a functional Gs but possesses β-R and C. When functional Gs from other sources is introduced into the cell membrane of cyc$^-$, it restores all the functions of the adenylate cyclase system. The membranes of these cells therefore serve as a convenient and specific assay system for Gs. When $G_{GTP\gamma S}$ or $G_{GPP(NH)P}$ is introduced into cyc$^-$ membranes, high spontaneous adenylate cyclase activity is produced as a result of the formation of the active complex G*C. When first characterized, this cell line showed almost no adenylate cyclase activity and it was accordingly termed AC$^-$ or cyc$^-$; only later did it become apparent that this was due to a lack of Gs and not of C. Locking, a dramatic shift of R from low affinity for the agonist to an extremely tight interaction.
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