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Discovery of G Protein Signaling

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Key Words

adenylyl cyclase, catecholamine, GTPase, cholera toxin, GTPase cycle, β-adrenergic receptor

Abstract

The mechanism of transmembrane signaling by the receptor-activated adenylyl cyclase was an enigma. It was suggested that hydrolysis of GTP is a turn-off mechanism that resets the active adenylyl cyclase to the inactive state. To test this hypothesis, we developed a specific GTPase assay and found that the catecholamine adrenergic agonists stimulated the hydrolysis of GTP. To resolve the question of how the hormone concurrently stimulates GTP hydrolysis and activates the adenylyl cyclase, we suggested the regulatory GTPase cycle. Thus, because the hormone facilitates the binding of GTP, which is subsequently hydrolyzed, the regulatory cycle results in a hormone-stimulated GTPase activity. This model also predicts that two mechanisms could account for stimulation of adenylyl cyclase activity—either by the familiar hormone stimulation of the activation reaction or by an inhibition of the turn-off reaction. Indeed, we showed that cholera toxin enhances adenylyl cyclase activity by inhibition of GTP hydrolysis. Finally, we also showed that the hormone-activated receptor stimulates adenylyl cyclase activity by facilitating the exchange of bound GDP for free GTP. Thus, we presented, for the first time, an explicit mechanism for receptor action.

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INTRODUCTION

I finished high school at age 16 ½, and four years of army service (my parents had formally consented to my joining the army ahead of time) then transformed me from an adolescent to an officer bearing great responsibility. My initial plans to become a naval officer did not work out. As usual, the army had other plans; I was ordered to do what I was trained for and served as an instructor in the basic officer school. My response was to leave the army at the end of my compulsory service and to study medicine. My reason was that, as a medical doctor, many more career opportunities would be open to me. Indeed, after the second year of medical school, I tried a change of course. I received a stipend, which allowed me to interrupt my medical studies and spend a year doing research at the Hebrew University in the Department of Chemical Microbiology, headed by Professor Shlomo Hestrin. At the end of this year, I was invited to enter a Ph.D. program and forget about medicine. Although I greatly enjoyed doing research, I decided to keep all my options open, so I returned to complete my medical studies.

At that time, the university also made fundamental changes. The Department of Chemical Microbiology was transferred from the medical school to the faculty of science, acquired the name of Biological Chemistry, and was relocated to an empty building right on the border between the old city of Jerusalem, then under Jordanian rule, and the Israeli part of Jerusalem. On the roof of the building, there was a sign "beware of snipers," but this looked normal to us. Naturally, at the time, this was not a populated part of Jerusalem. Professor Hestrin recognized that there was unlimited space at this location, and he embarked on an extensive conversion of the empty building into modern laboratories, a hobby that he enjoyed as much as research. Unfortunately, Professor Hestrin died suddenly; Mickey Schramm then became acting head of the new department.

By that time, my internship was finished, and the question of what to do next became a reality. I had offers of residencies in surgery and in internal medicine, as well as to return to the Department of Biological Chemistry. I learned, however, that in hospital medical practice you have very little freedom to make your own decisions and that you must do as you are told by your seniors. This is not the case in research, where you have complete freedom of choice in what you decide do. These considerations brought me back to the Department of Biological Chemistry, a decision that I never regretted.

In the department, Schramm believed there should be a central research theme, and he identified membrane biochemistry as

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an emerging field that would become important in the near future. Schramm developed the rat parotid gland as a model system to study exocytosis during enzyme secretion from exocrine glands. He discovered that this secretion is initiated by activation of the β-adrenergic receptor coupled to adenylyl cyclase, producing cyclic AMP that acts as a second messenger of the process (1). Although we made considerable progress in the study of the parotid gland, it was clear that the parotid was not suitable for studying the mechanism of transmembrane signaling. Schramm argued that we should concentrate all our efforts on one system and not spread ourselves too thin if we wished to understand the mechanism of adenylyl cyclase at the molecular level. Schramm spent considerable periods of time in Martin Rodbell's laboratory at the National Institutes of Health (NIH) in Bethesda, Maryland, and was very familiar with their studies of the rat liver glucagon-stimulated adenylyl cyclase (2, 3) and of the isolated fat cell system, whose adenylyl cyclase could be stimulated by six different hormones (4). Both of these systems, however, had high background activity and lacked hormone antagonists that could block hormone action. Work of Oye & Sutherland (5) showed that avian erythrocyte has an active β-adrenergic-stimulated adenylyl cyclase. These cells were terminally differentiated, having a nucleus but lacking internal membranes and protein biosynthesis activities. Schramm suggested that they could serve as a source of a simplified membrane preparation. After preliminary experiments, we settled on turkey erythrocytes, which were available in Israel in abundance all year round—not only on Thanksgiving Day. In retrospect, we realized that the simplified turkey erythrocyte membrane, with a low background of the multiple guanine nucleotide-binding proteins normally present in eukaryotic cells, was crucial to our studies on the role of guanine nucleotides in the regulation of adenylyl cyclase activity.

THE CATECHOLAMINE-STIMULATED GTPase

The turkey erythrocyte adenylyl cyclase activity proved stringently dependent on activation of the β-adrenergic receptor. In the absence of agonist, there was negligible adenylyl cyclase activity, whereas addition of isoproterenol, a selective hormone agonist of the β-adrenergic receptor, caused a 20–50-fold increase in cyclase activity. On its face, this was an ideal system to study the hormone stimulation of adenylyl cyclase.

At that time, we had at the department four scientists working with their students on the turkey erythrocyte adenylyl cyclase: Mickey Schramm, Alex Levitzki, Daphne Atlas, and myself. It seemed a little bit crowded, but this provided a critical mass for discussion and for learning from one another. Because everybody else was interested in the receptor and its mechanism of action, I decided to work on the role of guanine nucleotide in the adenylyl cyclase. In particular, I was influenced by the new discoveries about guanine nucleotide and their effects on the adenylyl cyclase that came from Rodbell and his colleagues. Rodbell discovered that GTP had some stimulatory effects on the glucagon-stimulated adenylyl cyclase activity of rat liver membranes. Its main effect, however, was on the binding of iodinated glucagon to rat liver membranes. The binding of labeled glucagons did not follow the activation of the adenylyl cyclase, and even more disturbing, the binding of glucagons was quasi irreversible because subsequent addition of excess unlabeled glucagon did not cause the release of prebound labeled glucagon from the membranes (2, 3, 6). Realizing that different compositions of the test system were used to monitor the binding of glucagon to the membranes and the assay of adenylyl cyclase activity, they found that ATP, which is used as a substrate for the adenylyl cyclase, enhances the release of prebound labeled glucagon. Testing other nucleotides, they found that GTP was effective in enhancing the release of glucagon from the liver membranes at 1000 times lower

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concentrations than that of ATP. Interestingly, the only other nucleotide able to reduce the binding of glucagon was GDP.

Trying to resolve the question of possible contamination of ATP by GTP, Rodbell and coworkers (2, 3, 6) synthesized AppNHp, an analogue of ATP that could serve as substrate for the adenylyl cyclase reaction but not for the phosphoryl transfer between ATP and GDP. Using AppNHp as a substrate, they showed marked stimulation of the adenylyl cyclase reaction by GTP. Rodbell also synthesized GppNHp to find out whether GTP acts as a phosphoryl donor or as an allosteric effector. In the rat liver preparation, both GTP and GppNHp increased the activity of the glucagon-stimulated adenylyl cyclase, a finding that was inconsistent with a reaction of phosphoryl transfer because GppNHp does not serve as a phosphoryl donor. A clue to the action of GppNHp came from studies in Rodbell's laboratory on the action of GppNHp in adenylyl cyclase systems other than the glucagon-stimulated adenylyl cyclase. These studies proved that GppNHp is a "superstimulator" of all adenylyl cyclase systems tested and, in many cases, was much more effective than GTP (7). Of particular importance, however, was a study by Schramm and Rodbell performed when Schramm spent a sabbatical in Rodbell's laboratory at the NIH. They showed that in frog erythrocytes, GppNHp differs from GTP not only quantitatively by giving higher activation of the adenylyl cyclase, but the activation by GppNHp was also qualitatively different than that caused by GTP (8). Whereas activation of basal adenylyl cyclase activity stimulated by GTP in the absence of hormone was minimal, activation by GppNHp was much larger and increased with time. Addition of the hormone caused practically instantaneous activation by GTP, whereas the hormone mainly decreased the lag time for activation by GppNHp. Furthermore, the activation by the hormone and GppNHp was resistant to the subsequent addition of the hormone antagonist or repeated centrifugal washing, retaining a persistent active state of the adenylyl cyclase that no longer required the presence of hormone for activity (8). These studies indicated that the differences between GTP and GppNHp could be due to the resistance of GppNHp to enzymatic hydrolysis, with the assumption that hydrolysis of GTP is a turn-off signal, resetting the active adenylyl cyclase to the inactive state. Although GTPase activity as part of the adenylyl cyclase was considered a possibility and was even tested, no real effort was made to verify that the measured GTPase was related to the adenylyl cyclase system.

At that time, Dan Cassel, a graduate student, came to my group, and he was assigned to develop a highly specific assay of GTPase activity in turkey erythrocyte membranes. Cassel was a superb student with excellent experimental skills and greatly contributed to the success of our adenylyl cyclase project. Our strategy in developing the GTPase assay was to take into consideration the high affinity and extreme selectivity for GTP in the stimulation of adenylyl cyclase activity. Plasma membranes typically contain abundant nucleoside triphosphatases that do not discriminate between ATP and GTP. We found that these enzymes have low affinity for nucleotides. Thus to reduce their interfering hydrolysis of GTP, we decreased the concentration of GTP in the GTPase assay to low levels that barely saturated the high affinity of the GTP effect on adenylyl cyclase. The second measure to suppress the nonspecific nucleoside triphosphatases was to add AppNHp, a hydrolysis-resistant analogue of ATP, at high concentrations. This analogue further suppressed the nucleoside triphosphatases, which do not discriminate between adenine and guanine nucleotides. Although both of the above measures reduced the nonspecific hydrolysis of GTP, considerable hydrolytic activity still remained. I told Cassel that, as part of good biochemical practice, we should analyze the reactants and products of our GTPase reaction. Using [γ-^{32}P] GTP as a substrate,

followed by chromatographic analysis, revealed that within one minute of incubation the membranes caused about an equal redistribution of ^{32}P in both GTP and ATP. Apparently the membrane contained nucleoside diphosphokinase, which in the presence of traces of ADP leads to redistribution of radioactivity between guanine and adenine nucleotides. The ^{32}P-ATP, which accumulates during the reaction, is probably hydrolyzed by specific ATPases and contributes significantly to liberation of radiolabeled inorganic phosphate, ^{32}Pi, in the GTPase assay. To eliminate this interfering activity, we added to the GTPase assay both ATP and the nucleoside triphosphate regeneration system that converted the endogenous ADP to ATP, thereby eliminating the acceptor for the transfer reaction. Indeed, these additions eliminated the transfer of the labeled phosphate to adenine nucleotides and thereby caused a significant decrease in nonspecific GTP hydrolysis (**Table 1**).

Membranes were incubated in the presence of 0.25 μM [γ^{32}-P] GTP. 5 mM $MgCl_2$, 50 mM imidazole-HCl buffer (pH 6.7) and the other additions, as indicated. The ATP regeneration system was 2 mM creatine phosphate and 3 units of creatine phosphokinase.

To test whether the remaining GTPase reaction after these additions was influenced by activation of the β-adrenergic receptor, we added isoproterenol to the assay system. Addition of this β-adrenergic selective catecholamine agonist caused a 30% to 70% increase in GTP hydrolysis. The catecholamine-stimulated GTPase reaction showed the same affinity for isoproteranol and GTP, as well as stereoselectivity for L-epinephrine and not D-epinephrine, as activators of the adenylyl cyclase through the β-adrenergic receptor (**Figure 1**). The effect of activation of the β-adrenergic receptor on the rate of the GTPase reaction was small. However, it was very reproducible and, despite the many additions, was easy to perform. The inset in **Figure 1** reveals that the increment

Table 1 Hydrolysis of GTP by turkey erythrocyte membranes

Additions	[γ^{32}-P] GTP hydrolysis (pmol/mg protein per min)[a]
None	1200 ± 50
0.5 mM App(NH)p	19.4 ± 0.2
0.5 mM App(NH)p + ATP regeneration	9.5 ± 0.2
0.5 mM App(NH)p + ATP regeneration + 0.1 mM ATP	7.4 ± 0.6

[a]The data are the mean ± half range of duplicate determinations. Reprinted with permission from Reference (9).

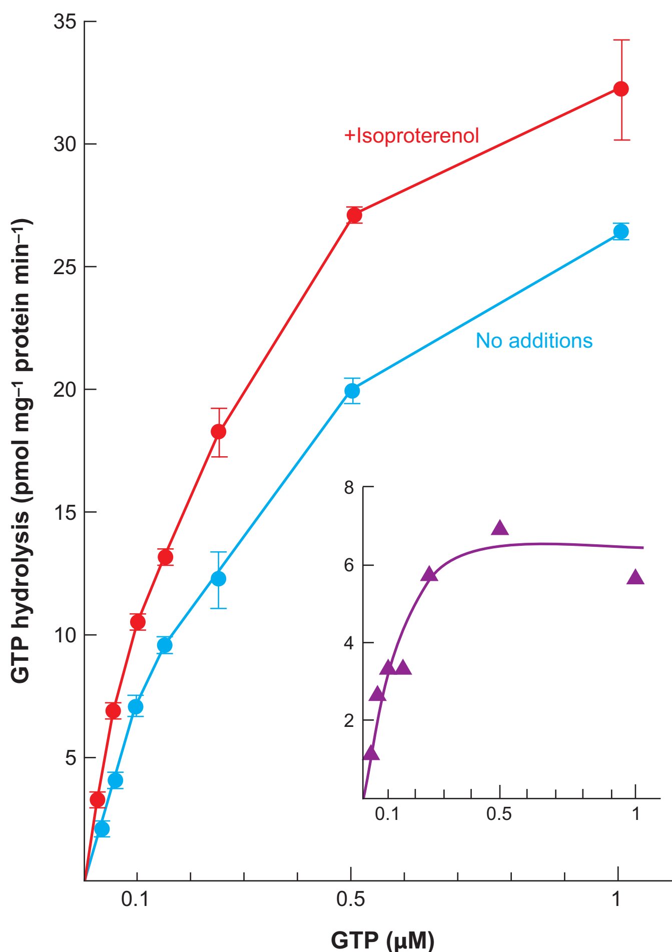

Figure 1

Rate of GTP hydrolysis by turkey erythrocyte membranes at various GTP concentrations. The inset shows the increment in GTP hydrolysis caused by addition of isoproterenol, i.e., the catecholamine-stimulated GTPase. Reprinted from Reference 9. Abbreviation: pmol, picomole.

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in GTPase activity, caused by addition of isoproterenol, saturates at submicromolar concentrations, showing that it indeed has a high affinity for GTP. We were, however, concerned whether the catecholamine-stimulated GTPase was mediated by direct action of the β-adrenergic receptor or was caused indirectly by cyclic AMP, which was formed during the GTPase reaction and, in turn, might activate the GTPase through the cyclic AMP-dependent kinase. To rule out this possibility, we added cyclic AMP directly to the GTPase assay in the absence of hormone and found that it had no effect. Furthermore, we found conditions that inhibited the catecholamine-stimulated production of cyclic AMP while preserving the catecholamine-stimulated hydrolysis of GTP, thus definitely showing that the catecholamine-stimulated GTPase was not dependent on production of cyclic AMP (9).

TROUBLES ARE GOOD FOR YOU

Once we finished the characterization of the catecholamine-stimulated GTPase, we submitted a manuscript containing our findings to *The Journal of Biological Chemistry*. We described the assay based on the high affinity and specificity of the GTPase reaction in turkey erythrocyte membranes and the properties of the catecholamine-stimulated GTPase activity that were revealed by this assay. We concluded with the proposal that the catecholamine-stimulated GTPase is a component of the turkey erythrocyte adenylyl cyclase system and suggested that the role of the GTPase is to reset the active adenylyl cyclase to the inactive state. When we received the comments of the reviewer, we were rather surprised to read the following two sentences: "This is a Prejudice and not Science. If the hormone has any effect on the GTPase, it should inhibit rather than stimulate the hydrolysis of GTP." My polite answer to the editor was that we were not in the business of arguing what the hormone should or should not do, but rather we were reporting what the hormone did do. This answer was to no avail. Consequently, the paper was sent to another journal (the *Biochimica et Biophysica Acta*) and was published without difficulty (9).

In retrospect, the comments by the reviewer were helpful to us. We realized that people do not understand how the hormone can, on the one hand, activate the adenylyl cyclase and, on the other hand, accelerate the reaction that turns it off. In our first paper, we did not explicitly explain how one could possibly account for these seemingly contradictory actions of the hormone because we felt that it should be supported by additional experiments. What we tried to do next was to demonstrate directly, by binding experiments, that activation of the receptor by the hormone increases the binding of hydrolysis-resistant GTP analogues to the membranes and that this binding blocks the catecholamine-stimulated GTPase activity. Direct binding of GppNHp did not show any increase in hormone-dependent binding of the nucleotide analogue because of a high background of hormone-independent binding; however, we were able to show that if hormone and GTP were added to a membrane preparation that was preactivated by hormone and GppNHp, there was a decrease in adenylyl cyclase activity and a hormone-dependent release of GppNHp from the membrane (10). We were more successful in another study (11) when we showed that if hormone and GTP were added to a membrane preparation that was preactivated by hormone and the hydrolysis-resistant analogue, GTPγS, a decrease in adenylyl cyclase activity did not occur; and as we expected, this addition also did not relieve the inhibition of the catecholamine-stimulated GTPase activity. The difference between the effects of GppNHp and GTPγS is probably because GTPγS has a 200-fold higher affinity for the adenylyl cyclase than does GppNHp. The supporting evidence that we obtained led us to think about the regulation of adenylyl cyclase by GTP in terms of two reactions: An "on"

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reaction after activation by the hormone-bound receptor owing to an increase in the accessibility of the regulatory site for GTP, followed by a "turn-off" reaction caused by hydrolysis of the bound GTP. We believed, however, that we needed more experiments before we could create such a model. These experiments came from our studies of adenylyl cyclase activation by cholera toxin (12) and from detailed kinetic analysis of the activation and turn-off reactions (13).

MECHANISM OF ADENYLYL CYCLASE ACTIVATION BY CHOLERA TOXIN

Regulation of adenylyl cyclase activity by on and off reactions predicts that it should be possible to activate the adenylyl cyclase either by accelerating the on reaction or by inhibiting the off reaction. The hormone-activated receptor activates the on reaction. We needed an example of an agent that activates the adenylyl cyclase by the second mechanism, of inhibition of the turn-off reaction.

The similarity between the effect of GTP on the adenylyl cyclase and on protein biosynthesis was known to us. Each step of protein synthesis binding of GTP facilitates association of the guanine nucleotide-binding factor with the ribosome to form an active complex, whereas hydrolysis of the bound GTP results in dissociation of the complex (14). Thus, each step generates a GTPase cycle. Interestingly, when I gave a short report on the catecholamine-stimulated GTPase in a Gordon conference and pointed to the possible mechanistic similarity between the adenylyl cyclase and protein biosynthesis, this concept was not understood. Immediately, somebody in the audience raised his hand and said, "The similarity that you are suggesting is probably unlikely because GppNHp inhibits protein biosynthesis, whereas it maximally stimulates the adenylyl cyclase activity." My response—"these two systems use two different assay conditions"—was not understood, and the listener's reply was, "Zvi, that is exactly what we are saying, these are two different systems." In the heat of the discussion, there was no time to explain that protein biosynthesis measures multiple successive incorporations of amino acids into the growing peptide chain. Therefore, freezing the system by a GTP analogue that was not hydrolyzed would result in inhibition, whereas freezing the adenylyl cyclase in the active state by a hydrolysis-resistant GTP analogue would result in stimulation. So we left it there, and the discussion moved on to other topics.

Another analogy between protein biosynthesis and the adenylyl cyclase was manifested by the action of certain bacterial toxins, the diphtheria and cholera toxins, and one more similarity was the finding that the effect of both toxins was dependent on NAD. The diphtheria toxin caused the inhibition of protein biosynthesis by ADP-ribosylation of the eukaryotic elongation factor 2 (15), whereas treatment with cholera toxin caused an NAD-dependent stimulation of the adenylyl cyclase by a mechanism, which at that time was unknown. The similarity between the two systems was too tempting to ignore. I found out that a joint Japanese-American conference on the study of cholera toxin was to be held soon in Atlanta, Georgia. To bring us up to date on the latest developments, I signed up, boarded an airplane, and arrived in Atlanta on the second day of the conference. Much to my surprise the Japanese scientist on the podium was lecturing in Japanese. It took me some time to find the earphones, which provided a simultaneous translation into English. The conference was mainly clinically oriented, so was mostly not relevant to my concerns, but I learned what I really needed. Michael Field (16) and his colleagues had developed conditions for the activation of membrane preparations of adenylyl cyclase by cholera toxin, using NAD, concentrated cell cytosol, and ATP. That was exactly what we needed for our studies, which were aimed at evaluating the effect of cholera toxin on the adenylyl cyclase and the catecholamine-stimulated GTPase of turkey erythrocyte membranes.

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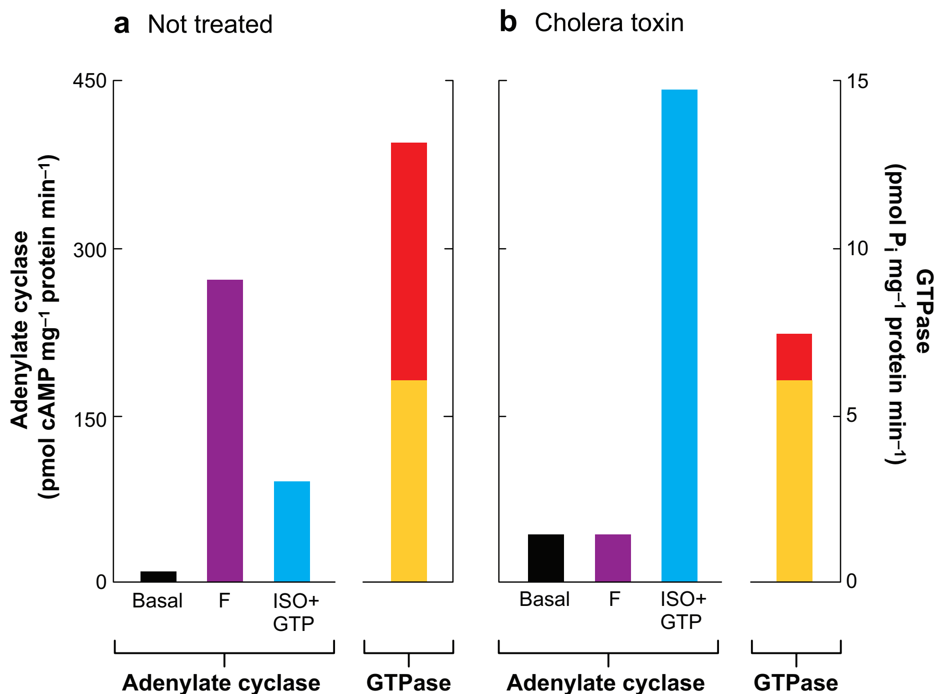

Figure 2

Cholera toxin treatment increases the ISO+GTP-stimulated adenylyl cyclase and simultaneously decreases the catcholamine-stimulated GTPase (*red bar*). The basal GTPase activity was not affected (*yellow bar*). Abbreviation: F, fluoride-stimulated adenylyl cyclase; ISO + GTP, isoproterenol plus GTP-stimulated adenylyl cyclase. Reprinted from Reference 12.

Treatment of turkey erythrocyte membranes with cholera toxin in the presence of erythrocyte cytosol, NAD, dithiothreitol, and ATP dramatically changed the profile of the adenylyl cyclase activities (**Figure 2**). The previously insignificant basal activity was increased by cholera toxin treatment about tenfold, and the response to the β-adrenergic agonist isoproteranol plus GTP was increased by fivefold, reaching the level of adenylyl cyclase activity determined in the presence of hormone and hydrolysis-resistant GTP analogues. The fluoride-stimulated adenylyl cyclase (marked F in **Figure 2**) was severely decreased by cholera toxin treatment. At that time, we did not understand the effect of cholera toxin on the fluoride-stimulated adenylyl cyclase, and it was not studied further by us. As shown in **Figure 2**, toxin treatment selectively inhibited about 80% of the catecholamine-stimulated GTPase activity (the red bar in **Figure 2**), whereas it had no effect on the basal GTPase activity. To test the cause-and-effect relationship between the toxin-induced inhibition of the catecholamine-stimulated GTPase and activation of the hormone-stimulated adenylyl cyclase, we studied these effects at different concentrations of cholera toxin. Indeed, increasing the concentration of the toxin caused progressive inhibition of the catecholamine-stimulated GTPase activity and elicited a parallel increase in the basal- and isoproterenol-plus GTP-stimulated adenylyl cyclase activities. A litmus test for the above cause and effect was based on the following argument. We anticipated that if cholera toxin activated the adenylyl cyclase through inhibition of the GTPase reaction, then after toxin treatment, GTP should act like a hydrolysis-resistant

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analogue and cause maximal and persistent activation of the adenylyl cyclase that is resistant to subsequent additions of the receptor antagonist propranolol. We indeed found that, in the cholera toxin-treated preparation, activation of the adenylyl cyclase by hormone plus GTP followed by the addition of propranolol to stop the activation reaction showed a slow decay of activity with a half-life of about three minutes. This decay was strikingly different in a membrane preparation that was not treated with cholera toxin. In this case, the adenylyl cyclase activity, following the addition of the antagonist propranolol, decayed within a few seconds and was too fast to be accurately measured (12).

These experiments thus indicated that the half-life of GTP at the regulatory site determines the extent of the adenylyl cyclase activity. Our first model describes the regulation of the adenylyl cyclase by on and off reactions as well as the effect of cholera toxin, as shown in **Figure 3**. At that time, we did not know that after hydrolysis of GTP, the product GDP remains bound to the membrane, whereas Pi is released into the medium. Finding an example of a second mechanism of activation of the adenylyl cyclase, mediated by an inhibition of the turn-off reaction by cholera toxin, was a turning point. Unlike the results of our previous studies, it was widely accepted and appreciated. In part, this was because we solved the mechanism of action of a major disease that remained unknown for many years.

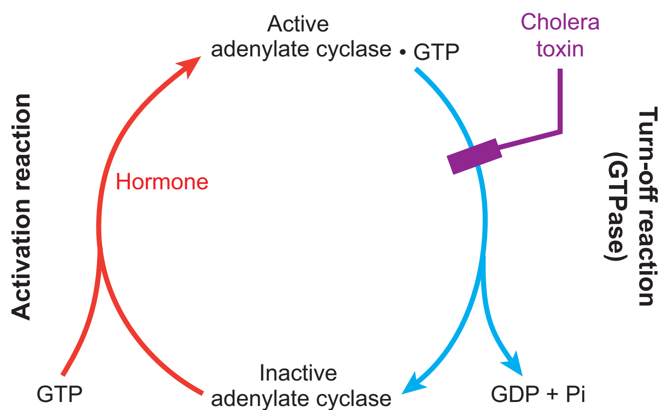

Figure 3

Model of cholera action on the regulatory GTPase cycle. Reprinted from Reference 12.

THE REGULATORY GTPase CYCLE

Our model suggested that adenylyl cyclase activity is controlled by a regulatory cycle consisting of two reactions: A hormone-facilitated formation of the active adenylyl cyclase-GTP complex and a subsequent turn-off reaction in which hydrolysis of the bound nucleotide resets the system to the inactive state. Although we provided extensive and multifaceted support for our model, we were not satisfied. We believed that our model should be tested by quantitative analysis—preferably by two independent methods, which could be compared with one another. To this end, we developed methods to assay separately the activation and the turn-off reactions, and their rate constants were derived from kinetic analysis of each reaction. Substitution of these rate constants in the steady-state equation of the regulatory GTPase cycle gives rise to the fraction of adenylyl cyclase, which is present in the active state under steady-state conditions. These values can be compared with direct measurements performed by assay of adenylyl cyclase activity in the presence of hormone plus GTP (representing the fractional active adenylyl cyclase) and in the presence of hormone plus a hydrolysis-resistant analogue, such as GppNHp or GTPγS (representing the total adenylyl cyclase) (8).

Determination of the Rate Constant of the Adenylyl Cyclase Activation Reaction

Activation of the adenylyl cyclase by hormone and GppNHp represents the net activation reaction because, unlike GTP, the analogue GppNHp is not hydrolyzed at the regulatory site and the turn-off reaction does not take

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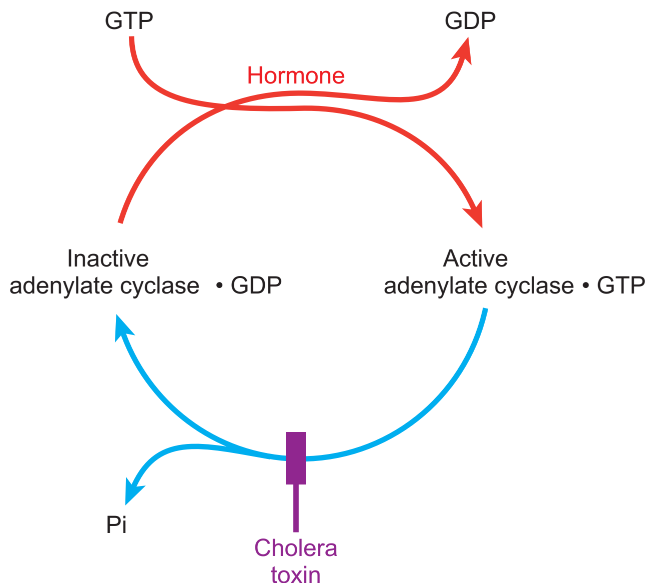

Figure 4

Model for hormonal regulation of adenylyl cyclase activity. The inactive adenylyl cyclase contains tightly bound GDP. The hormone-bound receptor acts as guanine nucleotide exchange factor, facilitating the exchange of bound GDP for free GTP, thereby activating the adenylyl cyclase. Subsequent hydrolysis of the bound GTP resets the system to the inactive state. Cholera toxin inhibits the hydrolysis of the bound GTP, thus increasing adenylyl cyclase activity. Abbreviation: Pi, inorganic phosphate. Reprinted from Reference 18.

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place (7). To monitor the activation reaction, we preequilibrated the membranes for two minutes with epinephrine and with all the other ingredients of the adenylyl cyclase assay system, except that only unlabeled ATP was present at this stage. The activation reaction was initiated by the addition of either GppNHp or GTPγS. At various time points after initiation of the activation reaction, aliquots were taken for assay of adenylyl cyclase activity in the presence of (α-^{32}P) ATP and propranolol. Propranolol stops further activation of the adenylyl cyclase, whereas the labeled ATP gives rise to labeled cyclic AMP, which in turn serves to measure the extent of adenylyl cyclase activity. Activation by epinephrine with either GppNHp or GTPγS followed a first-order kinetics with the same rate constant of 3 min^{-1} (13).

Decay of the Adenylyl Cyclase Activity as a Measure of the Turn-Off Reaction

If one assumes that hydrolysis of GTP at the regulatory site stops the adenylyl cyclase activity, it is not immediately obvious how the hormone, on the one hand, can increase the adenylyl cyclase activity and, on the other hand, can accelerate the hydrolysis of GTP that turns off the adenylyl cyclase activity. To reconcile these seemingly contradictory observations, we suggested that the hormone plus GTP-stimulated adenylyl cyclase system repeatedly oscillates between the inactive and active states. This results in a regulatory cycle in which GTP is continuously hydrolyzed. The hormone stimulates the hydrolysis of GTP by virtue of its role in initiating the regulatory cycle (**Figure 4**). One should, therefore, distinguish between a GTPase activity, meaning the operation of the whole regulatory cycle (which includes both the activation and turn-off reactions of the adenylyl cyclase) and a turn-off GTPase reaction, referring only to that part of the cycle in which hydrolysis of the bound GTP terminates the activity of the adenylyl cyclase (**Figure 4**). Currently these two GTPase activities are called the steady-state GTPase activity and the one-cycle GTPase activity, respectively.

In order to study the turn-off reaction, the decay of the adenylyl cyclase activity was measured in a system that was activated by epinephrine plus GTP, followed by addition of the β-adrenergic blocker propranolol to prevent further activation. Because propranolol prevents the regeneration of an active adenylyl cyclase-GTP complex, the decay of activity is a measure of GTP hydrolysis at the regulatory site. The addition of the antagonist resulted in a rapid decline of adenylyl cyclase activity, whereas the control system, which did not receive propranolol, maintained undiminished activity. As the decay process was too rapid to be accurately monitored, the rate constant of the decay was calculated from the total amount of cAMP formed during the

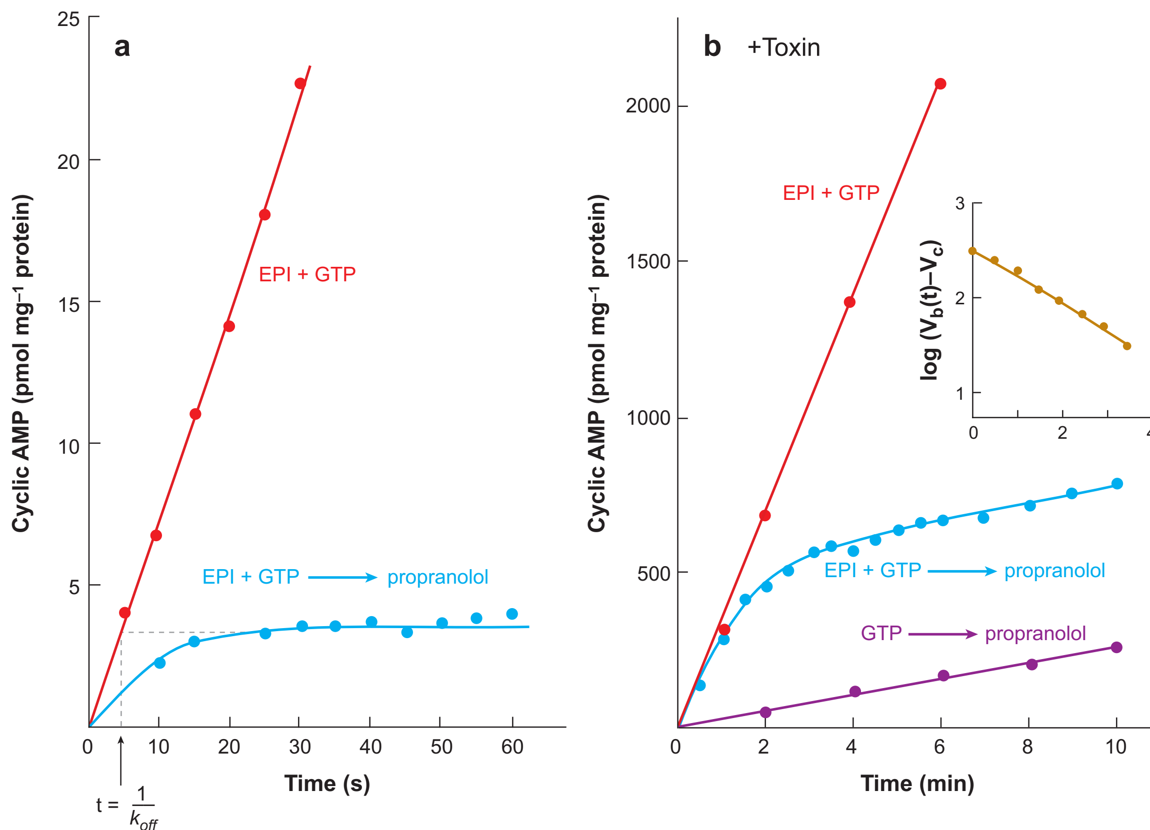

Figure 5

Decay of hormone plus GTP-stimulated adenylyl cyclase caused by addition of propranolol to membranes untreated (panel *a*) and treated (panel *b*) with cholera toxin. Abbreviation: EPI, epinephrine. Reprinted from Reference 13.

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decay process rather than from the individual points on the decay curve. This method was previously used by Kepes (17) to measure the degradation of β-galactosidase messenger RNA. **Figure 5** depicts assays of the decay of adenylyl cyclase as a measure of the turn-off reaction. In **Figure 5*a***, the assay was performed on untreated turkey erythrocyte membranes, whereas in **Figure 5*b***, membranes pretreated with cholera toxin were used. Calculation of the kinetic data showed that cholera toxin treatment caused a 96% inhibition of the turn-off reaction. The value of the turn-off reaction in the untreated membranes was 15 min^{-1}, much faster than the rate constant of the activation reaction. This explains why the hormone plus GTP cause only partial activation of the adenylyl cyclase and why its activity can be further manipulated. Cholera toxin, which inhibits the GTPase, activates the adenylyl cyclase, and many GTPase-activating proteins inhibit their cognate signaling systems.

Calculation of Steady-State Adenylyl Cyclase Activity from Measured Rate Constants

Addition of hormone plus GTP to the adenylyl cyclase preparation initiates both the activation and the turn-off reactions. The rate of activation diminishes with time as the

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amount of active enzyme decreases, while the rate of the turn-off reaction increases as the amount of adenylyl cyclase-GTP increases.

The steady-state equation gives the following ratio:

$$\frac{E_A}{E_{total}} = \frac{k_{on}}{k_{on} + k_{off}}.$$

E_A and E_{total} represent the active and total adenylyl cyclase, and k_{on} and k_{off} represent the rate constants of the activation and turn-off reactions.

Using the rate constants that we determined, we could calculate the fractional adenylyl cyclase activity at a steady state. We could also directly measure the fractional adenylyl cyclase activity as the ratio of the hormone-stimulated adenylyl cyclase activities in the presence of GTP and in the presence of its hydrolysis-resistant analogue, respectively. Comparison of the fractional adenylyl cyclase activity determined by these two methods gave very similar values. Furthermore, treatment of the adenylyl cyclase with cholera toxin caused a decrease of 96% in the rate constant of the turn-off reaction. In this case too, the activities calculated from the steady-state equation were in good agreement with those determined directly.

MECHANISM OF ADENYLYL CYCLASE ACTIVATION THROUGH THE β-ADRENERGIC RECEPTOR

We claimed that activation of the adenylyl cyclase by the β-adrenergic receptor is due to increased accessibility of the regulatory site to GTP. However, the detailed mechanism of this process was not determined, and the possibility that GTP first binds to the regulatory site and only then is transformed to the active state by the hormone-bound receptor was not ruled out. To determine the mechanism of receptor action, we studied the fate of the guanyl nucleotide in a preparation of turkey erythrocyte membranes that was incubated with (^{3}H)GTP plus isoproterenol and subsequently washed to remove hormones and free guanyl nucleotides. Further incubation of this preparation in the presence of the β-adrenergic agonist isoproterenol resulted in the release of a labeled nucleotide identified as (^{3}H)GDP. The catecholamine-induced release of (^{3}H)GDP was increased two to three times in the presence of the unlabeled nucleotides GTP, Gpp(NH)p, GDP, and GMP, whereas adenine nucleotides had little if any such effect. In the presence of Gpp(NH)p, the isoproterenol-induced release of (^{3}H)GDP and the activation of the adenylyl cyclase followed similar time courses (18). These findings indicate that the inactive adenylyl cyclase possesses tightly bound GDP produced by hydrolysis of GTP at the regulatory site. The hormone, through binding to its receptor, stimulates the adenylyl cyclase activity by inducing an "opening" of the guanine nucleotide site, resulting in dissociation of the bound GDP and its exchange for GTP, which is the abundant cellular-free guanine nucleotide under physiological conditions. The hormone-activated receptor thus acts as a guanine nucleotide exchange factor that under physiological conditions facilitates the exchange of bound GDP for free GTP and thereby causes activation of the adenylyl cyclase (**Figure 4**).

EPILOGUE

The text above describes experiments by Dan Cassel and me aimed at devising methods to unravel the role of guanine nucleotides in the regulation of adenylyl cyclase activity. Because of its focused approach, this prefatory article is not a review of the field and does not include other studies unless they were directly related to our studies. Within the span of four years, we arrived at the regulatory GTPase cycle as the control mechanism of the adenylyl cyclase activity. Subsequent studies by other scientists applied the emerging recombinant DNA technology to expand our knowledge of adenylyl cyclase and other signaling guanine

nucleotide-binding proteins, all with a regulatory GTPase cycle. These studies are beyond the scope of the this chapter.

A "take home lesson" from our studies of the adenylyl cyclase is that choosing the appropriate system to address your problem is crucial to the success of your project. In our case that system was the turkey erythrocyte membrane preparation, with its low level of background activities.

DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

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