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COMMENTARY

PERTUSSIS TOXIN IN THE ANALYSIS OF RECEPTOR MECHANISMS

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Neurotransmitters and hormones can induce their cellular effects by binding to and activating membrane bound receptors. The intervening events between the activation of receptors and changes in cellular activity are poorly understood. Some receptors contain in their structure ionic conductance channels. Activation of these receptors leads to increases in the permeability of the cell membrane to ions which can result in alterations in charge distribution across the plasma membrane and modifications in membrane excitability and cellular firing activity. Most receptors, however, do not have ionic conductance channels in their structure. These receptors regulate cellular activity by interacting with different effector systems. The enzymes adenylyl cyclase and phospholipase C are examples of cellular effector systems. They catalyze the synthesis of the second messengers cyclic AMP (cAMP), inositol trisphosphate, and diacylglycerol. Activation of these enzymes following receptor stimulation can increase the intracellular levels of these second messengers, thereby triggering a cascade of biochemical events leading to changes in cellular activity. Other cellular effectors are voltage-gated ionic conductance channels which, like the channels intrinsic to some receptors, control membrane permeability to ions and regulate membrane excitability and action potential generation. The regulation of the activity of cellular effectors by membrane bound receptors involves complex interactions between multiple proteins in the plasma membrane. In general, receptors do not come directly in contact with enzymes or ionic conductance channels. Instead, they couple to cellular effectors through intermediary proteins.

A class of such coupling proteins is the guanine nucleotide-binding regulatory proteins (G proteins). G proteins are a family of structurally related protein complexes involved in signal transduction [1, 2]. They share several common characteristics. Each G protein consists of three subunits, alpha (α), beta (β) and gamma (γ) which form a heterotrimer. The subunits are not covalently linked. The binding of GTP to a G protein is thought to be a major stimulus for the dissociation of the complex which is critical for the biological activity of the G proteins. The GTP binding site of G proteins resides in the α subunit [1]. In its resting, inactive state, the heterotrimer has GDP bound to the α subunit. Receptor activation of the G protein is believed to promote the release of GDP from the heterotrimer. GTP is then able to bind to the α subunit. This binding initiates the dissociation of the α subunit from the β/γ complex. The α subunit is then free to regulate the activity of effector systems such as the catalytic subunit of adenylyl cyclase. Presumably the α subunit interacts with cellular effector systems by binding to recognition sites in the effector systems, although such recognition sites in the effector systems have never been clearly identified.

The α subunit contains a GTPase [1, 3]. The GTPase catalyzes the conversion of the bound GTP to GDP. This results in a deactivation of the α subunit and facilitates its reassociation with the β/γ complex. As a consequence, receptor regulation of effector systems is terminated. Thus, G protein dissociation and reassociation are cyclic in nature and dependent on hormonal activation of membrane bound receptors and the intrinsic GTPase activity of the α subunit of the G proteins.

Most of the known functional specificity of the different G proteins resides in the α subunits (two forms of the β subunit, $G_{\beta 35}$ and $G_{\gamma 36}$, have been identified but it is not clear whether these subunits exert different functions; furthermore, γ subunit variability may exist but much less is known about the γ subunit compared to the α or β) [1, 4]. The variability in structure of the α subunits may be responsible for the ability of G proteins to distinguish and selectivity bind to different receptors as well as to differentially regulate effector systems. The α subunit of the G proteins, therefore, plays a critical role in the diversification of signal transduction across cell membranes.

Stimulatory G proteins

The initial evidence that G proteins were involved in coupling receptors to cellular effector systems came from studies examining the mechanisms through which hormones regulate adenylyl cyclase activity. Rodbell *et al.* [5, 6] first reported that glucagon stimulation of adenylyl cyclase activity requires the presence of GTP. A number of other investigators subsequently reported that other hormones and neurotransmitters stimulate adenylyl cyclase activity via a GTP-dependent mechanism [1]. Furthermore, it was shown that GTP modifies the characteristics of receptors by reducing the affinity of receptors for agonists [7]. Receptors themselves do not contain GTP binding sites, indicating that some other membrane component mediates the effects of GTP on receptors and their effector systems. The existence of GTP binding proteins was

further suggested from the results of studies employing mutants of the murine lymphoma cell line S49. *cyc*$^-$ Mutants of S49 lymphoma cells express the β-adrenergic receptor as well as a functionally active catalytic subunit of adenylyl cyclase, but β-adrenergic agonists cannot stimulate adenylyl cyclase activity in the mutant cells although they do stimulate adenylyl cyclase activity in wild type S49 cells [8–10]. These studies supported the hypothesis that a cellular component was needed to couple β-adrenergic receptors to the catalytic subunit of adenylyl cyclase in order for adenylyl cyclase activity to be regulated by hormones or neurotransmitters.

While a number of other studies had implicated a role of G proteins in mediating the cellular effects of hormones and neurotransmitters, it was not until G proteins were purified that clear evidence was provided that they couple receptors to the catalytic subunit of adenylyl cyclase as well as other cellular effector systems. Gilman and associates first purified a G protein that could reconstitute β-adrenergic receptor stimulation of adenylyl cyclase activity in *cyc*$^-$ S49 lymphoma cells [11]. This G protein was eventually termed G_s, since it mediated stimulation of adenylyl cyclase activity. Following the purification of G_s, the sequence of the α subunit of G_s ($G_{s\alpha}$) was determined [12], and the cDNA encoding this protein was cloned [12–15]. Multiple forms of $G_{s\alpha}$ exist and two mRNAs encode the different $G_{s\alpha}$s. The mRNAs result from the differential splicing of a common transcript [16]. Presently, there is no evidence to indicate that the various forms of $G_{s\alpha}$ have different functional activities.

Inhibitory G proteins

Other G proteins besides G_s are known to exist and are involved in mediating the actions of hormones and neurotransmitters on cellular effector systems. Neurotransmitters and hormones inhibit adenylyl cyclase activity, and this inhibition is mediated by G proteins that appear to be distinct from G_s [1]. This was suggested, in part, from the results of studies employing *cyc*$^-$ S49 lymphoma cells. These cells express somatostatin receptors and somatostatin inhibits adenylyl cyclase activity in *cyc*$^-$ membranes [17, 18]. The inhibition requires GTP and somatostatin can stimulate GTPase activity in *cyc*$^-$ membranes, suggesting that the effects of somatostatin on adenylyl cyclase activity are mediated by a G protein [17]. However, as discussed above, hormones cannot stimulate adenylyl cyclase activity in *cyc*$^-$ cells, suggesting that functional G_s is not present in these cells. Furthermore, studies using recombinant DNA probes have shown that *cyc*$^-$ cells do not express $G_{s\alpha}$ mRNA, indicating that $G_{s\alpha}$ is not synthesized in these cells [13]. Thus, inhibition of adenylyl cyclase activity in *cyc*$^-$ cells by somatostatin must be mediated by a G protein not containing $G_{s\alpha}$.

These and other studies lead to the identification of an inhibitory G protein, termed G_i. G_i has been purified, sequenced and cloned [14, 19–21]. It differs structurally from G_s in its alpha subunit. Three forms of $G_{i\alpha}$ have been identified [22]. They have been named $G_{i\alpha\text{-}1}$, $G_{i\alpha\text{-}2}$ and $G_{i\alpha\text{-}3}$. These forms of $G_{i\alpha}$ have over 85% structural homology, and no clear functional difference has been reported for them. Recombinant DNA studies have shown that these isoforms are encoded by different genes [22].

The mechanisms by which G_i inhibits adenylyl cyclase activity have been proposed to differ from those of G_s [1]. Thus, GDP is bound to the α subunit of G_i when G_i is in an inactive state. Receptor activation of G_i results in the release of GDP, and GTP is then able to bind to the α subunit. This promotes dissociation of the α subunit from the β/γ complex. Gilman [1] then proposed that the β/γ complex of G_i acts to sequester $G_{s\alpha}$ in order to inhibit the activation of adenylyl cyclase. This hypothesis suggests that the α subunit of G_i does not directly interact with the catalytic subunit of adenylyl cyclase. However, G_i is able to inhibit adenylyl cyclase activity in *cyc*$^-$ S49 cells devoid of functional $G_{s\alpha}$ [1]. This would suggest that $G_{i\alpha}$ is capable of interacting with the catalytic subunit of adenylyl cyclase, or some related protein, in order to inhibit cyclase activity. Thus, presently the mechanisms by which G_i inhibits adenylyl cyclase activity are controversial and not clearly established.

While $G_{i\alpha}$ and $G_{s\alpha}$ have structural homology, significant sequence variations do exist between the two classes of proteins [1, 23]. These structural variations may be responsible, in part, for their functional differences. One major difference that exists between $G_{s\alpha}$ and $G_{i\alpha}$ is their regulation by bacterial toxins [1, 2, 24]. The activity of $G_{s\alpha}$ can be regulated by cholera toxin. This agent catalyzes the ADP-ribosylation of an arginine residue in the vicinity of the GTP binding site of $G_{s\alpha}$ [23]. The ADP-ribosylation inactivates the GTPase and maintains the $G_{s\alpha}$ in a permanently active state. As a result, cholera toxin can induce long-term activation of adenylyl cyclase. The bacterial toxin, pertussis toxin, catalyzes the ADP-ribosylation of $G_{i\alpha}$. The site of ADP-ribosylation is a cysteine residue four amino acids from the C-terminus of $G_{i\alpha}$ [24]. Pertussis toxin catalyzed ADP-ribosylation of $G_{i\alpha}$ inactivates $G_{i\alpha}$ and blocks hormonal inhibition of adenylyl cyclase activity [25]. Pertussis toxin treatment of a number of cell types causes an enhancement of hormonal stimulation of adenylyl cyclase activity. This is not believed to be due to a modification of G_s, but rather to a blockade of the tonic inhibitory influence of G_i on adenylyl cyclase activity. In general, pertussis toxin is not believed to catalyze the ADP-ribosylation of $G_{s\alpha}$ in membranes, and cholera toxin does not catalyze the ADP-ribosylation of $G_{i\alpha}$ in membranes. As a result of these apparent selective actions of the bacterial toxins, they have been extensively used to identify the presence of $G_{s\alpha}$ and $G_{i\alpha}$ in membranes. Furthermore, they have been employed to examine the functional role of G_s and G_i in mediating the effects of different hormones and their receptors on the activity of various cellular effector systems.

Other G proteins regulted by pertussis toxin

In addition to regulating the activity of G_i, pertussis toxin has been shown to catalyze the ADP-ribosylation of other G proteins. In particular, pertussis toxin induces the ADP-ribosylation of the α subunit of G_o, a G protein found in high quantity in the brain [26, 27]. $G_{o\alpha}$ has been purified, sequenced

and cloned [14, 26, 27]. The gene encoding $G_{o\alpha}$ is different from the ones encoding the various $G_{i\alpha}$s [14]. Unlike $G_{i\alpha}$, the role of G_o in regulating adenylyl cyclase activity is not established. However, $G_{o\alpha}$ can mediate neurotransmitter and hormonal regulation of different ionic conductance channels and pertussis toxin treatment has been shown to attenuate modulation of ionic currents by G_o, suggesting that the ADP-ribosylation of $G_{o\alpha}$ does diminish the biological activity of this G protein subunit much like it does for $G_{i\alpha}$ [1, 2].

Multiple actions of G proteins

Besides its established role in inhibiting adenylyl cyclase activity, G_i is believed to induce other biological actions. In particular it can stimulate phospholipase C activity and, as a result, facilitate the hydrolysis of phosphatidylinositol (PI). GTP analogs have been shown in a number of permeabilized cell systems as well as cell-free systems to increase the hydrolysis of PI [28–30]. Hormonal stimulation of PI hydrolysis has also been shown in some cases to be attenuated by treatment with pertussis toxin [31–33]. Convincing evidence that pertussis toxin sensitive G proteins can mediate hormonal stimulation of phospholipase C activity and PI hydrolysis has come from the studies of Kikuchi *et al.* [34] who were able to reconstitute chemotactic peptide-stimulation of PI hydrolysis in pertussis toxin treated HL-60 cells with purified G_i. Interestingly, purified G_o also reconstituted chemotactic peptide-stimulation of PI hydrolysis, suggesting that multiple pertussis toxin sensitive G proteins may be involved in the hormonal regulation of phospholipase C activity.

Besides regulating enzyme activity, pertussis toxin sensitive G proteins have been suggested to couple membrane bound receptors to different ionic conductance channels. Dunlap and associates have shown that neurotransmitters including dopamine, norepinephrine and γ-aminobutyric acid can inhibit Ca^{2+} currents in neurons [35]. These effects are mimicked by intracellular injection of GTP analogs, and the effects of the neurotransmitters on the Ca^{2+} currents are attenuated by pertussis toxin treatment. Hescheler *et al.* [36] provided clear evidence for a role of pertussis toxin sensitive G proteins in coupling neurotransmitter receptors with Ca^{2+} channels. These authors reported that opiates inhibit a voltage-dependent Ca^{2+} current in neuroblastoma cells (a model neuronal system), and this effect was blocked by pertussis toxin. The opiate inhibition of the Ca^{2+} current could be reconstituted with purified G_i or G_o. $G_{o\alpha}$ was 10-fold more potent than $G_{i\alpha}$ in reconstituting the opiate inhibition of Ca^{2+} currents in pertussis toxin treated cells, suggesting that G_o may be a more effective mediator of the opiate regulation of Ca^{2+} channel activity in neurons than G_i. This would be consistent with the results of studies showing that G_o is present in greater amounts in some neurons than G_i.

Further support for a role of G_o in the regulation of Ca^{2+} currents in neurons has come from the studies of Harris-Warrick *et al.* [37] who examined the mechanisms by which dopamine inhibits Ca^{2+} currents in snail neurons. These investigators [37] reported that dopamine inhibition of Ca^{2+} currents in these neurons was mimicked by intracellular injection of GTP analogs and was blocked by intracellular administration of pertussis toxin. Furthermore, intracellular injection of an antibody directed against $G_{o\alpha}$ prevented dopamine from inhibiting the Ca^{2+} current. This is one of the first studies that have shown that antibodies generated against a particular G protein can block functional activity of the G protein. Harris-Warrick *et al.* [37] also showed that injection of purified $G_{o\alpha}$ into the snail neurons mimicked the inhibitory actions of dopamine on Ca^{2+} currents. These findings are the first clear evidence that endogenous G_o can couple a neurotransmitter receptor to a specific ionic conductance channel in neurons.

While G_o can inhibit Ca^{2+} channel activity, G_s has been shown to enhance Ca^{2+} currents. Yatani *et al.* [38] have shown in studies of skeletal muscle T-tubule membranes incorporated into lipid bilayers, that purified G_s or $G_{s\alpha}$ can increase voltage-dependent Ca^{2+} currents. Yatani and Brown [39] also showed that G_s mediates the stimulatory effects of β-adrenergic agonists on Ca^{2+} currents in cardiac myocytes. Interestingly, β-adrenergic agonists stimulate Ca^{2+} currents in myocytes through two different mechanisms [39]. β-Adrenergic receptor activation can lead to the dissociation of $G_{s\alpha}$ from G_s, and $G_{s\alpha}$ can directly interact with the Ca^{2+} channel, or some closely related membrane component in the myocyte, to induce a rapid stimulation of Ca^{2+} currents. β-Adrenergic agonists can also activate $G_{s\alpha}$ to stimulate adenylyl cyclase activity and subsequently increase cAMP-dependent protein kinase activity in cardiac myocytes. cAMP-dependent protein kinase can then catalyze the phosphorylation of Ca^{2+} channels or related proteins to increase Ca^{2+} channel activity. The two mechanisms appear to differ in their time course for stimulation of Ca^{2+} currents with the G protein interaction with Ca^{2+} channels occurring rapidly and the second messenger mediated response occurring more slowly. Both pathways of β-adrenergic control of Ca^{2+} channel activity involve G_s, suggesting that this G protein may act as a branch point for the differential regulation of Ca^{2+} currents in myocytes by catecholamines. Interestingly, this finding is one of the few reports suggesting that β-adrenergic receptors can regulate cellular responses through cAMP-independent mechanisms.

The reconstitution studies of Hescheler *et al.* [36], Harris-Warrick *et al.* [37], and Yatani *et al.* [38, 39] indicate that only the α subunits of G_i, G_o and G_s are involved in the modulation of Ca^{2+} currents. The differential modulation of Ca^{2+} currents by G_i and G_o versus G_s raises the question of whether such regulation can occur in the same cell and, if so, whether the α subunits of the G proteins can compete for similar recognition sites on the Ca^{2+} channels in order to regulate Ca^{2+} conductance.

In addition to their effects on Ca^{2+} currents, G proteins have also been shown to link neurotransmitter and hormone receptors to K^+ channels. Pfaffinger *et al.* [40] reported that in cardiac atrial cells, muscarinic agonists increase a voltage-sensitive K^+ current. This effect was mimicked by GTP analogs and blocked by pertussis toxin. The effects of

muscarinic agonists were independent of their ability to inhibit adenylyl cyclase activity in the atrial cells. Interestingly, Breitwieser and Szabo [41] showed that muscarinic agonists also reduce Ca^{2+} currents in atrial cells by acting through a pertussis toxin sensitive G protein. However, the effects on Ca^{2+} currents were independent of the stimulatory actions of muscarinic agonists on K^+ currents. The inhibition of Ca^{2+} channel activity by the muscarinic agonists was proposed to involve an inhibition of adenylyl cyclase activity that would result in a diminished stimulation of cAMP-dependent protein kinase activity and Ca^{2+} channel activity. Thus, muscarinic receptors in atrial cells can regulate two different ionic conductances by coupling with two different effector systems (the catalytic subunit of adenylyl cyclase and K^+ channels) via pertussis toxin sensitive G proteins. Such studies raise the question of whether the same G proteins mediate the different actions of acetylcholine in atrial cells or whether subtypes of muscarinic receptors couple to different G proteins which then regulate distinct effector systems.

To identify the G proteins coupling muscarinic receptors to the K^+ channels in atrial cells, Brown, Birnbaumer and associates [42–44] performed a series of biophysical studies on inside-out patches of atrial cell membranes using purified forms of various G proteins. In initial studies [42], purified G_i from human erythrocytes was shown to stimulate a K^+ current in atrial cells. Purified G_o or G_s had little effect on the K^+ current. While these findings tended to suggest that G_i selectively stimulated the K^+ current, Yatani *et al.* [42] were cautious in their interpretation of the results since their purified preparation of G_i was not able to inhibit adenylyl cyclase activity, which is an established characteristic of G_i. Conceivably, during the purification of the G protein, some modification of G_i occurred which prevented it from inhibiting adenylyl cyclase activity. Since this G protein preparation was able to stimulate K^+ channel activity in atrial cells, it raises the possibility that G_i inhibits adenylyl cyclase activity and stimulates K^+ channel conductance through different mechanisms. As a result of their uncertainty of the nature of the G protein purified from the human erythrocytes, Brown, Birnbaumer and associates [42] referred to the protein as G_k to signify that it is a G protein involved in regulating K^+ channel activity. Subsequent studies [43] showed that recombinant, purified isoforms of $G_{i\alpha}$ ($G_{i\alpha\text{-}1}$, $G_{i\alpha\text{-}2}$ and $G_{i\alpha\text{-}3}$) stimulated K^+ currents in atrial cell patches to the same extent as G_k. Such findings support the hypothesis that G_k and G_i are similar if not identical. In fact, Brown, Birnbaumer and associates eventually indicated that G_k and $G_{i\alpha\text{-}3}$ were identical. Further support for a role of endogenous α subunit of G_k or G_i in stimulating K^+ currents in atrial cells came from the studies involving the use of a monoclonal antibody that was generated against the α subunit of transducin but which also crossreacted with the α subunit of purified G_k from human erythrocytes [44]. Addition of the antibody to atrial cell patches blocked muscarinic stimulation of K^+ channel activity. The antibody did not crossreact with the β/γ complex of the G proteins indicating that the α subunit of G_k mediates muscarinic stimulation of K^+ channel activity. These findings indicate that the same muscarinic receptor could exert a dual regulation of both K^+ channels and adenylyl cyclase activity in the same cell.

While Brown, Birnbaumer and associates [42–44] have proposed that the α subunit of G_k or G_i stimulates K^+ currents in atrial cells, in apparent contrast, Clapham, Neer and associates [45, 46] have indicated that the β/γ complex is responsible for stimulating the K^+ channels. This hypothesis is intriguing for several reasons. First, the β/γ subunits of G_i, G_o and G_s are generally believed to be similar if not identical [1, 2]. However, G_o and G_s have been shown not to stimulate the K^+ channels of atrial cells to any extent. Thus, if the β/γ subunit stimulates K^+ currents in atrial cells, there must be some difference in the β/γ complexes of the G proteins. This is possible since our knowledge of the γ subunits of the G proteins is limited and potentially small variations in γ subunits could be responsible for the apparent selectivity of G_i or G_i-like proteins to regulate K^+ channels.

Another intriguing aspect of the findings of Clapham, Neer and associates is that muscarinic stimulation of K^+ channel activity in atrial cells is blocked by pertussis toxin. Pertussis toxin is generally believed to affect the activity of α subunits of G_i and G_o. This being the case, how could treatment of atrial cells with pertussis toxin prevent the β/γ complex from stimulating K^+ channel activity unless pertussis toxin also modifies the properties of the β/γ complex?

While these questions still remain unanswered, some clarification of the controversy between the two groups regarding the mechanism of G protein regulation of K^+ currents has occurred recently. Kim *et al.* [47], as well as Kurachi *et al.* [48], have shown that the β/γ complex does not activate K^+ channels directly, but instead regulates K^+ channel activity through an indirect mechanism involving phospholipase A_2 and arachidonic acid. Previous studies had shown that the β/γ complex can stimulate phospholipase A_2 activity in retinal cells [49]. Kim *et al.* [47] showed that an antibody against phospholipase A_2 that abolishes phospholipase A_2 activity blocked the β/γ complex stimulation of K^+ currents in atrial cell patches. Furthermore, arachidonic acid stimulated the K^+ current, and blockade of arachidonic acid synthesis with a lipoxygenase inhibitor prevented the β/γ complex stimulation of K^+ currents. Prostaglandins did not appear to be involved in the β/γ subunit stimulation of K^+ channel activity since the effects of the purified β/γ subunits were not attenuated by the cyclooxygenase inhibitor indomethacin.

Interestingly, the antibody against phospholipase A_2 and the lipoxygenase inhibitors did not eliminate muscarinic stimulation of the K^+ currents and therefore it was suggested by Clapham, Neer and associates [47] that the α subunit of a G protein endogenous to atrial cells has a major role in regulating K^+ channel activity. This suggests that activation of G_k or G_i-like proteins can stimulate K^+ channels through at least two mechanisms: one involving the α subunit acting directly on K^+ channels or related proteins to increase the K^+ current and the other involving the $\beta\gamma$ subunit stimulating phospholipase

A_2 activity and arachidonic acid synthesis to increase K^+ conductance. Since endogenous G_i or G_i-like proteins inhibit adenylyl cyclase activity in atrial cells and potentially also stimulate phospholipase C activity and inositol trisphosphate and diacylglycerol synthesis, activation of a single receptor and G protein complex could conceivably regulate four different effector systems simultaneously. Presumably this can allow acetylcholine to exert widespread influence over the activity of atrial cells.

Summary

G proteins play a critical role in signal transduction across cell membranes. Information about the diversity of G protein structure and function has provided valuable insights into the nature of the complex actions exerted by hormones and neurotransmitters on different cells and biological systems. A common finding of the biophysical and biochemical studies described above is that G proteins have the potential to couple neurotransmitter or hormone receptors to multiple cellular effector systems. When this occurs in a cell, it may allow a particular hormone or transmitter to regulate a variety of different cellular events simultaneously. Furthermore, it has become clear that different receptors can couple to the same G protein. When this occurs in the same cell, it may provide the basis for the convergent regulation of cell activity by various hormones or neurotransmitters. Thus, G proteins greatly diversify the manner by which hormones and neurotransmitters can regulate cells. As more information is available on the mechanisms by which G proteins recognize and interact with receptors and effector systems, we may be able to better understand the specific events involved in signal transduction and the subtle processes by which hormones and neurotransmitters can control cell activity.

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