Review Letter

# BIMODAL REGULATION OF ADENYLATE CYCLASE

Dermot M. F. COOPER

*Section on Membrane Regulation, Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20205, USA*

Received 2 November 1981

## 1. Stimulation of adenylate cyclase

The role of GTP in the stimulation of adenylate cyclase has received intense attention in the last 10 years. A number of excellent reviews of this area have appeared [1–3]. As a prelude to a discussion of inhibition of adenylate cyclase it seems appropriate to present a brief perspective on stimulation of adenylate cyclase.

Many hormones interact with cell surface receptors to transmit a stimulatory signal to the catalytic unit of adenylate cyclase through the intervention of a GTP-regulatory protein (termed $N_s$)[+]. By a mechanism that remains unclear (due largely to a limited knowledge of the components of the system), GTP both decreases the affinity of hormones for their receptors and synergistically amplifies hormonal stimulation of activity. In general, the non-hydrolysable GTP analogue, Gpp(NH)p, promotes the latter action more effectively than the native compound. These and allied findings have led to the development of a general hypothesis, which proposes that hormone binding to receptors leads to the release of previously bound GDP (an ineffective stimulator), which allows occupancy by GTP and thus attainment of a more active $R_s \cdot N_s \cdot C$ complex. On hydrolysis of GTP to GDP, the complex reverts to an inactive form, which coincides with the release of hormone [1–3].

Considerable gaps exist in our knowledge of stimulatory adenylate cyclase systems. Quantitative information is lacking on the relationship between hormone occupancy, GTP hydrolysis, and active complex formation. Similarly the relative stoichiometry of $R_s$:$N_s$:C is a subject for speculation. Whether the various elements exist in a preformed complex which is stabilized on interaction with regulatory ligands, or whether there is some degree of independent movement or collision, is unclear. Regulatory components in addition to those already identified may exist. For instance, cytoskeletal elements are candidates for supporting roles in these systems. The number of proteins comprising the $N_s$ unit appears to be either 2 or 3, depending on the source of the purified component. In addition an ADP-ribosylation factor, which permits the $N_s$ unit to be ADP-ribosylated by cholera toxin, may also be an integral component of the $N_s$ unit [4].

Notwithstanding the unanswered questions, the central role of the $N_s$ unit in mediating the stimulatory effects of hormones is clearly established and progress in understanding the functioning of this component provides a yardstick against which our understanding of inhibitory regulation can be evaluated.

## 2. GTP-dependent inhibition of adenylate cyclase

Early observations on fat cell membranes indicated that GTP at >1 μM could reduce adenylate cyclase activity [5–7]. Evidence had also been accumulating that cyclic AMP production could be decreased by a number of agents, such as adenosine and $PGE_1$ in adipocytes [8], or norepinephrine in platelets [9]. Isolated reports demonstrated that adenylate cyclase activity could be inhibited in various broken cell preparations by, for instance, muscarinic cholinergic

[+] Abbreviations are functional assignments, which may be represented by one or more distinct proteins: $R_s$ and $R_i$ are receptors for hormones/neurotransmitters evoking either stimulation or inhibition, respectively, of the catalytic activity, C; $N_s$ and $N_i$ are the GTP regulatory elements mediating either the stimulation or inhibition of activity, respectively

*Published by Elsevier Biomedical Press*
00145793/82/0000–0000/$02.75 

drugs [10], norepinephrine [9] and opiates [11]. These observations were drawn together when it was shown that inclusion of GTP in concentrations exceeding those required for the stimulation of adenylate cyclase by hormones, permitted inhibition of the enzyme by various putative neurotransmitters, for instance, epinephrine in platelets [12] and in neuroblastoma × glioma hybrids [13] and adenosine in fat cells [14]. The GTP requirement for these effects clearly established adenylate cyclase inhibition as a receptor-mediated event which was somewhat analogous to the stimulation of adenylate cyclase by hormones.

Indications that distinct GTP regulatory proteins might mediate stimulation and inhibition of adenylate cyclase came from a series of studies with the fat cell. This enzyme displayed a pronounced biphasic response to GTP; GTP up to 40 nM increased activity. The non-hydrolysable analogue Gpp(NH)p did not share the inhibitory response [16]. Treatment of fat cell membranes (or cells prior to membrane preparation) with either trypsin [15] or cholera toxin and NAD, or assaying in the presence of $Mn^{2+}$ resulted in the abolition of the inhibitory response to GTP. In contrast, treatment of membranes with *p*-hydroxymercuriphenylsulphonic acid eliminated stimulation but retained inhibition by GTP [16]. The functional association between the inhibitory response to GTP and GTP-mediated inhibition by adenosine analogues was established by the observation that conditions which eliminated inhibition by GTP also led to the loss of the ability of adenosine analogues to inhibit the enzyme in a GTP-dependent manner [16].

## 3. Dually regulated adenylate cyclase systems

A rapid growth in reports of GTP-mediated inhibition of adenylate cyclase by many putative neurotransmitter receptors has occurred in recent years. These include opiate [17], muscarinic cholinergic [18], $\alpha_2$-adrenergic [15] in neuroblastoma × glioma hybrids; muscarinic cholinergic in myocardium [19, 20]; dopamine (via a $D_2$ receptor) in intermediate pituitary [21]; adenosine, $PGE_1$ and nicotinic acid in adipocytes [22–24]; $\alpha_2$-adrenergic in platelets [12, 25]; opiate and adenosine in hippocampus [26]; opiates in striatum [27,28]; angiotensin and $\alpha_2$-adrenergic in liver [29]; and adenosine in brain cortex [30].

Common features shared by most of these systems are as follows*:

(i) Biphasic GTP kinetics are almost always encountered (in the absence of $Na^+$) [31];
(ii) GTP concentrations beyond those in the stimulatory range ($\sim 10^{-7}$ M) promote inhibition by putative neurotransmitters and related compounds [30,31];
(iii) Where GTP, in the absence of other ligands causes a decrease in activity, $Na^+$ (up to 100 mM) reverses this effect [22];
(iv) $Na^+$ amplifies inhibition by ligands in direct proportion to its reversal of the inhibition promoted by GTP alone [17,22];
(v) Gpp(NH)p does not promote inhibition by inhibitory hormones or neurotransmitters, even when it evokes a transient inhibition at early incubation times [31,32];
(vi) Inhibition by neurotransmitters is generally <60%, except when directed against basal activities when it may reach 80%;
(vii) Where multiple inhibitory effectors operate, their effects are non-additive [13,22,32];
(viii) As with stimulatory ligands, the binding of inhibitory ligands is modulated by GTP (see section 4);
(ix) Sodium ion and Gpp(NH)p modulate binding of inhibitory ligands, even though these agents may not affect the ability of the inhibitory ligands to attenuate activity (see section 4).

Little deviation from these general properties is encountered in a wide range of dually regulated systems.

## 4. Receptor binding of inhibitory ligands

Extensive studies have been performed on the binding of inhibitory ligands to their receptors. The early studies of Pert and Snyder [33] on the binding of opiates to brain receptors provided valuable insights for later studies on the inhibition of adenylate cyclase by opiates. In particular, monovalent cations (most effectively sodium) were shown to increase the receptor binding of antagonists and decrease that of agonists.

* The summary of the properties of dually regulated adenylate cyclase systems is drawn from studies in many laboratories. Detailed references are available in the review articles cited in this section

Subsequently, Blume [17] found that $Na^+$ amplified the inhibition of adenylate cyclase in neuroblastoma X glioma hybrid by opiates. Sodium ions have now been shown to modify the binding of inhibitory ligands in diverse systems, even in the absence of an effect of sodium on inhibition of adenylate cyclase by these ligands. For instance, in platelets, a decrease in the affinity at the α-adrenergic receptor was encountered [34,35], whereas the cation did not modify the inhibition by epinephrine of the adenylate cyclase activity [31,32]. This observation is readily understood in view of the lack of inhibition by GTP in the absence of inhibitory ligand. In the fat cell, a striking effect of sodium on inhibition is seen due to the marked inhibition of enzyme activity by GTP in the absence of inhibitory ligand [22,31]. The retention of a $Na^+$ effect on binding in situations where it shows no effect on activity may indicate separate loci for these 2 regulatory events; alternatively, an excess of receptors over catalytic elements would permit discrepant regulation of receptor binding and the function mediated by the receptors.

The sodium effect on binding is not constant with respect to all inhibitory ligands. For instance, in brain, the binding of both opiate agonists and antagonists is regulated by sodium [36]; whereas only α-adrenergic agonist binding is modified [37]. In contrast to the monovalent cations, $Mg^{2+}$ generally increases agonist affinity [34,38].

Guanine nucleotides are more consistent in the regulation of the binding of inhibitory ligands. GTP and Gpp(NH)p generally decrease the affinity of inhibitory ligands for their receptors [34,38]. This is directly analogous to the effects of guanine nucleotides on the binding of stimulatory ligands to their receptors. However, this observation conflicts with the inability of the non-hydrolysable GTP analogue to promote inhibition of adenylate cyclase. It might have been anticipated that, since only GTP, and not Gpp(NH)p, can promote adenylate cyclase inhibition by these ligands, the latter compound might not have shared the ability of GTP to modulate binding. The fact that this prediction is not fulfilled again raises the possibility of either separate loci for the regulation of binding compared with function or an excess of inhibitory receptors not in association with catalytic activity, which permits discrepant regulation of total binding compared with a small pool of receptors associated with the enzyme.

In tissues where it has been studied it appears that $Mg^{2+}$ can modify the effect of guanine nucleotides on inhibitory ligand binding. In both brain (α-adrenergic receptor binding) and fat cell membranes (adenosine receptor binding) at low magnesium concentrations, GTP increases the number of binding sites for the inhibitory ligands, whereas at higher cation concentrations, GTP decreases affinity for the ligands ([38], Cooper and Gill, in preparation).

The apparently wide diversity which exists in the regulation of the binding of inhibitory ligands to their receptors compared with that of stimulatory agents underlines our rather primitive understanding both of the interaction of inhibitory receptors with the putative inhibitory GTP regulatory components and of the precise transduction mechanisms which translate the binding event into an inhibitory response.

## 5. The role of GTP hydrolysis in inhibition of adenylate cyclase

The most persuasive evidence that GTP-hydrolysis plays a role in hormonally-mediated inhibition of adenylate cyclase is the observation that Gpp(NH)p, the non-hydrolysable analogue, will not promote inhibition under any assay conditions. A characteristic of dually regulated systems is a transient inhibitory response to low concentrations of Gpp(NH)p [7,26, 31,39]. Under such conditions, in the absence or presence of $Na^+$, inhibitory ligands will not affect activity. An obvious interpretation of this widely encountered finding is that GTP hydrolysis is an absolute requirement for inhibition. The hydrolysis product, GDP, is not required since GDP (or $GP(CH_2)P$), under conditions where care is taken to prevent phosphorylation to the triphosphate, will not promote inhibition ([31], Cooper and Schlegel, unpublished). These observations contrast with the ready interchange of GTP with Gpp(NH)p in stimulation of adenylate cyclase, where GTP hydrolysis seems not to be a stringent requirement for enzyme stimulation.

Supportive evidence for an obligatory role for GTP hydrolysis is available from studies with cholera toxin. As mentioned in section 2 cholera toxin treatment abolishes both GTP inhibition and that promoted by adenosine in a GTP-dependent manner in fat cell membranes [16]. Cholera toxin invariably enhances hormonal stimulation in stimulatory systems by a mechanism believed to involve the inhibition of a specific GTPase activity associated with the stimulatory ($N_s$) unit. Consequently, the observations with the fat cell

could be interpreted to imply that toxin treatment also inhibited a GTPase activity associated with the inhibitory ($N_i$) unit. However, this effect of cholera toxin is not universally encountered in dually regulated systems. Inhibitory effects were retained following toxin treatment of Chinese hamster ovarian, neuroblastoma X glioma hybrid cells and platelets [40–42].

In neuroblastoma X glioma and platelet membranes, a GTPase activity has been detected which can be stimulated by opiates and α-adrenergic agents, respectively [43,44]. Substrate specificities were not examined in these studies, thus a specific GTPase activity was not unequivocally demonstrated. Nevertheless, a 2-fold stimulation was achieved, and it is tempting to speculate that the GTPase activity measured is relevant to the inhibition of adenylate cyclase mediated by the receptors in these tissues. The rather complex assay mixtures involved may allow transphorylation of the terminal phosphate of GTP to ATP, which is also included in the assay. Neither group has attempted to determine the source of the $P_i$ released into the medium, therefore the possibility must be considered that inhibitory ligands may also be capable of stimulating an ATPase activity in plasma membranes in an analogous manner to that reported for insulin and catecholamines [45,46].

However, notwithstanding the reservations raised concerning these studies, the accumulated evidence from more indirect studies would have anticipated a more central role for a GTPase in inhibitory regulation than in the case of stimulatory regulation.

## 6. The relationship between $N_s$ and $N_i$

Definitive evidence is lacking on whether $N_s$ and $N_i$ are distinct proteins or merely functional notations. The broadly descriptive options which might be considered include:

(i) That the N unit (or complex, see section 1) is constant in all adenylate cyclase systems and that the functions associated with $N_s$ and $N_i$ merely reflect the association of the N unit with $R_s$ or $R_i$, respectively;

(ii) $N_s$ and $N_i$ are distinct regulatory protein complexes which share a common catalytic unit; or

(iii) $N_i$ may be a modified form of $N_s$ (e.g., an oligomeric form, an association with an exogenous factor or protein, such as calmodulin) which may or may not be stabilised or promoted upon interaction with $R_i$.

Differences in properties between the functional entities referred to as $N_s$ and $N_i$ are summarised in table 1. These differences justify the functional assignments and, with other evidence discussed herein, support the view that fundamental differences exist between the 2 regulatory systems. Nevertheless a full appreciation of their properties will become available only by further structural studies.

## 7. Structural studies on dually regulated adenylate cyclase systems

Apart from the preliminary studies on the fat cell

Table 1
Summary of the properties associated with the 2 GTP regulatory functions

| Property | $N_s$ | $N_i$ |
|---|---|---|
| GTP requirement ($ED_{50}$) | 10 nM, 1 μM[a] | 1 μM (3) |
| Sodium ions | – | – ↑ (4) |
| Cholera toxin | ↑ | –,↓ (3) |
| $Me^{2+}$ | ↑ | ↓ (3) |
| Gpp(NH)p | ↑ | –,↓ (3,4) |
| Cholera toxin-labeled bands | 1 or 2 | Additional bands (8) |
| Mild trypsin treatment | ↑,– | –,↓ (3) |
| Effect of GTP, Gpp(NH)p on binding | ↓ $K_d$ | ↓ $K_d$, ↑ R (5) |
| GTP on basal cyclase | –,↑ | ↓ (3) |
| Sodium effect on binding | – | ↓ $K_d$ (5) |

[a] When stimulatory systems are considered

*Notations:* –, no effect; ↑ amplification of the function; ↓, dampening of the function; numbers in parenthesis refer to sections where these features are discussed

indicating selective abolition and retention of 1 of the 2 effects mediated by GTP (see section 4), most attention has focussed on the receptors in dually regulated systems.

For instance, opiate receptors have been solubilized with full retention of their agonist specificity [47]. Another solubilised opiate receptor preparation retained the ability of $Na^+$ to modify binding [48], although no effects of GTP were detected. This important observation suggests that the sodium site may be associated with the receptor rather than the GTP regulatory unit.

The α-adrenergic receptor from liver has been solubilised and partially purified [49]. The irreversibly binding antagonist, phenoxybenzamine, was utilised to monitor the presence of the receptor through various purification procedures. Although phenoxybenzamine cannot readily be removed from the receptor preparation, this material is quite suitable for generating antibodies which may be utilised to identify and purify unoccupied receptors.

Prior incubation of platelet plasma membranes with $\alpha_2$-adrenergic agonists stabilises a higher $M_r$ form of the receptor than that observed following incubation with antagonists. This data may suggest that agonists stabilise interaction between receptor and $N_i$ unit [50].

### 7.1. *Cholera toxin*

Since cholera toxin with NAD modifies the functions ascribed to both $N_s$ and $N_i$, it is conceivable that with [$^{32}$P]NAD, protein bands additional to those encountered in stimulatory systems might be detected on SDS electrophoresis following exposure to the toxin of dually regulated systems. When a range of plasma membrane preparations were compared, those subject to dual regulation showed additional labelled bands; in fat cell and CHO, a 52 000 and a 54 000 $M_r$ band were detected; in platelet a 58 000 band was detected, in addition to the widely observed 42 000 $M_r$ band [51]. The relationship of these additional proteins to the $N_s$ unit remains to be established.

### 7.2. *Calmodulin*

The hippocampal adenylate cyclase is a dually regulated system (section 3), which can be inhibited by opiates and adenosine analogues in a GTP-dependent manner. Calmodulin appears to play a role in this system, since its removal by EGTA treatment results in the loss of inhibition by the opiates and adenosine. Addition of calmodulin restores the effect [26]. This behaviour does not seem to be generally applicable to inhibitory systems since the platelet and fat cell systems, for instance, are not affected by EGTA treatment [31]. However, in the case of the hippocampal system (and possibly other neural systems), calmodulin may provide a means for identifying inhibitory components.

### 7.3. *Ultrastructural studies*

Recent microscopic studies indicate that both opiate and cholinergic receptors occur in clusters on the cell surface [52,53]. Irradiation inactivation studies also indicated that very large structures mediated stimulation and inhibition of fat cell adenylate cyclase [54]. Such a situation is readily envisaged in view of the multiplicity of stimulatory and inhibitory neurotransmitters converging on a common pool of catalytic activity (see section 3). These structures, which if composed of heterogenous stimulatory and inhibitory receptors with their associated N units, would resemble multi-enzyme complexes and provide a ready means of achieving the non-additive stimulation and inhibition of adenylate cyclase by different neurotransmitters discussed in section 3.

## 8. Future directions

A number of putative neurotransmitters and peptides which have been identified in brain as yet do not have a measurable function in isolated membrane preparations. It seems likely that some of these compounds, including histamine, serotonin, GABA, glutamine, ACTH, substance P, α-MSH, will turn out to utilise GTP inhibitory pathways.

Whether the catalytic unit of adenylate cyclase is the same in dually regulated as in stimulatory systems must be determined. It is conceivable that the complexity of catalytic units varies as a function of the degree of regulation to which they are subject.

Further progress in understanding the structural nature of these systems will require a combination of approaches, including ultrastructural studies, solubilisation and reconstitution of components, identification of mutants lacking one or other of the regulatory elements, coupled with the fusion/complementation approach pioneered by Orly and Schramm [55].

A perplexing question, which must reflect a fundamental property of inhibitory systems, arises from the partial inhibition evoked by inhibitory neurotransmit-

ters. Since inhibition ranges from 20–80% maximally [31,32], the physiological significance of this regulation may be doubted. However, partial inhibition of adenylate cyclase combined with the presence of phosphodiesterase in intact cells can result in substantial reduction of intracellular cAMP levels. The striking inhibition of cAMP production in the fat cell by adenosine and its analogues (leading to a marked inhibition of lipolysis) and in platelets by epinephrine (resulting in platelet aggregation) must reflect such a situation.

The finding of angiotensin II- and α-adrenergic-inhibition of adenylate cyclase in liver [29], which had been considered a simple stimulatory system, raises some intriguing possibilities. Inclusion of high concentrations of EDTA was required during all stages of the preparation of the plasma membranes for the observation of this effect. Now, it has long been known that both GTP and $Na^+$ would effect angiotensin binding to adrenal cortex membranes [56] (without EDTA treatment). Thus the possibility is raised that an RN complex existed for angiotensin which had not been linked to adenylate cyclase, but to some other process, prior to the chelator treatment. Certainly a number of receptors mediating inhibition of adenylate cyclase have also been implicated in either calcium transport or phosphatidylinositol metabolism, e.g., muscarinic cholinergic, $\alpha_2$-adrenergic and angiotensin [57]. The findings discussed above raise the possibility that switching of the function served by an RN complex can occur physiologically in addition to the experimental means presented above.

In [58] increased adenylate cyclase activity was described following prolonged exposure of neuroblastoma × glioma hybrid cells to morphine without alteration in receptor number. The situation was presented as a model system for tolerance and addiction to opiates. Current appreciation of the existence of stimulatory and inhibitory GTP regulatory protein interactions may provide an understanding for the basis of this observation.

Receptor-mediated inhibition of Gpp(NH)p-activated adenylate cyclase activity by progesterone in *Xenopus* oocytes and by α-mating factor in yeast [59–61] has been reported. In both cases fundamental developmental changes correlate with these inhibitions. It is possible that modified forms of $N_i$ with less severe restrictions on the terminal diphosphate bond of the guanine nucleotide mediate these effects.

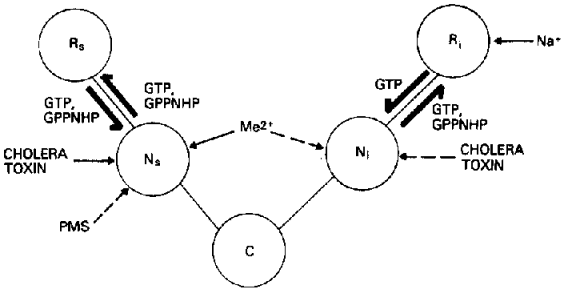


Fig.1. Schematic representation of dually regulated adenylate cyclase with suggested sites of action of various regulators. In this scheme broken arrows indicate inhibition or suppression of a process; solid arrows indicate promotion or enhancement of a process; the heavy directional lines between R and N units indicate the guanine nucleotides which promote communication from R to N or from N to R. PMS, *p*-hydroxymercuripheylsulphonate.

## 9. Conclusions

A schematic model (fig.1) summarises the functional components involved in dual regulation of adenylate cyclase and the site of action of some modifiers of activity. The central role of GTP is evident. Appreciation of the potential importance of GTP has proven to be the key to our current appreciation of these systems. Evidence is accumulating that separate GTP-regulatory proteins are associated with inhibition and stimulation from measurements of both activity and regulation of binding to the 2 classes of receptor. The next few years will see increasing appreciation not only of the physiological importance, but also insights into the structural elements of these systems.

## Acknowledgements

I would like to acknowledge the useful comments of my colleagues Drs D. L. Gill, R. Honnor and R. T. Simpson on this manuscript. I would also like to thank Dr M. Rodbell for his continuing support and encouragement and Ms Bonnie Richards for her expert secretarial assistance.

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