Activation and inhibition of adenylate cyclase by hormones

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Basic features of the hormone-dependent adenylate cyclases

The adenylate cyclase system is composed of three basic units (Ross & Gilman, 1980): the receptor R facing the outside of the cell and binding the hormone or neurotransmitter, the GTP regulatory unit N (or G/F), and the catalytic unit C. Both N (G/F) and C face the cytoplasm and react with GTP and MgATP respectively. Hormone binding to R and GTP binding to N induce activation of C to its cyclicAMP-producing state C'. Thus

\[
\text{ATP} \xrightarrow{\text{Hormone}} \text{cyclicAMP} + \text{PP}_\text{i}
\]

The hormonal signal is terminated upon GTP hydrolysis to GDP and P_i at the GTP regulatory site. Reactivation requires the removal of GDP, binding of a new GTP molecule and the continued presence of hormone. Thus, the steady-state level of activated adenylate cyclase is determined by the relative rate of conversion of the inactive enzyme into its active form (the 'on' reaction) and the rate of the GTPase deactivation (the 'off' reaction). This 'on-off' cycle seems to operate in most or all of the eukaryotic hormone-dependent adenylate cyclases, which possess also the characteristic hormone GTPase activity.

The ratio \( \frac{[N' \cdot C']}{[N' \cdot C]^\text{total}} \) can be measured by monitoring the rate at which activated adenylate cyclase molecules decay to their basal inactive state by either flooding the system with a hormone antagonist (Cassel & Selinger, 1976; Levitzki, 1977) or a GTP antagonist \( \text{p[NH}^\text{PP}G \) (or \( \text{S[pppG} \)). \( k_{\text{on}} \) can be measured by monitoring the rate at which activated adenylate cyclase molecules decay to their basal inactive state by either flooding the system with a hormone antagonist (Cassel & Selinger, 1976; Levitzki, 1977) or a GTP antagonist \( \text{p[NH}^\text{PP}G \) (or \( \text{S[pppG} \)). Such quantitative measurements have been performed extensively on the \( \beta \)-receptor (Cassel & Selinger, 1977; Tolkovsky & Levitzki, 1978a; Cassel et al., 1979; Arad & Levitzki, 1979) and adenosine receptor (Tolkovsky & Levitzki, 1978b; Braun & Levitzki, 1979) dependent adenylate cyclase in turkey erythrocytes. These measurements have shown that eqn. (2) describes well the hormone-sensitive adenylate cyclase in that system.

This simple model accounts for a number of properties of the system such as: (a) the increase in adenylate cyclase activity induced by cholera toxin; Cholera toxin catalyses the ADP-ribosylation of N, reducing \( k_{\text{off}} \) (Cassel & Selinger, 1977) and thus increasing \( [N' \cdot C'] \); (b) partial agonism; Partial agonists are 'partial' because of decreased \( k_{\text{on}} \) with unaltered \( k_{\text{off}} \) (Arad & Levitzki, 1979), and thus reduced \( [N' \cdot C'] \).

Collision coupling

In the turkey erythrocyte membrane system the rate of adenylate cyclase activation (\( k_{\text{on}} \)) is proportional to the concentration of the \( \beta \)-adrenergic receptor, namely, the receptor acts catalytically (Tolkovsky & Levitzki, 1978a; Arad et al., 1981). More recently, the catalytic role of the receptor has also been shown in reconstituted systems (Citi & Schramm, 1980). This finding suggests that one agonist-bound receptor molecule can react consecutively with a number of adenylate cyclase molecules, also within the two-dimensional array of the membrane. Hence, the mechanism can be formulated as follows:

\[
\text{HR} + N \xrightarrow{\text{GTDP}} C \xrightarrow{HR \cdot N \xrightarrow{\text{GTDP}}} C' \xrightarrow{\text{HR} + N \xrightarrow{\text{GTDP}}} C'
\]

where the species \( HR \cdot N \xrightarrow{\text{GTDP}} C' \) does not accumulate to more than a few per cent of the total enzyme concentration. Reducing the concentration of N, guanyl nucleotide (Tolkovsky et al., 1982) and of C (Tolkovsky & Levitzki, 1978a) does not affect the basic finding that the 'on' reaction in the presence of \( \text{p[NH}^\text{PP}G \) is always first order and that the rate constant \( k_{\text{on}} \) is always a saturating function of the hormone concentration [H]. These findings further support the view that N is tightly associated with C at all times. Furthermore, lubrol-PX-solubilized adenylate cyclase still appears as a tight NC complex at a concentration of 0.1mg/ml, which is about one-tenth of the concentration within the membrane (Neer et al., 1980). Only in ionic detersants such as cholate in the presence of high salt does C dissociate from N (Ross, 1981).

The finding of 'collision coupling' itself immediately raises questions such as (a) which of the components is mobile, the receptor agonist complex (HR) or the GTP unit together with the catalytic unit-NC; (b) are cytoskeletal elements involved and
therefore the relative movement is affected not only by 'fluidity' of the membrane but also by anchoring to the cytoskeleton; (c) do these findings apply to the intact living cell?

**Criticism of 'collision coupling'**

Experiments directed at some of these questions have already been performed. Thus, increasing membrane 'fluidity' by cis-vaccenic acid and N-NBF-phosphatidylethanolamine induce an increase in the rate of adenylate cyclase activation by the \( \beta \)-receptors (Orly & Schramm, 1975; Rimon et al., 1978). Recent experiments, however, in which the diffusion coefficient of N-NBD-phosphatidylethanolamine inserted into turkey erythrocyte membranes was shown to be independent of added cis-vaccenic acid and were interpreted to mean that cis-vaccenic does not change membrane fluidity (Henis et al., 1982a). The main criticism of this experimental approach is that the authors did not look at the diffusion coefficients of the \( \beta \)-receptor, the cyclase components or any other protein within the membrane as a function of added cis-vaccenic acid. Furthermore, it is not to be expected that cis-vaccenic acid, which is close in size to the phospholipids themselves, will affect strongly their lateral mobility.

In support of the view that protein mobility is essential for hormone activation are the experiments in which it was found that immobilization of membrane proteins by cationized ferritin strongly inhibits the \( \beta \)-agonist-dependent activity but has almost no effect on the NaF and pNPPG-dependent activities (Atlas et al., 1980). These results seem to contradict recent measurements of NBF-alpenolol fluorescence on Chang liver cells, which were taken to mean that \( \beta \)-adrenergic receptors are immobile (Henis et al., 1982b). These experiments, however, are inconclusive, first, because a fluorescent antagonist that does not activate the cyclase system was used, and secondly, if indeed the apparent dissociation constant \( K_d \) for NBF-alpenolol used in the study is 0.33nM, why is it necessary to generate specific fluorescence at a 120-fold excess of NBF-alpenolol? Concentrations 10-fold lower should be sufficient. Cherksy et al. (1981) have shown, using fluorescence techniques, that 0.1 nM (+)-alpenolol is sufficient to saturate a significant portion of the \( \beta \)-receptors in frog cell membranes, as expected from the finding that \( K_d \) for that ligand is also in that range. In addition, the same authors have shown, using fluorescence techniques, that agonist binding to \( \beta \)-adrenergic receptors of frog erythrocytes mobilizes them (Cherksy et al., 1980), in contrast with the findings of Henis et al. (1982a). Clearly the key experiments that must be performed should make use of very specific fluorescent ligands with very high affinity. Furthermore, to avoid both difficulties in experimentation and interpretation, a high-affinity fluorescent agonist is highly desirable.

**The role of GTP in adenylate cyclase**

Adenylate cyclase stimulation and inhibition by neurohormones depends on GTP. The half-maximal GTP concentration required for enzyme stimulation as in the 10–50nM range, whereas the half-maximal GTP concentration required for full expression of the inhibitory activity is in the range of 200 to 600nM (Table 1). Interestingly, the half-maximal GTP concentrations for the hormone-dependent GTPase coupled to adenylate cyclase are also in the range of 200 to 300nM (Table 1). It therefore seems that the current view on the role of GTP in hormone-dependent adenylate cyclase must be revised (Braun et al., 1982). We suggest that the high affinity GTP site of the cyclase-inhibitory agents enhance \( k_{in} \) and stimulate sodium-pH-dependent low-\( K_d \) GTPase activity. These results do not agree with our findings that adrenaline has no effect on either basal or prostaglandin \( \text{E}_2 \)-dependent GTPase in purified human platelet membranes under the same experimental conditions whereby 44% inhibition of adenylate cyclase by \( \alpha \)-adrenaline is observed (Lester et al., 1982; M. L. Steer, S. Braun, H. A. Lester & A. Levitzki, unpublished work). Furthermore, in the purified platelet membrane preparation no effect of \( \alpha \)-agonists on either \( K_d \) or \( k_{in} \) is observed under conditions where cyclase activity is inhibited (M. L. Steer, S. Braun, H. A. Lester & A. Levitzki, unpublished work). We therefore suggest that the mechanism of hormone inhibition is not through the stimulatory GTPase cycle but rather by functional uncoupling of the stimulatory GTP regulatory site from the catalytic unit. Apparently, the fruitful coupling of the inhibitory hormone receptor to the adenylate cyclase system requires the occupancy of both a high-affinity GTP site involved in adenylate cyclase activation and the low-affinity GTP site, which probably represents still another GTPase site.

<table>
<thead>
<tr>
<th>System</th>
<th>Adenylate cyclase activation</th>
<th>Hormone-dependent GTPase</th>
<th>Adenylate cyclase inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey erythrocyte</td>
<td>( 14^\text{th} (\beta) )</td>
<td>220 ± 20%</td>
<td>600% (\text{a})</td>
</tr>
<tr>
<td>Human platelets</td>
<td>40l (prostaglandin ( \text{E}_2 ))</td>
<td>350 ± 50%</td>
<td>300% (\text{a})</td>
</tr>
<tr>
<td>Rat liver</td>
<td>~40%</td>
<td>N.D.</td>
<td>360%</td>
</tr>
</tbody>
</table>

* Braun et al. (1982).
† Cassel & Selinger (1976); Lester et al. (1982).
‡ Lester et al. (1982); Aktories & Jakobs (1981).
§ Steer et al. (1982); Jakobs et al. (1978).
\( \ast \) Calculated from Kimura & Nagata (1979).
** Kimura & Shimada (1980)

Table 1. The apparent dissociation constants for GTP in adenylate cyclase

N.D. not determined.
β-adrenoceptor coupling to adenylyl cyclase

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There have been substantial developments over the last few years in our understanding of the nature of hormone and neurotransmitter receptors and their association with the membrane-bound enzyme adenylyl cyclase. Much emphasis has been placed on α- and, in particular, β-adrenoceptors, since, unlike many hormone receptors, earlier intensive classical pharmacological studies have led to the development of a multitude of pharmacological agents that have been crucial in the characterization of adrenoceptor subtypes and in the mechanism of receptor–effector coupling. For example, the availability of selective competitive antagonists, full and partial agonists, has proved critical in the dissection of components involved in the coupling of these adrenoceptors to adenylyl cyclase and in the regulation of this transmembrane signalling process.

It is now clear that the β-adrenoceptor complex consists of a number of separate, though interacting, protein entities [receptor recognition site, one or possibly more guanine nucleotide-binding proteins (N-proteins or G-proteins) and the adenylyl cyclase unit (for review, see Ross & Gilman, 1980; Limbird, 1981). The crucial role of guanine nucleotides in transferring information from the receptor to adenylyl cyclase is now widely recognized (Limbird, 1981) and it now seems likely that alterations in the efficiency of this receptor–effector coupling may be an important mechanism by which cells regulate their responsiveness to catecholamines (Su et al., 1980; Iyengar et al., 1981). Although the precise mechanisms of β-catecholamine-induced stimulation of adenylyl cyclase are not fully understood, it is widely accepted that binding of an agonist to the receptor promotes the exchange of GDP by GTP at the G-protein and that the GTP bound form of the protein activates the cyclase. Activation of the enzyme is turned off by the hydrolysis of bound GTP to GDP and P, by GTPase activity associated with the G-protein (Cassell et al., 1977). There is much evidence compatible with this mechanism; for example, non-hydrolysable GTP analogues can cause persistent activation of cyclase (Londos et al., 1974), and cholera toxin, which greatly enhances GTP-induced activation of the cyclase, has been shown to inhibit GTPase activity in turkey erythrocytes (Cassell & Selinger, 1977) possibly by ADP-ribosylation of components of the G-protein (Johnson et al., 1978).

More recently, on the basis of the above evidence and on the behaviour of β-adrenoceptor agonists and antagonists in receptor binding assays, a ternary complex model of β-adrenoceptor–cyclase coupling has been proposed (Kent et al., 1980; De Lean et al., 1980). In this model it is believed that agonists induce or stabilize a complex of agonist, receptor and G-protein and that generation of this ternary complex is an absolute pre-requisite for cyclase activation. Further it seems probable that the extent by which an agonist induces or stabilizes the ternary complex relates directly to the relative efficacy of the agonist in terms of cyclase activity, and there is direct evidence that the G-protein that induces high-affinity binding of agonists to the β-receptor also conveys nucleotide-dependent stimulation to the catalytic unit (Stadel et al., 1981). In practical terms within a ligand binding assay, the availability of agonists to promote the formation of a receptor–G-protein complex corresponds to the ability of the agonist to form a high-affinity state of the receptor and is the basis for the shallow displacement curves that are characteristic of agonists competing against labelled antagonist ligands. Predictably, addition of guanine nucleotides to the assay results in a shift of the agonist competition curve to the right with steepening. This corresponds to the ability of guanine nucleotides to dissociate the receptor–G-protein complex resulting in a homogeneous population of receptors with lower affinity for agonists.

It should, however, be emphasized that this ternary complex model has been largely derived from data obtained from frog and avian erythrocytes and that there is evidence that these β-adrenoceptor systems may differ from those found in mammalian tissues. For example, we have recently provided evidence that the pharmacological characteristics of frog or chick erythrocytes do not strictly correspond to either the ββ or ββ-subclasses associated with mammalian tissues (Dickinson & Nahorski, 1981a). Moreover, extensive studies of the pharmacological characteristics of β-adrenoceptor binding sites in several mammalian tissues have revealed the co-existence of ββ- and ββ-adrenoceptors in many tissues (for review, see Nahorski, 1981). Since there is increasing evidence that both subtypes may be present on the same cell and mediate, under certain circumstances, the same physiological function (O'Donnell & Wanstall, 1981), it would seem critical to evaluate whether both subtypes display similar mechanisms of coupling to adenylyl cyclase.

Previous work from this laboratory has established that the non-subtype selective β-adrenoceptor antagonist 1H[3H]iodoalprenolol specifically labels sites in rat and rabbit lung that possess properties indicative of an interaction with β-adrenoceptors. Moreover, the use of highly selective ββ- or ββ-agonists and computer-assisted curve fitting has established that, whereas rat lung possesses predominantly ββ-sites (20% ββ, 80% ββ-receptors predominates in rabbit lung (60% ββ, 40% ββ). (Rugg et al., 1978; Dickinson & Nahorski, 1981). Examination of the effect of GTP on ββ-rich rat lung membranes revealed the agonist-specific effects of this nucleotide. Thus the occupation curve of the antagonist propranolol had a slope factor of close to unity and was totally unaltered by GTP. Salbutamol, which is a partial agonist at rat lung adenylyl cyclase, (maximum stimulation 56% of that of the full agonist isoprorenaline), generated a displacement curve of low overall slope (slope factor, nH – 0.8) in the absence of GTP. GTP decreased the overall ICσ (concentration producing 50% inhibition) of salbutamol by 2-fold and in the presence of the nucleotide the slope of the occupation curve approached unity. The full agonist isoprorenaline was markedly affected by GTP, resulting in a 3.3-fold decrease in overall affinity and steepening of the displacement curve (Fig. 1). The binding data were subjected to