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COMMENTARY

THE MODE OF COUPLING OF ADENYLATE CYCLASE TO HORMONE RECEPTORS AND ITS MODULATION BY GTP

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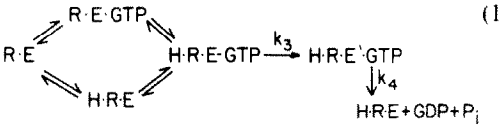
The discovery of the hormone sensitive adenylate cyclase in the late 1950's by Sutherland and coworkers [1, 2] led them to suggest that the system is comprised of a regulatory unit; the receptor, and the catalytic unit—the enzyme [2]. When the hormone or neurotransmitter binds to the receptor on the surface of the cell, the catalytic unit which is embedded in the membrane becomes activated through a conformational change transmitted from the receptor to the enzyme moeity. The exact mechanism of this coupling was not known at that time and became the subject of intensive and interesting studies by a number of laboratories.

The role of GTP

Rodbell and colleagues [3–5], through their studies on the glucagon sensitive adenylate cyclase from rat hepatic membranes, came to the conclusion that adenylate cyclase exhibits the properties of an allosteric regulatory enzyme. These investigators have established that in numerous adenylate cyclases [6] the guanyl nucleotide GTP controls the transmission of the message from the hormone receptor to the enzyme and, therefore, suggested [3, 5] that the adenylate cyclase system is composed of three interacting units. These units are: (1) a discriminator site for hormone binding (the receptor); (2) a transducer unit which possesses a nucleotide regulatory site having high specificity for GTP and (3) a catalytic function which converts ATP to cyclic AMP. The simultaneous occupancy of the receptor by the agonist and the binding of GTP to its regulatory site induce the activation of the catalytic moeity, namely, the adenylate cyclase. The requirement for both GTP and hormone to induce the activation of adenylate cyclase has since been demonstrated for many, if not all, of the hormone and neurotransmitter dependent adenylate cyclases.

The net result of such a relationship between the GTP and the hormone is that the action of these two ligand is synergistic in nature [7, 8]. In order to study in more detail the role of GTP in the activation of adenylate cyclase, the non-hydrolyzable analogues of GTP, such as guanylyl-methylene-diphosphate (GppCH$_2$p) [3] and later guanylyl-imido-diphosphate (GppNHp) [9], were introduced. Using these compounds it became indeed possible to demonstrate that the activation of adenylate cyclase by the hormone and the guanyl nucleotide is synergistic in nature [7, 8].

It was found that when GppNHp is used as the regulatory ligand instead of GTP, the enzyme is activated to a permanently active state [7, 10–14]. Furthermore, in the case of the β-adrenergic dependent adenylate cyclase, it was demonstrated that the permanently active state of the enzyme can be reversed back to its inactive state when the permanently active state is exposed to GTP in the presence of the hormone *l*-epinephrine [8, 15, 16]. This interesting finding suggests that only when GTP occupies the regulatory site, the activated form of the enzyme can revert back to its inactive form. Thus, both the process of enzyme activation and the process of enzyme deactivation are agonist dependent [15]. Indeed, both the process of enzyme activation by GppNHp and hormone and the process of reversal of the enzyme from its permanently active state to its inactive form are specifically inhibited by β-adrenergic antagonists such as propranolol [7, 8, 16, 17]. Recently it was found that turkey erythrocyte membranes possess β-adrenergic receptor dependent GTPase [18] which is specifically blocked by propranolol and which cannot induce the hydrolysis of the GTP analogues GppNHp or guanosine 5′-(γ-thio)-triphosphate (GTPγS). This finding establishes the hypothesis [15, 19] that the process of enzyme activation involves the following steps:



(1)

where R is the receptor, E the enzyme, k_3 is the rate of enzyme activation, and k_4 is the rate of enzyme deactivation. In the activated state, GTP is hydrolyzed at the regulatory site to GDP and phosphate, leading to the deactivation of the enzyme [15]. Only the activated form of the enzyme, E′, is responsible for the synthesis of cAMP from ATP. Thus, cAMP will be produced by the system as long as the enzyme can be activated to the E′ form, namely, the continuous occupancy of the receptor by the hormone and a continuous supply of GTP. When the concentration of either one of these two ligands drops, the adenylate cyclase activity decays.

From the scheme described in equation 1, it is also clear that when GTP is substituted by non-hydrolyzable analogues, such as GppNHp or guanosine 5′-(γ-thio)-triphosphate, GTPγS [19], the enzyme is 'stuck' in a permanently active state R.E′.GppNHp, even when the hormone is removed from the receptor [7, 8, 10–14]. This model also

predicts [15, 16] that the enzyme can be reverted back from its permanently active state only when GTP replaces GppNHp from the regulatory site. Indeed, it has been demonstrated that R.E'. GppNHp can be reverted back to R.E only when the permanently active enzyme is exposed simultaneously to hormone and GTP or ATP [16, 17, 20].

The individual rate constants k_3 and k_4 were measured in the turkey erythrocyte catecholamine dependent adenylate cyclase and found to be: $k_3 = 0.7\ min^{-1}$ [8] and $k_4 = 6\ min^{-1}$ [20]. The absolute values of k_3 and k_4 may change from one preparation to the other, depending on the degree of coupling between the receptor and the enzyme. The ratio k_4/k_3, however, is constant. Using the scheme described in equation 1, one can therefore calculate that only a fraction of the total (E_T) adenylate cyclase is in its activated form E' under steady state conditions in the presence of GTP:

$$[E'] = \frac{[E_T]}{1+k_4/k_3} = \frac{[E_T]}{1+(6/0.7)} \sim 0.1[E_T] \qquad (2)$$

Since in the presence of GppNHp $k_4 = 0$, *all* the enzyme is converted to its active form, and thus specific activity of the adenylate cyclase is 10-fold or higher [8] than in the presence of GTP [7, 8, 10–14]. Another line of evidence in favor of the general scheme described in equation 1 comes from studies on the mechanism of the irreversible activation of adenylate cyclase by cholera toxin [21]. It has been shown that cholera toxin induces an increase in the extent of adenylate cyclase activation by hormone and GTP [22, 23]. This activating effect of the cholera toxin is probably due to the inhibition of the GTPase step [23]. Thus, the effect of cholera toxin is to decrease the value of the rate constant k_4 (equations 1 and 2). This effect on k_4 explains the increase in the hormone stimulated activity in the presence of GTP subsequent to cholera toxin treatment, since the value of the denominator in equation 2 decreases. Furthermore, it explains why the effect of GTP on the cholera toxin treated membrane is reversible, unlike that of GppNHp [22].

Recently, direct evidence for the existence of a transducer entity possessing a GTP regulatory site as been obtained in the laboratory of Professor Ernst J. M. Helmreich from Würzburg, West Germany. Thomas Pfeuffer was able to solubilize [24] the β-adrenergic receptor dependent adenylate cyclase from pigeon erythrocytes and separate the catalytic unit from the GppNHp binding protein, and to demonstrate, that the activation of the enzyme requires the guanyl nucleotide binding protein and the guanyl nucleotide GppNHp [24]. It is not clear, however, at this stage, whether this protein represents also the GTPase, although recent experiments suggest this hypothesis (Cassel and Pfeuffer, personal communication). The complete separation of the adenylate cyclase complex into its components—the receptor, the transducer, and the enzyme—and their complete reconstitution, has yet to be demonstrated.

The physical relationship between the receptor and the enzyme

An interesting question which has only recently attracted the attention of investigators in the field is the architectural organization of the adenylate cyclase system and its distribution on the membrane surface [25]. Although it is quite clear that the enzyme adenylate cyclase and its receptor are separate macromolecules [26], little is known about their stoichiometry, their organization within the membrane, and the mode of coupling between them. Four major types [20, 25] of coupling mechanisms between the receptor and the enzyme can be considered:

(1) *The precoupled mode.* In this model it is assumed that the receptor and the enzyme are permanently attached to each other, and the sequence of events upon activation follows equation 1. The scheme described in equation 1 can be represented in a simplified form:

$$H+R\cdot E \underset{K_H}{\rightleftharpoons} H\cdot R\cdot E \xrightarrow{k_3} H\cdot R\cdot E' \qquad (3)$$

The guanyl nucleotide has been omitted from all schemes for the sake of simplicity. Since in the presence of GppNHp no reversion occurs, one can study by simple means the process of enzyme activation as described in the various schemes. When GppNHp is used, the enzyme remains in its active form E'.

(2) *The dissociation model.* In this model it is assumed that the receptor and the enzyme are precoupled but dissociate upon hormone binding prior a concomitant to enzyme activation:

$$R\cdot E+H \rightleftharpoons H\cdot R\cdot E \rightarrow HR+E' \qquad (4)$$

(3) *The floating receptor model.* In this case the receptor and the enzyme are in equilibrium with the receptor–enzyme complex [27, 28] according to the following scheme:

$$R+E \rightleftharpoons R\cdot E \underset{K_H}{\overset{H}{\rightleftharpoons}} H\cdot R\cdot E \rightarrow H\cdot R\cdot E' \rightleftharpoons HR+E' \qquad (5)$$

The two forms of the activated enzyme E' and H·R·E' can be reverted back to the inactive form of the enzyme E and H·R·E when GTP occupies the regulatory subunit.

(4) *The collision coupling mechanism.* This mechanism is an extreme case of the floating receptor mechanism [20, 25] and is described by the following equation:

$$HR \overset{E\cdot k_1}{\rightleftharpoons} (H\cdot R\cdot E \rightleftharpoons H\cdot R\cdot E') \xrightarrow{k_4} HR+E' \qquad (6)$$

The difference between model 4 and model 3 is in the assumption that the activated form of the enzyme is exclusively E' and that the concentration of the species H·R·E, R·E and H·R·E' are negligible. Namely, the process of enzyme activation is *bimolecular* in nature and occurs during the

Table 1. The mode of hormone binding and the kinetics of adenylate cyclase activation as predicted by the various models

Model	Mode of hormone binding	Kinetics* of cyclase activation	Effect of reducing $[E_T]$ on the activation kinetics	Effect of reducing $[R_T]$ on the activation kinetics
1 Precoupled	Non-cooperative	First order	No change in the rate of activation; proportional reduction in maximal response	No change in the rate of activation; proportional reduction in maximal response
2 Dissociation	Negatively cooperative	Deviates from first order	Proportional reduction in the rate of activation and in maximal response	Proportional reduction in the rate of activation and in maximal response
3 Equilibrium-floating	Negatively cooperative	Deviates from first order	As in Model 2	As in Model 2
4 Collision-coupling	Non-cooperative	First order	No change in the rate of activation; proportional reduction in maximal response	Proportional reduction in the rate of activation; no change in the maximal response

* The kinetics of enzyme activation can be followed in the presence of GppNHp as a function of time and as a function of hormone concentration. Under these conditions, the enzyme is activated to a permanently active state. Thus, the rate of its activation can be monitored with great precision by incubating the membrane with GppNHp and the agonist, stopping the reaction at desired time points by adding excess antagonist [7, 8] and examining the specific activity attained. This table is a summary of a more elaborate analysis presented elsewhere [20].

transient encounter of the hormone-bound receptor and the enzyme.

The four different models have different predictions concerning both the mode of hormone binding and the kinetics of enzyme activation. A summary of these predictions is given in Table 1.

Detailed binding studies on the β-adrenergic receptor of turkey erythrocytes [25,29,30] have shown that the binding of either agonist or antagonist is non-cooperative. Furthermore, the binding of antagonists such as [^{3}H]propranolol [30] or [^{125}I]hydroxybenzylpindolol [20] to the β-receptor adenylate cyclase complex in its permanently active form, namely, subsequent to GppNHp and hormone activation, is also non-cooperative. Detailed displacement experiments [25] revealed that the binding of agonists subsequent to activation by hormone and GppNHp is non-cooperative as well. Both the dissociation model and the equilibrium floating model (Table 1) can be rejected since both models predict negatively cooperative hormone binding. Furthermore, these two models can be rejected also because the kinetics of enzyme activation in the presence of GppNHp and *l*-epinephrine is first order (Fig. 1), whereas both models predict non-first order kinetics of enzyme activation. Thus, the two diametrically opposed models, the precoupled model and the collision coupling model (Table 1), account equally well for both the binding data and the kinetic data. Closer examination of Table 1 reveals, however, one fundamental difference between the predictions of the precoupled model and the collision coupling model. The precoupled model predicts that a reduction in the concentration receptors will result in a proportional reduction in the maximal level of enzyme activation, whereas no change is expected in the rate constant of activation (k_3 of equation 1). This conclusion is easily reached if one examines the equation which describes the rate of enzyme activation to its permanently active state:

$$[H \cdot R \cdot E'] = [RE]_T\left\{1-\exp-\left(\frac{k_3[H]}{K_H+[H]}t\right)\right\}$$
$$= [RE]_T\{1-\exp(-k_{obs}t)\} \qquad (7)$$

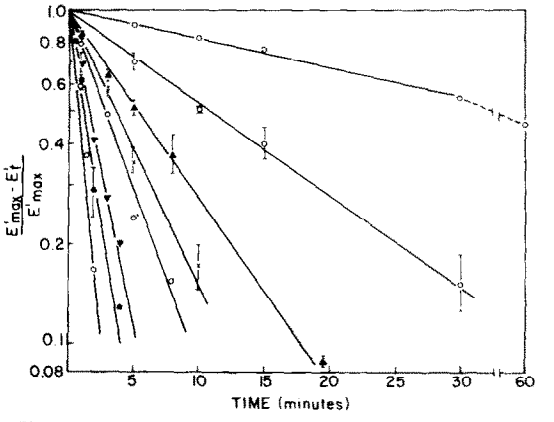


Fig. 1. *Time course of adenylate activation by GppNHp and l-epinephrine: A pseudo first order representation.* Turkey erythrocyte membranes were incubated at 37° with 1.0×10^{-4} M GppNHp (saturating) and increasing concentrations of *l*-epinephrine. At times indicated, the activation reaction was stopped by the addition of saturating concentrations of propranolol, and adenylate cyclase activity was measured. The *l*-epinephrine concentrations used were (from top to bottom): ○—○, 1.0×10^{-8} M; ○—○, 1.0×10^{-7} M; ▲—▲, $4.0 \times .0^{-7}$ M; ×—×, 8.0×10^{-7} M; ○—○; 1.0×10^{-6} M; ▼—▼, 4.0×10^{-6} M; ●—●, 8.0×10^{-6} M; ○—○, 2.0×10^{-5} M.

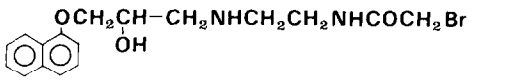


Fig. 2. *The β-adrenergic receptor affinity label*
The synthesis of the β-adrenergic receptor affinity label *N*-(2-hydroxy-3-(*l*-naphthyloxy)-propyl)-*N'*-bromoacetyl-ethylenediamine is described elsewhere. The affinity label irreversibly blocks the specific binding of [^{3}H]-propranolol [31, 32] and of [^{125}I]hydroxybenzylpindolol [20] to the β-adrenergic receptors.

$[RE]_T$ is the total receptor–enzyme concentration, k_3 the intrinsic rate constant of enzyme activation, and K_H the receptor–hormone dissociation constant. At saturating hormone concentrations: $k_{obs} = k_3$.

In contrast, the collision coupling mechanism predicts that upon reduction in the receptor concentration, a proportional decrease in the observed rate constant (k_{obs}) of enzyme activation occurs, whereas the maximal level of enzyme activation remains unchanged and equals the total enzyme concentration:

$$[E'] = [E_T]\left\{1-\exp\left(-k_1[R_T]\frac{[H]}{K_H+[H]}\right)t\right\} = [E_T]\{1-\exp(-k_{obs}t)\} \quad (8)$$

At saturating hormone concentrations: $k_{obs} = k_1[R_T]$. Thus, a diagnostic experiment was immediately performed on the turkey erythrocyte membrane in order to decide which of these two diametrically opposed models describes the mode of coupling between the receptor and the enzyme. The concentration of β-adrenergic receptors on the turkey erythrocyte membranes was progressively reduced by treatment with a specific β-adrenergic receptor affinity label [31, 32] described in Fig. 2. Then the membranes were subjected to activation by saturating concentrations of epinephrine and GppNHp. It was found that the progressive decrease in receptor concentration resulted in a proportional decrease in the rate constant of activation, but the same level of maximal activation was achieved as predicted by equation 8 (Fig. 3, Table 2). The final level of permanent activation was found to be always equal to that which is found in the absence of affinity label treatment, namely, that the final concentration of E' always equals $[E_T]$, as predicted by equation 8.

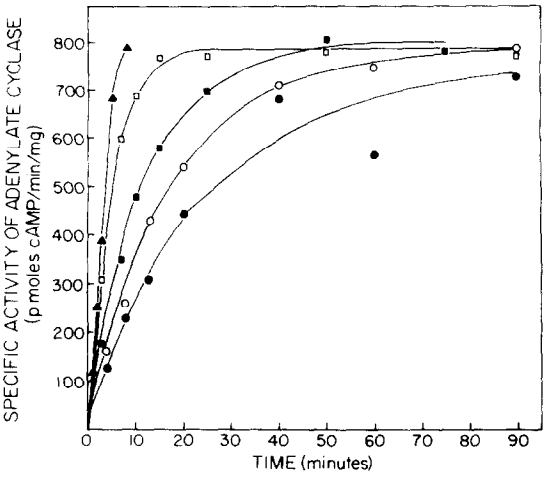


Fig. 3. *The effect of affinity label treatment on the activation kinetics of adenylate cyclase by l-epinephrine and GppNHp*
Membranes were treated with increasing concentrations of affinity label, as described in Table 2, and then subjected to activation by saturating concentrations of GppNHp and of *l*-epinephrine.
▲—▲, Untreated membranes; □—□, 1.67×10^{-5} M affinity label; ■—■, 4.34×10^{-5} M affinity label; ○—○, 1.0×10^{-4} M affinity label; ●—●, 2.3×10^{-4} M affinity label.

Furthermore, from equation 8 it is apparent that from the dependence of the observed first order rate constant k_{obs} on hormone concentration, one can determine the hormone–receptor dissociation constant K_H and compare this value with those measured independently by direct binding measurements. The close correspondence between the K_H values obtained by the two methods [20] also supports the validity of the 'collision-coupling' model. It should be emphasized that the kinetic predictions of the 'collision-coupling' model with respect to the mode of enzyme activation by hormone and GTP, are indistinguishable from those predicted by a model assuming a permanent coupling between the receptor and the enzyme. Thus, the level of active enzyme, as predicted by the

Table 2. The effect of affinity label on the activation rate of adenylate cyclase by *l*-epinephrine and GppNHp

Concentration of affinity label applied (M)	[^{125}I]hydroxybenzylpindolol* binding		Rate constant, k_{obs}, of enzyme activation by *l*-Epinephrine and GppNHp†	
	Extent pmoles/mg	Per cent of maximum	$k_{obs} = k_1[R_T]$ (min^{-1})	Per cent of maximum
0	1.12	100	0.374	100
1.67×10^{-5}	0.64	58	0.217	58
4.34×10^{-5}	0.34	30	0.178	47.6
1.0×10^{-4}	0.15	13.3	0.051	13.7
2.3×10^{-4}	0.105	9.3	0.030	8.1

* Affinity label treatment results in the loss of [^{125}I]hydroxybenzylpindolol binding sites but no change in the affinity of the remaining sites towards the antagonist.

† The kinetics of adenylate cyclase activation by *l*-epinephrine and GppNHp is first order in each case, and no change in mechanism occurs upon receptor modification. The kinetic experiments were performed at saturating concentrations of *l*-epinephrine and GppNHp, thus $k_{obs} = k_1[R_T]$ (see equation 8).

'collision-coupling' mechanism, in the presence of GTP rather than GppNHp, is given by:

$$[E'] = \frac{[E_T]}{1 + \frac{k_4}{1 + k_1[R_T]}} \quad (9)$$

where k_4 is the rate constant characterizing the GTPase step (as in equation 2) and the product $k_1[R_T]$ characterizes the activation step (instead of k_3 in equation 2). The 'collision coupling' model also explains rather well the nature of the permanently active state of adenylate cyclase obtained in the presence of hormone and GppNHp. If one examines the scheme presented in equation 6, it is immediately apparent that the activated state of the enzyme E' *is no longer* coupled to the receptor. Thus, *the addition of the antagonist propranolol does not change the fate of E'*. If E' possesses GppNHp at its regulatory site, the enzyme will *remain* permanently active, whereas if E' possesses GTP at its regulatory site, the latter will be hydrolyzed with the concomitant deactivation of the enzyme [15, 16, 20].

The topographical distribution of receptor and enzyme on the membrane

It should be stressed that the 'collision-coupling' mode of activation has far reaching implications with respect to the *topographical distribution* of the receptor molecules and the enzyme molecules. Thus, if k_1 in the scheme described in equations 6 and 8 depict the *collision* between the receptor and the enzyme, one should be able to correlate this bimolecular rate constant to the diffusion coefficients which characterize the movement of the two entities within the membrane matrix. Since the number of receptors per turkey erythrocytes has been accurately determined [29, 33–35] and since it is reasonable to assume [30] that the number of receptors is similar to the number of enzyme molecules, one can calculate the expected value of k_1 if one can estimate the diffusion coefficient of the receptor and/or of the enzyme. Using these calculations (Hanski and Levitzki, submitted for publication), one has to conclude that probably the receptors and the enzyme *are not randomly distributed* in the membrane but rather *confined to domains*. Although so far we have no direct evidence that the β-adrenergic receptors are confined to domains in turkey erythrocytes, suggestive evidence does exist for other cells which possess β-adrenergic receptors. Using the fluorescent β-adrenergic blockers [36], 9-aminoacridino propranolol (9-AAP) and the dansyl analogue of propranolol (DAPN) [37, 38], Atlas *et al.* found that cells known to possess β-adrenergic receptors reveal dotted fluorescence when exposed to these compounds. Even if one argues that the fluorescent antagonist or the β-adrenergic agonist (which is monovalent!) induces the formation of clusters of receptors, it does not invalidate the argument as enzyme activation occurs subsequent to hormone binding.

It should be emphasized that although it was demonstrated that in the case of the β-adrenergic receptor and the adenylate cyclase the collision-coupling mechanism applies, other modes of coupling are feasible [20, 25, 27, 28]. Thus, using similar experimental techniques, we were able to show that the adenosine receptor of turkey erythrocytes is permanently acoupled to the enzyme [39] and thus activates the enzyme, according to the precoupled model. Furthermore, it was possible to show [17, 39] that the *same* adenylate cyclase molecule can be activated either by an adenosine receptor to which it is permanently coupled or by a collision with the β-adrenergic receptor. The two modes of adenylate cyclase activation exemplified by the adenosine receptor and by the β-adrenergic receptor are, in fact, only two out of four different modes of receptor to enzyme coupling (see above). It seems, however, that at least in the case of receptors coupled to adenylate cyclase, the two models of activation described here are the most prevalent ones.

The remaining question to be dealt with in this context is: what is the topographical relationship of the transducer entity to the hormone receptor and the enzyme? Detailed kinetic analysis on the mode of adenylate cyclase activation as a function of GppNHp concentration in the turkey erythrocyte system suggests very strongly (Tolkovsky and Levitzki, in preparation) that the guanyl nucleotide regulatory protein (the transducer) is tightly coupled to the catalytic unit of the adenylate cyclase. More direct evidence of this point came from studies by Pfeuffer [24], who showed that the pigeon erythrocyte cyclase is associated with the regulatory GTP binding protein even subsequent to deterrent solubilization.

The coupling of adenylate cyclase to two receptors

In a number of cells such as the fat cell, it was shown [40, 41] that the adenylate cyclase is stimulated by a number of hormones including glucagon, ACTH, secretin, and catecholamines. An intriguing question is whether *all* receptors are linked to *one* pool of adenylate cyclase or each of the receptors is linked to its own adenylate cyclase. In a detailed study performed by us [17, 39] recently, we have shown that the adenylate cyclase of the turkey erythrocyte represents a *single pool* of enzyme capable of interacting with both the adenosine receptor or with the β-adrenergic receptor. We have shown, however [39], that the action of adenosine and *l*-epinephrine is not additive but rather *competitive*. In addition, we were able to show [39] that the *two receptors* are coupled to a *single* adenylate cyclase molecule through a common GTP regulatory unit. The relationship between the adenylate cy-

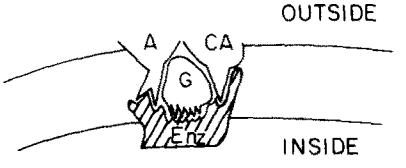


Fig. 4. *The structure of the adenylate cyclase system in turkey erythrocytes*

CA = Catecholamine receptor; Ad = adenosine receptor; G = the GTP regulatory unit.

clase, the GTP regulatory unit, and the receptors for adenosine and epinephrine are organized as presented schematically in Fig. 4.

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