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The Attenuation of Epinephrine-Dependent Adenylate Cyclase by Adenosine and the Characteristics of the Adenosine Stimulatory and Inhibitory Sites

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SUMMARY

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Turkey erythrocyte and rat caudate adenylate cyclase [ATP pyrophosphatase-lyase (cyclizing) EC 4.6.1.1] respond to β-receptor adenergic agents and to adenosine agonists. In both systems single adenylate cyclase responds to the two receptors. In both tissues the effect of the two agonists is not additive and the enzyme can be activated only by one agonist at a time.

The adenosine receptor is permanently coupled to the adenylate cyclase moiety and therefore the portion of the enzyme that is in its active form is directly proportional to the degree of saturation of adenosine sites. In contrast, the β-receptor is not coupled to the enzyme and activates the latter by a biomolecular collision between the two species that occurs during the diffusion of the hormone bound β-receptor. It follows that increasing concentrations of adenosine strongly enlarge the part of cyclase molecules governed by the adenosine receptor and, accordingly, reduce the free enzyme pool, accessible to β-receptor agonists, whereas hormone-occupied β-receptor cannot influence the pool of adenylate cyclase accessible to the adenosine receptor. Hence at saturating concentrations of adenosine only the adenosine-induced activity will be expressed and the combined effect of the two ligands is independent of β-agonist concentration. In turkey erythrocyte adenylate cyclase adenosine is a much poorer agonist than a β-agonist. Therefore, increasing adenosine concentrations *inhibit* β-agonist-stimulated cAMP production. The caudate nucleus adenylate cyclase presents a diametrically opposite situation where adenosine is a more potent agonist than epinephrine and thus, in the presence of adenosine, cAMP production is increased above that achieved by β-agonists. Caudate nucleus cyclase but not turkey erythrocyte cyclase possesses also an adenosine inhibitory site distinct from the stimulatory site. The ligand specificities of these two types of adenosine sites are different. The possible physiological role of the adenosine-induced modulation of hormonal response is discussed.

INTRODUCTION

The combined effect of adenosine and different hormones on adenylate cyclases from various tissues is widely reported (1-

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7). The vast diversity of observations concerning the combined effect of adenosine with other hormones [synergistic (1), additive (7), non-additive (5) and inhibitory (2, 4, 6) responses] demonstrate the complexity of the interrelationships between adenosine and other hormones in terms of their combined effect on the production of cAMP. Clearly, when the effect of hormones and adenosine is studied at the level of whole tissues or intact cells, a number of extracellular and intracellular events may contribute to the final outcome of the combined action of adenosine and hormone. Such events are: uptake and reuptake of adenosine (8), release of endogenous adenosine (9), metabolism of adenosine and of adenine nucleotides (10), adenosine and hormone-modulated release of neurotransmitters (11), the influence of cations (12), α-adrenergic receptors (7, 13), to name a few.

The combined action of adenosine and hormones on broken cell preparations reveals a more coherent picture. In homogenates or membrane preparations, adenosine has mostly an inhibiting effect on hormone-dependent adenylate cyclase activity (3, 4, 14). This inhibitory effect has been observed in a large number of the known hormone and neurotransmitter-dependent adenylate cyclases.

Many adenylate cyclase systems reveal two effects of adenosine: stimulatory at low concentrations and inhibitory at higher concentrations (1, 2, 10, 15). These observations led to the concept of an adenosine stimulatory site, mediated by an extracellular receptor—the "R" site— and an adenosine inhibitory site—the "P" site—presumably intracellular (16, 17). These two sites were shown to exhibit different ligand specificities (16, 18). In this communication we report on the properties of these two types of sites in rat *caudate nucleus* and compare the "R" sites of the rat system to that of the turkey erythrocyte system. Since in the turkey system the "P" site is absent, it was possible to explore the interrelationships between the adenosine receptor and the β-adrenergic receptor, both of which are coupled to a common pool of adenylate cyclase (19). This competitive relationship between the two receptors (19) leads, as we shall demonstrate, to the inhibition of the β-adrenergic response by adenosine through the adenosine stimulatory site. The possible physiological relevance of the inhibition of a hormonal signal through the stimulatory adenosine receptor "R" site and that of the inhibitory site are discussed.

MATERIALS AND METHODS

Materials. Adenosine, 2′-deoxyadenosine, N^6-phenylisopropyladenosine, adenosine deaminase, *l*-epinephrine bitartrate, phosphocreatine, creatine phosphokinase, guanosine 5′-triphosphate (GTP) sodium salt (Type I) from equine muscle, and theophylline were supplied by Sigma.

Adenosine 5′-triphosphate (ATP) disodium salt synthesized by phosphorylation of adenosine was obtained from Sigma and purified, as previously described (20).

[α-^{32}P]ATP triethylammonium salt (13.5 Ci/mmole) was purchased from Radiochemical Centre (Amersham, England).

5′-Guanylyl-imidodiphosphate (Gpp-NHp) was supplied by ICN Pharmaceuticals (Cleveland, Ohio, USA).

4-(3-butoxy-4-methoxybenzyl)-2-imidazolidine (RO20-1724) was a generous gift from Dr. H. Sheppard of the Roche Institute (Nutley, New Jersey, USA).

Turkey erythrocyte and rat caudate nucleus membranes. Turkey erythrocyte membranes were prepared and stored, as previously described (21).

Adenylate cyclase from caudate nucleus was prepared in the form of membranes, as follows: 10 male rats weighing 300 to 350 g were killed by decapitation. Brains were removed and the caudate nucleus was separated on a cold plate at 4°. Tissues were homogenized, using a Dounce homogenizer (10 strokes) in 30 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 2 mM $MgSO_4$ at 4°. The homogenate was centrifuged at 1,000 × *g* for 15 min. The pellet was discarded and the supernatant was centrifuged at 12,000 × *g* for 10 min. The resulting pellets were resuspended in the same buffer (5 ml) using a Dounce homogenizer, and applied to a sucrose gradient (15 ml of 45% w/w sucrose, lower layer, 20 ml of 25% w/w sucrose, upper layer), and cen-

trifuged at 13,500 × *g* for 45 min at 4°. The membrane fraction between the two sucrose layers was collected, washed with the same buffer, recentrifuged, and stored frozen under liquid nitrogen in the same buffer, containing 25% w/w sucrose.

For adenylate cyclase activity measurements, membranes were washed with 20 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 2 mM $MgSO_4$, recentrifuged at 10,000 × *g* for 10 min and suspended in the same buffer at a suitable concentration. There were no changes in adenylate cyclase activity after two months of storage under liquid nitrogen.

Protein was determined according to Lowry *et al.* (22), using bovine serum albumin as a standard.

Adenylate cyclase activity. Adenylate cyclase activity was measured according to Salomon *et al.* (23). The assay mixture contained 20 mM Tris-HCl, pH 7.4; EDTA, 1 mM; [α-^{32}P]ATP, 1 mM; $MgSO_4$, 5 mM; phosphocreatine, 2.2 mg/ml; creatine phosphokinase, 0.2 mg/ml; and phosphodiesterase inhibitor R020-1724, 0.3 mM. Rat caudate adenylate cyclase assays contained also 1 mM GTP.

All experimental values are means of triplicate determinations, and typically two independent determinations were performed. The range of variations between the determinations was less than 10%.

THEORY

We have already shown (19, 20) that the modes of coupling between the adenosine receptor and the β-adrenergic receptor to turkey erythrocyte adenylate cyclase are very different. The β-receptor is physically separate from the enzyme and activates the latter by a "collision-coupling" mechanism, whereas the adenosine receptor is permanently attached to the enzyme. The mechanism of enzyme activation by the two receptors is given by the schemes:

$$\begin{array}{ccccc} H\cdot R_H + R_A\cdot E & \xrightarrow{k_1} & (H\cdot R_H\cdot R_A\cdot E) & \longrightarrow & R_AE' + H\cdot R_H \\ \| K_H & \uparrow & & & \| K_H \\ H + R_H & & \xrightarrow[k_{off}]{} & & H + R_H \end{array} \quad (1)$$

for the "collision coupling" mechanism, and

$$\begin{array}{ccccc} A\cdot R_A\cdot E & \xrightarrow{k_1} & A\cdot R_AE' & \rightleftharpoons^{K_A} & R_AE' + A \\ \| K_A & \uparrow & \xrightarrow{k_{off}} & & \\ A + R_AE & & \xrightarrow[k_{off}]{} & & \end{array} \quad (2)$$

for the adenosine mode of activation. R_H and R_A are the β-receptor and the adenosine receptor, respectively; K_H and K_A are the dissociation constants characterizing the binding of the two ligands to their respective receptors. The pseudo-first order rate constant of enzyme activation by the β-receptor is given by k_1 $[R_H]_T$ where $[R_H]_T$ is the total β-receptor concentration (19). The first order rate constant for cyclase activation by adenosine is given by k_3. k_{off} characterizes the termination of the hormonal signal by the GTPase (24, 25). By studying the kinetics of accumulation of the permanently activated state of the enzyme, E', induced by the agonist plus GppNHp ($k_{off} = 0$), it was demonstrated that the two receptors operate on a *common* adenylate cyclase pool through a common GTP regulatory site (19). Furthermore, it was shown that the two receptors *compete* with one another (19). Namely, when $H\cdot R_H$ collides with an enzyme species already possessing adenosine on its receptor, the former is not capable of augmenting the effect of adenosine and, in fact, is not recognized by the cyclase moiety. Similarly, if adenosine binds to its receptor during the transient formation of the ternary complex $H\cdot R_H\cdot R_A\cdot E$, it is unable to augment the effect of the β-agonist. Thus, the interrelationship between the two receptors is competitive in nature.

The competitive relationship between the two receptors predicts that the *l*-epinephrine-induced activity will be *inhibited* by adenosine in the presence of GTP, namely when the GTPase turn-off reaction (schemes 1 and 2) takes place. This is due to the fact that adenosine is a much less potent agonist than the β-agonists: k_3

equals 0.13 k_1 $[R_H]_T$. We shall now derive the equations that characterize the interrelationship between adenosine and β-agonists in the turkey system. From Eq. 1 and 2 it is apparent that the steady state concentration of the activated form of the enzyme, namely the sum of $R_A \cdot E'$ and $A \cdot R_A \cdot E'$ can be obtained from the expression:

$$k_{off}([R_A \cdot E'] + [A \cdot R_A \cdot E']) = k_1 [H \cdot R_H][R_{\dot{A}}E] + k_4[A \cdot R_{\dot{A}}E] \quad (3)$$

In the turkey erythrocyte system the fraction of the adenylate cyclase in its activated form is small (24, 25) compared to the total amount of enzyme, namely:

$$[R_A \cdot E'] + [A \cdot R_A \cdot E'] \ll [R_A \cdot E]_T \quad (4)$$

where the sum $[R_A \cdot E'] + [A \cdot R_A \cdot E']$ is the concentration of adenylate cyclase in its active form and $[R_A \cdot E]_T$ is the total cyclase concentration. Therefore, a good approximation will be:

$$[R_A \cdot E]_T \sim [R_A \cdot E] + [A \cdot R_A \cdot E] \quad (5)$$

but since

$$[A \cdot R_{\dot{A}}E] = \frac{[R_A \cdot E]_T[A]}{K_A + [A]} \quad \text{or} \quad [R_{\dot{A}}E] = \frac{[R_A \cdot E]_T K_A}{K_A + [A]} \quad (6)$$

one can rewrite Eq. 3 as:

$$[R_{\dot{A}}E'] + [A \cdot R_A \cdot E'] = \frac{\frac{k_1}{k_{off}}[R_H]_T[R_{\dot{A}}E]_T[H]}{K_H + [H]} \cdot \frac{K_A}{K_A + [A]} + \frac{\frac{k_3}{k_{off}}[R_A \cdot E]_T[A]}{K_A + [A]} \quad (7)$$

The term

$$\frac{\frac{k_1}{k_{off}}[R_H]_T[R_A \cdot E]_T[H]}{K_H + [H]}$$

is the specific activity of the β-agonist dependent adenylate cyclase activity in the *absence* of adenosine and is designated V_H. The term

$$\frac{\frac{k_3}{k_{off}}[R_{\dot{A}}E]_T[A]}{K_A + [A]}$$

is the adenosine dependent cyclase specific activity V_A. Thus, Eq. 7 can be rewritten as:

$$v_{obs} = V_H \frac{K_A}{K_A + [A]} + V_A \quad (8)$$

In the turkey erythrocyte system $V_{H(max)} >> V_{A(max)}$ and thus it is expected that adenosine should attenuate the *l*-epinephrine-dependent activity. Equation 8 predicts that at *any* β-agonist concentration adenosine will *inhibit* cyclase activity where the maximal specific activity attained at saturating adenosine component will be the characteristic V_A. When $V_{A(max)} > V_{H(max)}$ eq. 8 predicts that the adenosine effect will be stimulatory even at saturating hormone concentrations. As will be shown in a later section this situation exists in the caudate nucleus adenylate cyclase.

Equation 8 suggests that adenosine influences epinephrine-induced adenylate cyclase activity, whereas epinephrine has no effect on adenosine-induced activity. This asymmetry is due to the different mode of coupling of the two receptors to the cyclase. According to the "collision coupling" model only a small fraction of the enzyme is at any given time associated with the β-receptor $[H \cdot R_H \cdot R_A \cdot E] << [R_A \cdot E]_T$ so it cannot reduce the pool of the cyclase subjected to adenosine-induced activation. In contrast, the concentration of adenosine-occupied precoupled receptor-enzyme complex might be large, and at saturating concentrations of adenosine all the cyclase pool is occupied by adenosine and $[A \cdot R_A E] = [R_A \cdot E]_T$. Under these conditions no free cyclase is available to interact with the hormone-occupied β-receptor and only adenosine-induced activation can be expressed.

A more convenient form of Eq. 8 can be obtained by substituting

$$V_A = \frac{V_{A(max)}[A]}{K_A + [A]}$$

(where

$$V_{A(max)} = \frac{k_3}{k_{off}} [R_A \cdot E]_T$$

the maximal velocity induced by adenosine, see eq. 7). Thus:

$$V_{obs} = V_H \frac{K_A}{K_A + [A]} + v_{A(max)} \frac{[A]}{K_A + [A]} \quad (9)$$

RESULTS

The combined action of adenosine and l-epinephrine on turkey erythrocyte adenylate cyclase. Adenylate cyclase activity as a function of adenosine concentration in the presence of different constant *l*-epinephrine concentrations, is presented in Fig. 1. The data were fitted to the equation:

$$v_{obs} - 0.32\ V_H = 0.68\ V_H \frac{K_A}{K_A + [A]} + V_A \quad (10)$$

which is similar to Eq. 8 derived above. The difference between Eq. 8 and Eq. 10 stems from the fact that only a fraction of the adenylate cyclase pool in turkey erythrocytes is adenosine-sensitive, whereas all of the pool is epinephrine-sensitive (19). The adenosine-insensitive cyclase pool was found to be 30–32% of the total epinephrine-sensitive pool in a large number of preparations of turkey erythrocyte membranes. The activation of 68–70% of the cyclase pool can be achieved by all R-site agonists such as adenosine, 2-cl-adenosine and phenylisopropyladenosine. Thus Eq. 8 should be corrected to yield Eq. 10. v_{obs} is the measured specific activity, V_H is the specific activity in presence of *l*-epinephrine alone; 0.32 V_H is the fraction of adenylate cyclase that cannot couple to the adenosine receptor; 0.68 V_H is therefore the fraction of adenylate cyclase which can couple to the receptors. K_A is the adenosine receptor dissociation constant, as determined from the adenosine dependence of adenylate cyclase activity (20) or from the adenosine concentration-dependence of adenylate activation to its permanently active state in the presence of GppNHp (19) and [*A*] is the adenosine concentration.

The maximal specific activity for the adenosine-dependent specific activity was found to be 7.1 pmole cAMP mg/min and the value of K_A was found to be 9.0 μM at 25°, in good agreement with previous reports (19, 20). Figure 7 depicts the correspondence between the observed specific activity in the presence of *l*-epinephrine and adenosine combined and the theoretical value predicted by Eq. 10. Similar experiments were conducted, using two adenosine analogues, N^6-phenylisopropyladenosine and 2-deoxyadenosine. These two ligands affected *l*-epinephrine activity in a manner identical to that of adenosine, and in all three cases the inhibition of the *l*-epinephrine-dependent cyclase activity is best described by Eq. 8. In addition, in all three cases the ligands inhibit *l*-epinephrine-dependent adenylate cyclase activity.

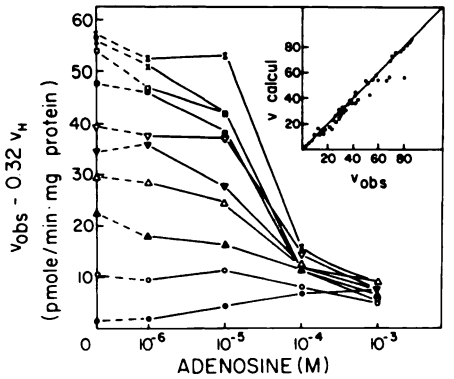

FIG. 1. *Inhibition of l-epinephrine stimulated turkey erythrocyte membrane adenylate cyclase by adenosine*

Adenylate cyclase activity in turkey erythrocyte membranes was assayed at 36° for 20 min. The assay mixture contained 0.4 mg/ml of membrane protein, 2 mM ATP, ATP regeneration system, 0.3 mM R020-1724, and different concentrations of adenosine and/or *l*-epinephrine. The activity was expressed in pmole cAMP/mg protein/min. Values are the means of triplicate determinations. Data were plotted with correction for the pool of adenylate cyclase common to the adenosine receptor and the β-receptor. For further explanations, see text. -●-●- Adenosine alone, -○-○- 1.0×10^{-6} M epinephrine, -▲-▲- 2.5×10^{-6} M *l*-epinephrine, -△-△- 5.0×10^{-6} M *l*-epinephrine, -▼-▼- 7.5×10^{-6} M *l*-epinephrine, -▽-▽- 1.0×10^{-5} M *l*-epinephrine, -■-■- 2.5×10^{-5} M *l*-epinephrine, -□-□- 5.0×10^{-5} M *l*-epinephrine, -×-×- 7.5×10^{-5} M *l*-epinephrine, -*-*- 1.0×10^{-4} M *l*-epinephrine. Insert: The correlation between observed adenylate cyclase activity and the calculated values, obtained from Eq. 10.

Table 1 summarizes the kinetic parameters for adenosine, 2-deoxyadenosine, phenylisopropyladenosine, and *l*-epinephrine.

The effect of adenosine and its analogues on the permanently active cyclase. Turkey erythrocyte adenylate cyclase, in its permanently active form, obtained by treatment with *l*-epinephrine and GppNHp, as previously described (19), was found not to be inhibited by adenosine or its analogues at concentrations up to 0.5 mM.

Properties of caudate nucleus adenylate cyclase. Rat caudate nucleus membranes were found to exhibit basal activity of 86 ± 8 pmole cAMP/mg/min at 30° in the presence of 1 μM GTP. All steady state measurements were performed in the presence of 1 μM GTP. Maximal specific activity was observed subsequent to the activation of the enzyme to its permanently active state by 1×10^{-5} GppNHp, and was found to be 216 ± 20 pmole cAMP/mg/min. This value is attained by either GppNHp alone or in the presence of adenosine and GppNHp. The kinetics of activation of cyclase by GppNHp to its permanently active form was found to be first order, as was previously found for the turkey erythrocyte system (19, 20). The first order rate constants for cyclase activation by GppNHp alone and by GppNHp plus saturating adenosine are $k = 0.406$ min^{-1} and $k = 0.801$ min^{-1}, respectively. Dopamine was found not to stimulate the cyclase activity of the membrane preparation. *l*-Epinephrine at 100 μM (saturating concentrations) was found to facilitate the rate of GppNHp-induced activation by 30 to 40% but without affecting the maximal specific activity attained. Thus, in this system, in contrast to the turkey erythrocyte system, the maximal specific activity of the permanently activated enzyme can be attained in the presence of GppNHp alone, and in the absence of added β-agonist.

The effect of adenosine on rat caudate nucleus membrane adenylate cyclase. Figure 2 depicts the effect of adenosine on adenylate cyclase of caudate nucleus membranes. It can be seen that the adenosine effect is biphasic: stimulatory up to 70 μM and inhibitory at higher concentrations. Theophylline abolishes the stimulatory response but not the inhibitory response. N^6-phenylisopropyladenosine exhibits exclusively the stimulatory effect (Fig. 3), whereas 2′-deoxyadenosine exhibits only the inhibitory effect (Fig. 4). Theophylline inhibits the N^6-phenylisopropyladenosine-induced activation (Fig. 3) but not the 2′-deoxyadenosine-induced inhibitory effect (Fig. 4).

The inhibitory effect of adenosine on permanently activated adenylate cyclase. Turkey erythrocyte adenylate cyclase permanently activated by pretreatment with *l*-epinephrine (100 μM) and GppNHp (100 μM) was found to be unaffected by adenosine even at a concentration of 0.5 mM. In contrast, a marked inhibition of the permanently active adenylate cyclase from caudate nucleus membranes by adenosine and by 2-deoxyadenosine was found (Fig. 2, 4, 5) whereas phenylisopropyladenosine had no effect (Fig. 3).

Figures 2 and 4 also show that the inhibitory effect of adenosine and of 2′-deoxyadenosine cannot be described by a simple Michaelian hyperbola but reveals site heterogeneity or negative cooperativity (26), as is shown by the Eadie-Hofstee plots (Fig. 5). The adenosine inhibitory effect is not due to competition with the ATP site since the effect is non-competitive (26). Indeed, the K_m for ATP is identical in the presence and in the absence of adenosine (Fig. 6) and the adenosine effect is exclusively on the catalytic efficiency of the system (k_{cat}). This

TABLE 1

Kinetic parameters for turkey erythrocyte adenylate cyclase

Ligand	Dissociation constant (μM)	Maximal specific activity (pmole cAMP/mg/min[a])
Adenosine	9.0 ± 2.0	7.1 ± 0.20
Phenylisopropyladenosine	13.0 ± 3.5	6.4 ± 0.15
2′-Deoxyadenosine	100.0 ± 15.0	1.5 ± 0.03
l-Epinephrine	6.0 ± 0.3	58.0 ± 1.50

The values are those for the system at 36° (see text).

[a] Values after subtraction of basal activity (= 0.6 pmole/mg/min).

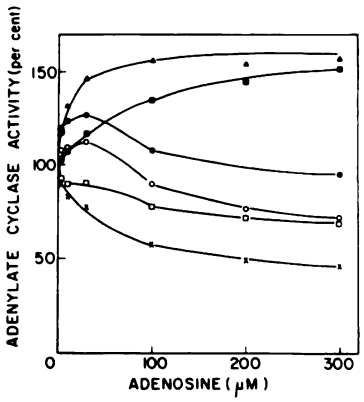

FIG. 2. *The effects of adenosine on adenylate cyclase activity from caudate nucleus membranes*

Ten minute assays of adenylate cyclase at 30° were performed, as described in MATERIALS AND METHODS. Assay mixtures contained 0.07 mg protein/ml. The values are expressed in percentage of basal activity (without addition of adenosine). The values are the means of triplicate determinations. Basal activity was always between 77 and 84 pmole cAMP/ml/min at 30°. -●-●- Rat caudate homogenate (basal activity 66 pmole/mg/min). -O-O- Rat caudate membranes (basal activity = 84 pmole/mg/min). -□-□- Adenosine dependence in the presence of 250 μM theophylline (basal activity = 81 pmole/mg/min). -x-x- The response of GppNHp induced permanently activated rat caudate cyclase to adenosine. The enzyme was activated to its permanently active form by preincubation with 0.1 mM GppNHp for 15 min at 30°. The specific activity was 209 pmole cAMP/min/mg. -▲-▲- "Ccrrected" dose-response curve (for further details see DISCUSSION). The curve was obtained by dividing the values of activity (-O-O-) by the relative inhibition observed by the same adenosine concentrations on the GppNHp permanently activated enzyme (-x-x-). -■-■- "Corrected" dose response curve in presence of 250 μM theophylline.

is readily apparent from the Eadie-Hofstee plot (Fig. 6) describing the response to ATP in the absence and in the presence of adenosine. The "high" affinity site has an apparent dissociation constant of 5 μM for adenosine and 3 μM for 2'-deoxyadenosine, leading to a maximal inhibition of 26%, whereas "low" affinity site has a dissociation constant of 53 μM for adenosine and 34 μM for deoxyadenosine. Maximal inhibition was found to be 62% (Table 2).

The combined action of N^6-phenylisopropyladenosine and l-epinephrine on caudate nucleus adenylate cyclase. Figure 7 shows the combined action of l-epinephrine and N^6-phenylisopropyladenosine on caudate membrane adenylate cyclase. In contrast to turkey erythrocyte membrane, this adenosine derivative has an activation effect on the l-epinephrine-dependent adenylate cyclase.

Nevertheless, the difference between V_A and v_{obs} decreases with increasing concentrations of the adenosine analogue, and approaches zero at saturating concentrations of N^6-phenylisopropyladenosine, as in the case of turkey erythrocyte membranes (Fig. 1).

From Eq. 9 it is, however, apparent that an activation effect of the adenosine compound should be observed when $V_{A(max)} > V_H$. Indeed, $V_{A(max)}$ is found to be 116 ± 8

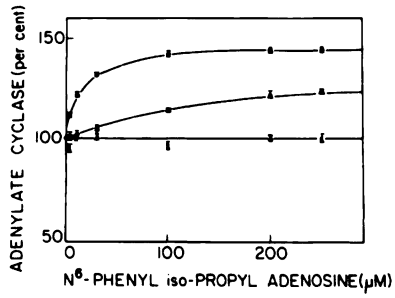

FIG. 3. *The effects of N^6-phenylisopropyladenosine on rat caudate membrane adenylate cyclase*

Assay conditions and basal activities as in Fig. 2. -●-●- Adenylate cyclase activity of untreated membranes. -O-O- Membranes in presence of 250 μM theophylline. -x-x- Membranes pretreated with 0.1 mM GppNHp.

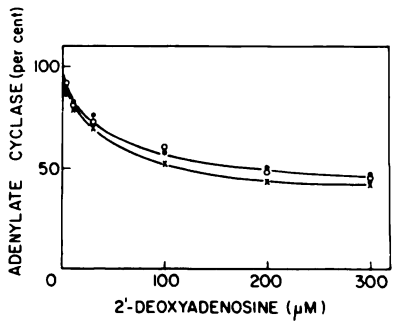

FIG. 4. *The effect of 2'deoxyadenosine on rat caudate membrane adenylate cyclase*

Assay conditions and basal activities as in Fig. 2. -●-●- Adenylate cyclase activity of native membranes. -O-O- Activity in the presence of 250 μM theophylline. -x-x- Membranes pretreated with 0.1 mM GppNHp.

pmole/mg/min and V_H = 105 ± 1.0 pmole/mg/min for the caudate membrane preparation. Using these data and inserting K_A = 7.0 μM one finds that the data fits very well with Eq. 9. The combined action of *l*-epinephrine and N^6-phenylisopropyladenosine on caudate membranes cannot be accounted for by a model which assumes that two agonist-receptor complexes compete with a pool of cyclase by a collision-coupling mechanism. Such a relationship between the two receptors would result in an additive effect of the two ligands (19).

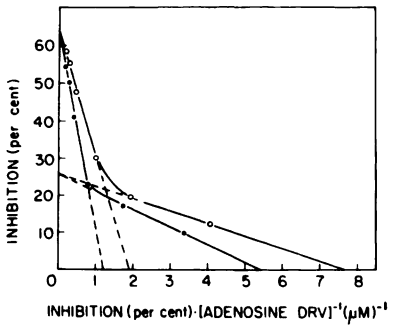

FIG. 5. *An Eadie-Hofstee plot of the inhibition of permanently activated adenylate cyclase from rat caudate by adenosine and 2'-deoxyadenosine*

Replot of data from Figs. 2 and 4. -●-●- Adenosine. -○-○- 2'-deoxyadenosine.

DISCUSSION

Turkey erythrocytes possess receptors for adenosine and *l*-catecholamines, both of which are coupled to a *common* pool of adenylate cyclase. Both ligands stimulate adenylate cyclase activity and yield normal non-cooperative adenylate cyclase dose response curves. Adenosine has no inhibitory effect on turkey erythrocyte adenylate cyclase in contrast to many other systems, including the caudate nucleus adenylate cyclase, where adenosine by itself has a biphasic effect: *stimulatory* at low concentrations (1, 2, 10, 15) and *inhibitory* at higher concentrations.

These findings led to the concept that many adenylate cyclases possess two types of adenosine recognition sites: the stimula-

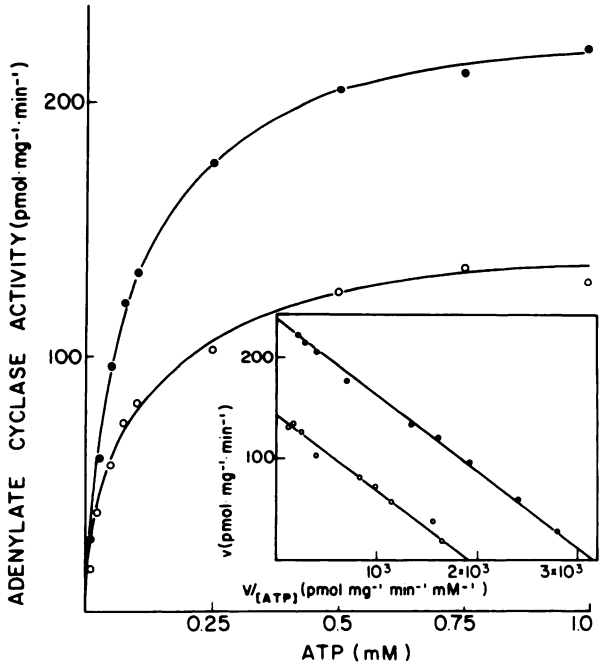

FIG. 6. *Dose response of permanently activated rat caudate adenylate cyclase to ATP*

Rat caudate membranes were preincubated for 15 min at 30° with 10^{-4} M GppNHp, and adenylate cyclase activity was assayed with different concentrations of ATP at 30°. -●-●- No adenosine added. -○-○- 0.1 mM adenosine added. Insert: Eadie-Hofstee plot of the same data.

TABLE 2

The dissociation constant of adenosine and its analogues to the adenosine receptor and the adenosine inhibitory site in turkey erythrocyte membranes and rat caudate nucleus membranes

Compound	Adenosine receptor		Adenosine inhibitory site (caudate nucleus)
	Turkey erythrocyte	Rat caudate nucleus	
	(μM)	(μM)	(μM)
Adenosine	9.0 ± 2.0	7.0 ± 1.0	4.8(I), 53.3(II)[d]
N^6-Phenylisopropyladenosine	13.0 ± 3.5[a]	8.0 ± 1.0[a]	—
2'-Deoxyadenosine	100.0 ± 15.0[b]	—[c]	3.4(I), 33.7 (II)[d]

All values apply to 36° (turkey erythrocytes) and 30° (caudate).

[a] Full agonist.

[b] Very weak agonist.

[c] No stimulatory effect could be measured.

[d] By extrapolation of Eadie-Hofstee plot (Fig. 5).

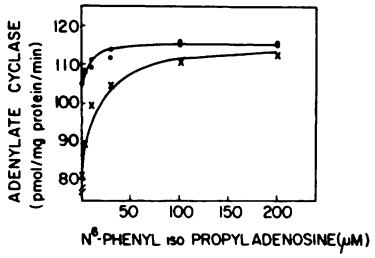

FIG. 7. *Activation of l-epinephrine-stimulated rat caudate adenosine adenylate cyclase by N^6-phenylisopropyladenosine*

Adenylate cyclase activity was measured as in Fig. 2. -●-●- In presence of 1 mM *l*-epinephrine. -○-○- Calculated values, obtained from Eq. 9. -×-×- No epinephrine added.

tory receptor site, the "R" site, and the inhibitory "P" site (16). Since the "R" site possesses a higher affinity toward adenosine compared with the "P" site, a biphasic dose response curve is to be expected. Turkey erythrocytes seem to lack the "P" inhibitory site and to possess only the "R" stimulatory site, whereas caudate nucleus membranes possess both. Alternatively, one can argue that turkey erythrocytes possess also a "P" site but that the latter is uncoupled to the cyclase. In the absence of a direct binding assay for either of these two sites, a choice between these two possibilities cannot be made at this point. It is interesting that even in the absence of an inhibitory site for adenosine in turkey erythrocytes, the latter is found to *inhibit* adenylate cyclase activity when present *together* with the more efficient stimulatory ligand *l*-epinephrine (Fig. 1). This effect, however, is due to the *competition* between the two receptors for a common pool of adenylate cyclase catalytic units.

Figure 1 demonstrates that adenosine strongly inhibits *l*-epinephrine-dependent adenylate cyclase activity. The adenosine effect fits quantitatively to Eq. 8 (Fig. 1), which assumes a competitive relationship between the adenosine receptor and the β-receptor with respect to adenylate cyclase. These findings corroborate previous experiments (19) that demonstrated that the rate of adenylate cyclase activation by *l*-epinephrine and GppNHp to the permanently active state is inhibited by adenosine, although the latter is an agonist by itself. The attenuation of the *l*-epinephrine effect on the turkey erythrocyte adenylate cyclase is due to the fact that adenosine is a much less powerful agonist than epinephrine. Thus, even in the absence of an adenosine inhibitory site, it is to be expected that adenosine will inhibit the action of *another* hormone if the two compete for a common pool of adenylate cyclase.

Theoretically it is even possible that adenosine will inhibit the *basal* activity in systems that exhibit it, if the binding of adenosine to the enzyme-bound receptor yields a species of cyclase with a low turnover number, as compared with the basal state. It should therefore be apparent that whenever adenosine inhibits basal adenylate cyclase activity or hormone-dependent adenylate cyclase activity, this does not necessarily mean that an inhibitory site for

adenosine exists in the system. As is shown in this study and a previous study (20), no adenosine inhibitory site is recognized in turkey erythrocytes.

The R-site-oriented adenosine analogue N^6-phenylisopropyladenosine activates *l*-epinephrine-induced adenylate cyclase in caudate nucleus membranes (Fig. 7). This effect, however, is of the same nature as the inhibitory effect described in the previous section. This combined effect of adenosine and *l*-epinephrine on rat caudate nucleus membrane is also best accounted for by Eq. 9, which assumes that both agonists act on a common pool of cyclase. Indeed, the only difference between the turkey erythrocyte system and the rat caudate nucleus system is that in the former $V_{A(max)} < V_{M(max)}$ whereas in the latter case $V_{A(max)} > V_{A(max)}$. In both cases the maximal specific activity in the presence of the hormone and adenosine combined approaches that induced by adenosine alone at increasing concentrations of the latter. This behavior is fully accounted for by the competitive relationship between the two receptors.

Thus adenosine may either increase hormonally-induced cAMP production or attenuate its production depending on the relative efficacy of the two ligands. The use of an identical molecular mechanism to achieve opposite effects in different tissues may have a physiological significance and therefore may be related to the role of adenosine in regulating cAMP production in the large variety of tissues where its action is well recognized.

Adenosine induces a biphasic effect on rat caudate nucleus adenylate cyclase. The stimulatory effect at low adenosine concentrations is followed by an inhibitory effect at higher concentrations (Fig. 2). This behavior was reported earlier and our results confirm previous observations (10). The biphasic behavior is due to the existence of a stimulatory adenosine receptor and an inhibitory site distinct from the adenosine receptor site. These two types of sites were defined as the "R" site for the stimulatory site and the "P" site for the inhibitory site by Londos and Wolff (16).

The biphasic effect can indeed be separated into two curves: a stimulatory one characteristic of the stimulatory adenosine receptor site (the "R" site) and an inhibitory one characteristic of the adenosine inhibitory site (Fig. 2). The dissociation constant for adenosine to the receptor site is 7.0 ± 1.0 μM (Table 2) close to values reported for other systems including turkey erythrocytes (2, 5, 18–20). The dissociation constant for adenosine to the inhibitory site is 53 μM which is similar to the value reported by Londos and Wolff (16) for the adipocyte system. The GppNHp permanently activated enzyme exhibits only the adenosine inhibitory action (Fig. 2). This curve was used to obtain the adenosine stimulatory curve by dividing the experimentally observed adenylate cyclase activity (in the absence of GppNHp, Fig. 2) by the relative inhibition of the permanently activated adenylate cyclase at the same adenosine concentration (Fig. 2).

These two types of adenosine sites exhibit different ligand specificities (16, 18). Thus, theophylline competes effectively for the adenosine receptor site but has no significant effect on the inhibitory "P" site (Figs. 2, 4). Furthermore, N^6-phenylisopropyladenosine induces only stimulation that is competitively inhibited by theophylline (Fig. 3). In contrast, 2′-deoxyadenosine is recognized exclusively by the adenosine inhibitory site (Fig. 4), and its effect is not antagonized by theophylline. These results are in good agreement with those of Londos and Wolff (16).

Permanently activated cyclase from caudate nucleus is strongly inhibited by adenosine and 2′-deoxyadenosine (Figs. 2 and 4) but not by phenylisopropyladenosine (Fig. 3). These results indicate that in the permanently activated state the rat caudate nucleus adenylate cyclase only the inhibitory "P" sites are expressed. 2′-Deoxyadenosine was found to be ineffective as an inhibitor of the *l*-epinephrine-induced adenylate cyclase activity in turkey erythrocytes but was potent as adenosine in inhibiting the adenylate cyclase activity of rat caudate membranes. These findings indicate that 2′-deoxyadenosine exerts its effect mostly through the inhibitory "P" site. A summary of the dissociation constants for adenosine and its analogues in turkey

erythrocyte adenylate cyclase and the rat caudate nucleus adenylate cyclase is given in Table 2.

The inhibitory effect of adenosine is noncompetitive with ATP (Fig. 6) and is a pure V_{max} effect. At this point it is not yet clear what is the mechanism of the adenosine-induced inhibition. The dose response curves for adenosine and its analogues are non-cooperative for the "R" site (Figs. 2, 3) and (20). The dose response curve for adenosine and 2′-deoxyadenosine inhibition reveal site heterogeneity or negative cooperativity (Fig. 5). The origin of this phenomenon is not yet clear.

Londos and Wolff report (16) that solubilized adipocyte adenylate cyclase can be inhibited by the "P" type adenosine ligands. This latter result indicates that the adenosine inhibitory site is tightly coupled to the adenylate cyclase.

The affinity of adenosine to its receptor "R" site is in the range of 7.0 to 9.0 μM in a number of systems studied, including the turkey erythrocyte and rat caudate nucleus membranes (5, 18–20). Similarly, the range observed for the affinity of adenosine and its analogues to the "P" inhibitory site is 25 to 50 μM (16, 18). Interestingly, turkey erythrocytes exhibit exclusively the adenosine stimulatory effects. The absence of a "P"-type response results either from its absence or from an uncoupling between existing "P" sites and the cyclase moiety. In the absence of radioactively labeled ligands, to directly assay the adenosine binding sites it is possible to discriminate between these two possibilities.

Adenosine, as compared with other hormones, is a rather poor agonist. For example, in the turkey erythrocyte system, adenosine induces only 13% of the effect of the agonist *l*-epinephrine. Therefore, in the presence of adenosine the effect of *l*-epinephrine is diminished. The inhibition of a hormonal response, as that of the β-agonist dependent response by adenosine (Fig. 1), is significant at the 10^{-6} to 10^{-5} M range. In a system where adenosine is a more efficient agonist as compared with other hormones or neurotransmitters operating on the same pool of cyclase, the effect of adenosine may become stimulatory, as in the case of caudate nucleus membranes. As the concentrations of adenosine observed physiologically are in the micromolar range (27), it seems that the attenuation of hormone action by adenosine occurs through the extracellular "R" receptor site for adenosine. It is worth noting that the increase in adenosine concentration in heart muscle during contraction is from about 0.5 to 3.0 μM, slightly below the midpoint concentration of the adenosine response (~6 to 9 μM), where the change in response is most sensitive to fluctuations in the concentration. The inhibitory site for adenosine, the "P" site, binds adenosine in the range of 50 to 100 μM, which is at least 10-fold higher than the recognized adenosine concentrations *in vivo* (27). Intracellular adenosine is usually phosphorylated rapidly or secreted from the cell, so that its concentration is well below the range of 1.0 μM. Therefore, it may very well be that the intracellular "P" inhibitory site for adenosine is a pure *in vitro* phenomenon and never becomes operational *in vivo*.

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