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RELATIONSHIP OF THE INHIBITION OF EPINEPHRINE-STIMULATED ADENYLATE CYCLASE IN TURKEY ERYTHROCYTES BY ADENOSINE TO ITS "R-" AND "P-SITE"-MEDIATED EFFECTS

MICHAEL E. NEWMAN*

Department of Biological Chemistry, Hebrew University of Jerusalem, Institute of Life Sciences, 91904 Jerusalem, Israel

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Abstract—Adenosine and its analogs inhibited epinephrine-stimulated adenylate cyclase activity in turkey erythrocyte membranes in a manner suggesting the presence of both "R-" and "P-site" effects. The existence of the "R-site" receptor-mediated effect implies that, even when the degree of coupling of the adenosine receptor to the enzyme is low, the mode of coupling is permanent.

An adenosine receptor has been shown to be present in membranes derived from turkey erythrocytes [1, 2] and was suggested to be permanently coupled to the catalytic subunit of adenylate cyclase [3], while the catecholamine receptor activates the enzyme by a diffusion-dependent bimolecular collision, termed the "collision coupling" mechanism [4].

Londos and Wolff [5] proposed the existence of two distinct adenosine-sensitive sites on adenylate cyclase, an intracellular P-site requiring integrity of the purine ring mediating inhibition, and an extracellular R-site requiring integrity of the ribose ring mediating activation of the enzyme. A biphasic action for adenosine has indeed been found in many systems, e.g. human and pig platelets [6, 7], rat striatum [8], and human astrocytoma cells [9]. In addition, in both mouse brain cultures [10] and rat cerebral cortex [11] a second R-type site, active at very low concentrations of adenosine or its analogs and mediating enzyme inhibition, has been found. The R-site is therefore now considered to be subdivided into activatory receptors, named A_2 or R_a [10, 12], and inhibitory receptors named A_1 or R_i [10, 12], either of which or both may be coupled to a given adenylate cyclase.

In turkey erythrocyte membranes, adenosine was found to inhibit epinephrine-stimulated cyclase activity, an action apparently mediated by analogs active at the R-site [13]. This phenomenon was explained by the weaker agonist potency of adenosine compared to catecholamine, together with the permanent coupling of the adenosine receptor to 68–70% of the cyclase molecules present. Recently, however, doubt has been cast on this explanation by the finding that P-site analogs of adenosine [14], which operate independently of receptor activation and guanyl nucleotide binding, were able to inhibit isoproterenol-activated and permanently activated cyclase in the same preparation, while R-site analogs were ineffective. In this communication the effects of various adenosine analogs have been re-examined to characterize more fully the nature of the receptor involved, and it is concluded that, although in present membrane preparations the degree of coupling of the adenosine receptor and, therefore, the maximum extent of adenosine-mediated activity, is less than previously described [2, 13], interaction between the two receptors does occur and accounts for part of the effect seen when catecholamines and adenosine are present simultaneously.

MATERIALS AND METHODS

Turkey erythrocyte membranes were prepared and stored as previously described [15], and adenylate cyclase was measured according to Salomon *et al.* [16]. The assay mixture contained 50 mM Tris–HCl, pH 7.4; 2 mM $MgCl_2 \cdot 6H_2O$; 1 mM EDTA; 0.5 mM [α-^{32}P]ATP; 2.2 mg/ml phosphocreatine; 0.2 mg/ml creatine phosphokinase; and 0.3 mM RO-20-1724. Assays were terminated after 20 min at 37° by addition of 0.8 ml 6.25% TCA. Kinetic analyses (see Table 3) were performed by non-linear regression analysis using a Wang micro-computer. N^6-Methyl adenosine was obtained from P-L Biochemicals Inc. (Milwaukee, WI); 2′,5′-dideoxyadenosine, 1-epinephrine and 5′-guanylyl-imidodiphosphate (GppNHp) from ICN Pharmaceuticals (Cleveland, OH); 5′-*N*-cyclopropyl carboxamide adenosine (A-40823) from Abbot Laboratories (North Chicago, IL); and N^6-(L-2)phenylisopropyl adenosine from Boehringer (Mannheim, F.R.G.). Adenosine, 2′-deoxyadenosine, 2-chloroadenosine, theophylline, phosphocreatine, creatine phosphokinase and ATP were all obtained from Sigma Chemical Co. (St. Louis, MO). [α-^{32}P]ATP was obtained from the Radiochemical Centre (Amersham, U.K.), or the Nuclear Research Center (Beer-Sheva, Israel).

* Present address: Department of Research, Jerusalem Mental Health Center, Ezrath Nashim Hospital, P.O.B. 140, Jerusalem, Israel.

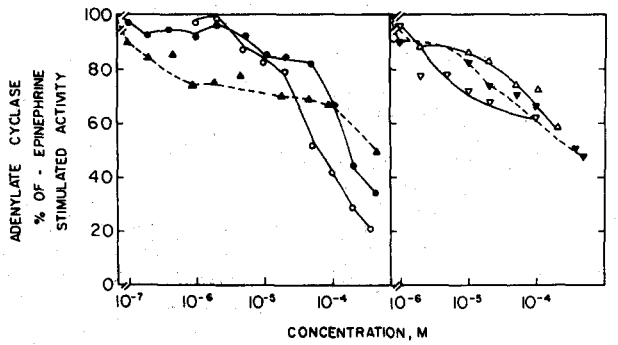

Fig. 1. Inhibition of epinephrine-stimulated activity by adenosine and its analogs. Turkey erythrocyte membranes (60–100 μg protein) were incubated with adenylate cyclase assay cocktail and adenosine analogs, as shown, for 20 min at 37°. Activity with 1 mM epinephrine was 47.2 ± 7.5 pmoles/min/mg protein and was taken as 100%. ●——●, Adenosine; △——△, N^6-phenylisopropyl adenosine; ▽——▽, 5′-*N*-cyclopropyl carboxamide adenosine; ▼---▼, 2-chloroadenosine; ▲---▲, 2′-deoxyadenosine; ○——○, 2′,5′-dideoxyadenosine.

Table 1. Dissociation constants and maximal activities of adenosine analogs obtained by inhibition of epinephrine-stimulated activity

Compound	K_{dis} (μM)	Maximal inhibition (%)
Adenosine	67 ± 20	51 ± 15
2-Chloroadenosine	14.5 ± 1	50 ± 2
N^6-Methyl adenosine	115	35
N^6-Phenylisopropyl adenosine	77 ± 12	41 ± 1
5′-*N*-Cyclopropyl carboxamide adenosine	11 ± 8	36 ± 3
N^6-Dimethyl adenosine	150	35
Adenosine-*N*-oxide	36	24

Values are shown as means ± S.D. of two or three experiments or single determinations, each experiment representing a curve such as those shown in Fig. 1

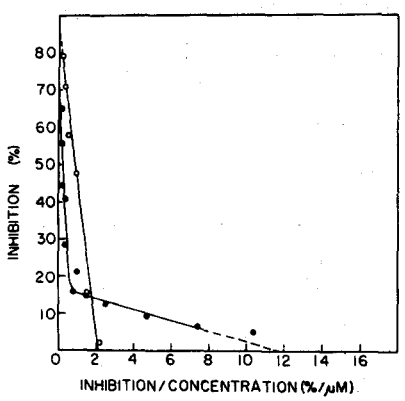

Fig. 2. Eadie–Hofstee plot of the inhibition of epinephrine-activated adenylate cyclase by adenosine and 2′,5′-dideoxyadenosine. Data are replotted from Fig. 1 and derived values are given in Table 2. ●——●, Adenosine; ○——○, 2′,5′-dideoxyadenosine.

RESULTS

Basal adenylate cyclase activity in this preparation was 2.73 ± 0.83 pmoles/min/mg protein and epinephrine-stimulated activity 47.2 ± 7.5 pmoles/min/mg protein (mean ± S.D. of 15 observations in each case). Adenosine on its own or any of its analogs had very little effect on basal activity, except for 5′-*N*-cyclopropyl carboxamide adenosine, which produced a slight stimulation. Adenosine or its ribose-modified (P-site) analogs 2′-deoxyadenosine or 2′,5′-dideoxyadenosine reduced epinephrine-stimulated activity by about 60% of that obtained with epinephrine alone (Fig. 1, left-hand panel), while purine-modified or R-site analogs reduced activity by only about 40% (Fig. 1, right-hand panel). The dissociation constants of the various analogs estimated from these data are given in Table 1, and show the relative potencies of the purine-modified analogs to be in the order 5′-*N*-cyclopropyl carbox-

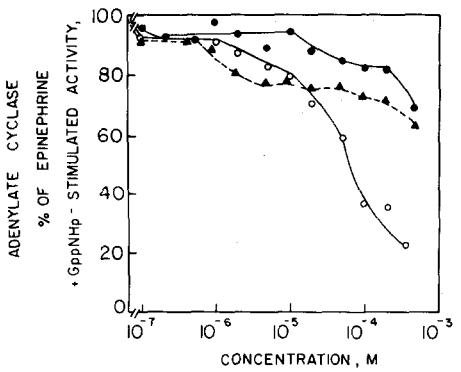

Fig. 3. Inhibition of GppNHp and epinephrine-stimulated adenylate cyclase activity by adenosine and its analogs. Each experiment was conducted by pre-incubating approximately 1 mg washed membranes with epinephrine and GppNHp to final concentrations of 1 and 0.1 mM respectively in 50 mM Tris, 2 mM $MgCl_2$, 1 mM EDTA buffer, pH 7.4, for 20 min at 37°, and then adding 50-μl aliquots of this suspension to tubes containing assay cocktail and various concentrations of the adenosine analogs studied, as follows: ●——●, adenosine; ▲---▲, 2′-deoxyadenosine; ○——○, 2′,5′-dideoxyadenosine. Assays were for 20 min at 37°. Activity of the permanently activated membranes measured after 20 min incubation following the preincubation was 406.6 ± 25.5 pmoles/min/mg protein (mean ± S.D. of seven observations) and was taken as 100%.

amide adenosine > 2-chloroadenosine > adenosine-*N*-oxide > adenosine > N^6-phenylisopropyl adenosine > N^6-methyl adenosine = N^6-dimethyl adenosine.

Closer examination of the curves for inhibition of epinephrine-stimulated activity by adenosine and some of its analogs showed a biphasic pattern in some cases, reflected by a non-linear Eadie–Hofstee plot. Both adenosine and 2′-deoxyadenosine showed this behavior, as illustrated for adenosine in Fig. 2, while the pure P-site analog 2′,5′-dideoxyadenosine gave a single line. Dissociation constants calculated from these data by linear regression analysis are shown in Table 2, indicating that in the case of adenosine the values calculated from Fig. 1 (Table 1) represent the low-affinity site only.

Further information was provided by studying the kinetics of activation of the enzyme to its permanently active form in the presence of the non-hydrolyzable GTP analog GppNHp. Both adenosine and 5′-*N*-cyclopropyl carboxamide adenosine caused a maximum activation of only 33–38% of that seen with epinephrine (Table 3), although the rate constants characterizing the adenosine activation process were very similar to those found previously [2], with lag times in the range of 12–17 min, as compared with 0.3–1 min for epinephrine. When membranes were permanently activated by preincubation with GppNHp and epinephrine before assay (Fig. 3), the inhibitory effects of both adenosine and 2′-deoxyadenosine were reduced relative to those seen with epinephrine alone, although the inhibitory effect of 2′,5′-dideoxyadenosine was maintained.

Table 2. Dissociation constants and maximal activities of adenosine analogs obtained by inhibition of epinephrine-stimulated activity

	High-affinity component			Low-affinity component		
Compound	K_{dis} (μM)	Maximal inhibition (%)	Correlation coefficient (r)	K_{dis} (μM)	Maximal inhibition (%)	Correlation coefficient
Adenosine	1.2	16.0	0.97	68.0	69.7	0.93
2′-Deoxyadenosine	0.2	31.9	0.96	33.8	55.2	0.68
2′,5′-Dideoxyadenosine				42.1	82.3	0.95

Values were obtained by linear regression analysis of data from several experiments, plotted by the Eadie–Hofstee method as shown in Fig. 2.

Table 3. Kinetic data for activation of turkey erythrocyte adenylate cyclase by GppNHp in the presence of various compounds

Compound	k_{on} (min^{-1})	Maximal activity (pmoles/min/mg protein)
Epinephrine (1 mM)	1.29 ± 0.38	410.1 ± 17.7
Adenosine (10 μM)	0.081 ± 0.013	156.4 ± 13.7
5′-*N*-Cyclopropyl carboxamide adenosine (90 μM)	0.059 ± 0.016	134.2 ± 22.1

Curves were fitted to the time course points for accumulation of cyclic AMP by non-linear regression analysis, and the values given represent the constants ± S.E. derived from the curves of best fit.

DISCUSSION

The order of potency of R-site analogs of adenosine in inhibiting epinephrine-stimulated activity of adenylate cyclase in turkey erythrocyte membranes was very similar to that found by Van Calker *et al.* [10] and by Londos *et al.* [12] for stimulation of adenylate cyclase in mouse brain glial cells and other systems, by activation of the A_2 or R_a receptor. The most potent analog in this system, 5′-*N*-cyclopropyl carboxamide adenosine, is only slightly structurally different from that characterized by Londos *et al.* [12] as having the greatest potency at the adenosine activatory site, 5′-*N*-ethyl carboxamide adenosine, and was indeed more potent in displacing bound ^{3}H-labeled 2-chloroadenosine from rat brain synaptic membranes [17], an action considered to take place at adenosine receptors in the CNS.

Both adenosine and 2′-deoxyadenosine showed a biphasic pattern of inhibition, and it is tempting to speculate that the high-affinity components represent interaction with the R-site (compare K_a value of 0.5 μM in rat striatum [8]), while the low-affinity components represent the P-site (compare K_i values of 70 μM for adenosine and 50 μM for 2′-deoxyadenosine in rat striatum [18], with maximal inhibition being 65% in both cases). However, in rat caudate nucleus membranes [13], P-site activity was found to have two components, with K_i values of 4.8 and 5.3 μM for adenosine, and 3.4 and 34 μM for 2′-deoxyadenosine, and a similar phenomenon may be present here.

The degree of coupling of the adenosine receptor to adenylate cyclase in these membranes was investigated by determining the relative degree of activation attainable with adenosine or its analog in the presence of GppNHp (Table 3). The value of just under 40% obtained agrees with that found by Lad *et al.* [14] both for activation of the enzyme by GppNHp alone after pretreatment with adenosine and GMP, as compared to pretreatment with isoproterenol and GMP, and for activation by adenosine and GTP or 5′-*N*-ethyl carboxamide adenosine and GTP in the presence of cholera toxin. It therefore seems reasonable to assume that the 40% inhibition of epinephrine-stimulated activity observed with "R-site" adenosine analogs is due to their interaction with the adenosine receptor. The failure of Lad *et al.* [14] to observe this phenomenon may be related to the very low activities ($\simeq$ 4 pmoles/mg protein) they obtained with the conditions adopted of 5 μM isoproterenol and 10 μM GTP at 30°.

When membranes were permanently activated by preincubation with GppNHp and epinephrine before assay (Fig. 3), adenosine caused only about 20% inhibition of maximally stimulated activity. This inhibition is a pure "P-site" phenomenon, since interaction with the receptor must take place via the guanyl nucleotide binding site, which is fully occupied. This provides further evidence that the remaining 40% of the inhibition seen with epinephrine, when membranes were not preactivated, may be due to a receptor-mediated action. Lad *et al.* [14] performed similar experiments in which membranes were pretreated with GMP and isoproterenol, and then assayed with various adenosine analogs in the presence of 10 μM GppNHp. Their failure to see inhibition of activity with R-site adenosine analogs under these conditions was, therefore, due to prevention of adenosine receptor interaction with the permanently activated enzyme by the fully occupied guanyl nucleotide binding site, and not as claimed to all inhibitory effects being mediated by the P-site only. The persistence of the "R-site" effect, even in the presence of the much more potent stimulating ligand, epinephrine, observed in this study, adds further support to the concept that adenosine receptors are permanently coupled to at least part of the pool of adenylate cyclase molecules in turkey erythrocyte membranes.

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