Biochemical Pharmacology, Vol. 37, No. 10, pp. 2085–2089, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
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5′-DEOXY-5′-METHYLTHIOADENOSINE: A NUCLEOSIDE WHICH DIFFERENTIATES BETWEEN ADENOSINE RECEPTOR TYPES

RAVI MUNSHI,*† ALEXANDER S. CLANACHAN and HANS P. BAER‡
Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

(*Received* 26 *March* 1987; *accepted* 8 *October* 1987)

Abstract—The activities of an endogenous nucleoside, 5′-deoxy-5′-methylthioadenosine (MTA), on adenosine sensitive sites such as adenosine A_1 and A_2 receptors and the P-site, as well as on purine nucleoside transport, have been studied. This nucleoside competitively antagonized the A_2 receptor-mediated stimulation of neuroblastoma adenylate cyclase, produced a GTP-dependent and 8-*p*-sulfophenyltheophylline-sensitive inhibition of adenylate cyclase activity in rat cerebellar membranes, and decreased the spontaneous contractile activity of isolated segments of rabbit jejunum. MTA was neither active at the P-site nor did it diminish the binding of [^{3}H]nitrobenzylthioinosine, a nucleoside transport inhibitor. We conclude that (a) MTA is an agonist at the adenosine A_1 receptor but an antagonist at the A_2 receptor, and (b) the adenosine receptor which causes relaxation of rabbit jejunum is not a neuroblastoma-type A_2 receptor which activates adenylate cyclase.

The existence of two types of adenosine receptors, A_1 and A_2, has been well documented [1]. Both receptor types are linked to the enzyme adenylate cyclase. The A_1 receptor, which is found in brain [2, 3], fat [4] and heart [5], mediates inhibition of adenylate cyclase activity. The A_2 receptor, on the other hand, stimulates the enzymic activity and is present in fibroblasts [6], liver [7], platelets [8] and striatum [9]. The A_1 and A_2 receptors may be differentiated further on the basis of agonist potency orders; most adenosine agonists have higher affinity at A_1 than A_2 receptors. Furthermore, the rank order potency of adenosine agonists at A_1 receptors (*R*-phenylisopropyladenosine > adenosine > *N*-ethylcarboxamidoadenosine) is opposite to that at an A_2 receptor. Methylxanthines, such as theophylline and caffeine, are antagonists at both adenosine A_1 and A_2 receptors. Besides methylxanthines, the nucleoside derivative 5′-deoxy-5′-methylthioadenosine (MTA§) has been reported to be a competitive antagonist of A_2 receptors in human fibroblasts [6]. While examining the structure–activity relationship of adenosine analogs to produce smooth muscle relaxation [10], we noted what appeared to be agonist activity of MTA on the smooth muscle of rabbits, i.e. MTA decreased spontaneous contractile activity. The results of the present study indicate that MTA is an agonist of A_1 receptors of rat cerebellum but an antagonist of A_2 receptors on neuroblastoma cells. The adenosine receptors of rabbit jejunum differed from both of these well characterized receptor subtypes.

* To whom all correspondence should be addressed.
† Present address: Department of Physiology, Box 449, Jordan Hall, University of Virginia Medical Center, Charlottesville, VA 22908.
‡ Present address: Biomedical Research, Box 3354, Riyadh 11211, Saudi Arabia.

§ Abbreviations: MTA, 5′-deoxy-5′-methylthioadenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; *R*-PIA, N^6-*R*-phenylisopropyladenosine; *R*-(^{125}I)HPIA, N^6-*R*-[3-(^{125}I)iodo-4-hydroxy]phenyl-isopropyladenosine; and 8-PST, 8-*p*-(sulfo)phenyltheophylline.

MATERIALS AND METHODS

Preparation of neuroblastoma cell membranes. Mouse neuro 2a neuroblastoma cells grown in suspension culture were homogenized in 10 vol. of a buffer containing 0.25 M sucrose, 10 mM Tris·HCl (pH 7.5) and 1 mM $MgCl_2$ using a Potter–Elvehjem homogenizer with a teflon pestle. The homogenate was centrifuged at 10,000 *g* for 20 min at 2°. The pellet was washed three times, resuspended in the same buffer, and stored frozen in liquid nitrogen.

Preparation of rat cerebellar membranes. Cerebellar membranes were prepared as described earlier for the whole brain [11]. Briefly, dissected cerebellar tissue was homogenized in 8 vol. of a buffer containing 0.25 M sucrose, 1 mM dithiothreitol and 50 mM Tris·HCl (pH 7.4). The homogenate was centrifuged at 1500 *g* for 20 min (2°). The pellet was discarded, and the supernatant fraction was recentrifuged at 15,000 *g* for 15 min. The pellet was washed twice in the incubation buffer (50 mM Tris·HCl and 1 mM dithiothreitol), redispersed in the same buffer, and stored frozen under liquid nitrogen. Protein was determined by the method of Lowry *et al.* [12].

Assay of adenylate cyclase activity. Adenylate cyclase from neuroblastoma cell membranes was measured by the method of Baer [13]. The reaction mixture, containing 0.1 mM [α-^{32}P]ATP, 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM cyclic AMP, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 0.04% bovine serum albumin, and

16–20 μg neuroblastoma membrane protein in a total volume of 50 μl, was incubated at 37° for 20 min. Rat cerebellar membrane adenylate cyclase was measured in the same manner but using [α-^{32}P]2-deoxyATP as the substrate [14]. The reaction mixture containing 0.1 mM [α-^{32}P]2-deoxyATP, 50 mM Tris·HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM 3′,5′-cyclic 2-deoxyAMP, 10 mM creatine phosphate, 72 units/ml creatine phosphokinase, 10 μM GTP, 5 units/ml adenosine deaminase, 0.04% bovine serum albumin, and 10–30 μg membrane protein, in a total volume of 50 μl, was incubated at 20° for 20 min.

N^6-R-[3-(^{125}I)Iodo-4-hydroxy]phenylisopropyladenosine [R-(^{125}I)HPIA] binding to rat cerebellar membranes. R-(^{125}I)HPIA was synthesized and used in binding assays as described earlier [11]. Briefly, R-(^{125}I)HPIA (1.2 nM) was incubated with 50 μg cerebellar membrane protein in buffer containing 50 mM Tris·HCl (pH 7.4) and 2 mM $MgCl_2$ in a total volume of 200 μl at 30° for 2 hr, and the bound and free radioligand were separated by vacuum filtration through pre-wetted and pre-cooled GF/B glass fiber filters. The filter papers were washed twice with 5 ml of ice-cold buffer, dried, and counted in a Beckman gamma-counter with a counting efficiency of 73%.

Contractility studies with rabbit small intestine. Portions of the small intestine (jejunum, 2-cm lengths) were excised as described earlier [10] and mounted longitudinally in 10-ml organ baths under an initial tension of 1 g. Isometric force was measured by means of Grass FTO3C force transducers. Following a 30- to 60-min stabilization period, increasing concentrations of the nucleoside were added to the baths. The tissues were washed between successive doses of nucleoside. Concentration–response curves for the nucleoside were generated in paired strips in the absence and presence of 100 μM 8-PST which was added 15 min before nucleoside additions.

[^{3}H]Nitrobenzylthioinosine ([^{3}H]NBMPR) binding to rat cerebellar membranes. The binding of [^{3}H]NBMPR was measured as described earlier [15]. Specific binding was calculated as the difference between total binding and the binding in the presence of 100 μM unlabeled NBMPR (nonspecific component).

Data analysis. The antagonist potency (K_b) was calculated from the Schild equation [16]: $(A'/A) - 1 = B/K_b$, where A and A' are the EC_{50} values for the agonist in the absence and presence, respectively, of a certain concentration, B, of the antagonist. K_i, the inhibitory constant, was calculated from the equation provided by Cheng and Prusoff [17]: $K_i = IC_{50}/(1 + F/K_d)$ where, IC_{50} is the inhibitor concentration for half-maximal inhibition, F is the free concentration of the radioligand, and K_d is the dissociation constant of the radioligand–receptor complex. The data from contractility experiments were analyzed as follows: semi-logarithmic concentration–response curves for each individual strip were plotted and log EC_{50} values (with 95% confidence limits) were calculated. Values of log EC_{50} obtained in the absence and presence of 100 μM 8-PST were compared by Student's t-test for paired data, and the differences were considered significant when $P < 0.05$.

Materials. Carrier-free Na^{125}I (2000 Ci/mmol) was obtained from the Edmonton Radiopharmaceutical Center, University of Alberta, Edmonton, Canada; [α-^{32}P]ATP and [α-^{32}P]2-deoxyATP (each 25 Ci/mmol) were from ICN, Irvine, CA, USA. Adenosine deaminase (Type III), creatine phosphokinase, dithiothreitol, 5′-deoxy-5′-methylthioadenosine and bovine serum albumin were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. NECA, GTP, creatine phosphate, cyclic AMP and 3′,5′-cyclic 2-deoxy-AMP were purchased from Boehringer Mannheim, Mannheim, F.R.G., and 8-PST was from RBI Chemicals, Natick, MA, U.S.A.

RESULTS

To measure the antagonistic effect of MTA on A_2 adenosine receptors, we studied neuroblastoma membrane adenylate cyclase preparations. NECA increased adenylate cyclase activity from a basal level of 8.5–10 pmol/mg/min by 134% (Fig. 1), with an EC_{50} of 0.5 μM (0.14–1.4, 95% confidence limits, N = 4). By contrast, MTA did not affect the basal enzyme activity up to a concentration of 100 μM. However, at that concentration, MTA shifted the concentration–response curve for NECA to the right, in a competitive fashion, with an EC_{50} for NECA of 12.0 μM (1.3–112, 95% confidence limits). These EC_{50} values were significantly different based on Student's t-test ($P < 0.05$). The antagonist potency of MTA, calculated from the Schild [16] equation, was 4.4 μM.

Rat cerebellar membrane preparations were used to study the effect of MTA on A_1 adenosine receptors. In this preparation, MTA produced a dose-dependent inhibition of adenylate cyclase activity (IC_{50} = 0.09 μM with 95% confidence limits of 0.021–0.43) but only in the presence of GTP (Fig. 2). This inhibitory effect of MTA on the cerebellar enzyme activity was competitively antagonized (IC_{50} = 10.4 μM, 95% confidence limit of 3–42) by the adenosine receptor antagonist, 8-PST (100 μM).

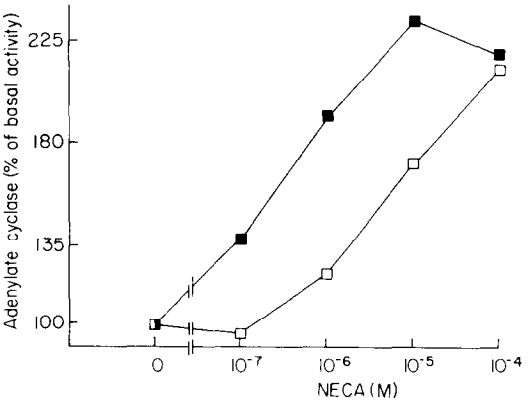

Fig. 1. Effect of NECA on adenylate cyclase activity in neuroblastoma cell membranes. Effects of NECA were measured in the absence (■) and presence (□) of 100 μM MTA. Mean values of three different experiments are shown (SEM values were below 15%). For details, see Materials and Methods.

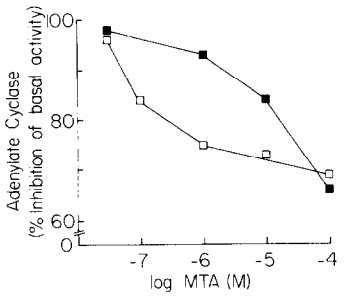

Fig. 2. Effect of MTA on the adenylate cyclase activity in rat cerebellar membranes. Adenylate cyclase activity was measured as described in Materials and Methods. Key: (□) MTA alone and in the presence (■) of 100 μM 8-PST. Each curve represents means from three different assays each performed in triplicate. The SEM values were below 12%.

MTA also displaced the specific binding of *R*-(^{125}I)HPIA to A_1 receptors in cerebellar membranes (Fig. 3), with an affinity (K_i = 121 nM) approximately 50-fold lower than that of *R*-PIA (K_i = 2.4 nM).

MTA and NECA decreased the spontaneous activity of isolated segments of rabbit jejunum. Although less potent than NECA (EC_{50} = 0.11 μM with 95% confidence limit of 0.06–0.21), MTA (EC_{50} = 110 μM with 95% confidence limit of 52–230) was a full agonist of the smooth muscle response, i.e. it produced 100% relaxation at the highest concentration used (Fig. 4). This was unlike NECA which produced only 80% of the maximum relaxation. Furthermore, the smooth muscle relaxant response to both NECA and MTA was antagonized by 8-PST (100 μM), and the EC_{50} values for both NECA and MTA, in the presence and absence of 8-PST, were significantly different ($P < 0.05$). However, the antagonist potency of 8-PST versus the two agonists, NECA and MTA, was markedly different. 8-PST was approximately 26-fold more potent versus NECA (K_b = 3.7 μM) than versus MTA (K_b = 95.7 μM).

The interaction of MTA with the nucleoside transporter was studied utilizing [^{3}H]NBMPR binding to rat cerebellar membranes. Both dipyridamole and

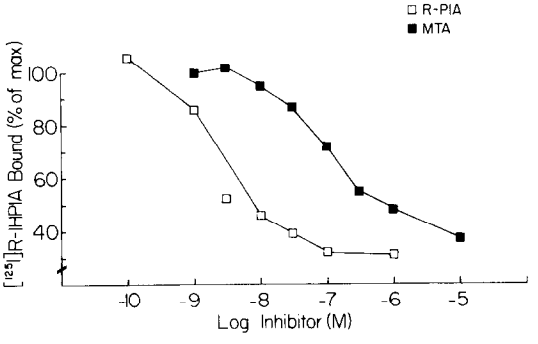

Fig. 3. Inhibition of the total binding of *R*-(^{125}I)HPIA to rat cerebellar membranes by MTA. The equilibrium binding was measured as described in Materials and Methods. Values are means of triplicate determinations (SEM values were below 6%).

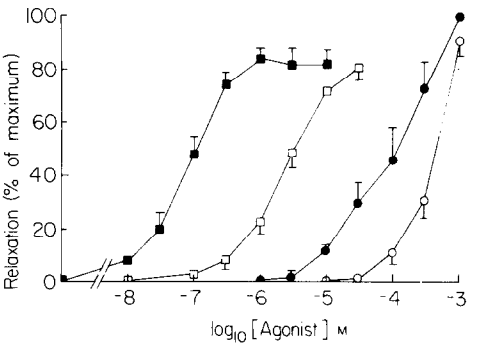

Fig. 4. Effects of MTA and NECA on the spontaneous contractility of isolated small intestine from rabbit in the presence and absence of 100 μM 8-PST. Tissues were isolated and tested as described in Materials and Methods. The curves show NECA alone (■) and in the presence of 8-PST (□), MTA alone (●) and in the presence of 8-PST (○). Each curve represents means ± SEM of four different experiments.

R-PIA displaced the bound radioligand, at a concentration of 100 μM. In contrast, MTA had only a marginal effect (Table 1) on [^{3}H]NBMPR binding (8% inhibition at a concentration of 100 μM).

DISCUSSION

MTA has been shown to act as a competitive antagonist of adenosine A_2 receptor stimulated adenylate cyclase activity in cultured intact VA13 fibroblasts [6]. A similar effect of MTA on neuroblastoma cell adenylate cyclase activity was demonstrated in this study. The potency of MTA as an antagonist of A_2 receptors in neuroblastoma membranes (K_b = 4.4 μM) was comparable (K_b = 8.2 μM) to that reported in VA13 fibroblasts [6], suggesting that the two receptors are the same. The binding of the adenosine A_1 receptor agonist [^{3}H]cyclohexyladenosine to guinea pig brain membranes had been reported to be displaced by MTA in a competitive fashion [18]. [^{3}H]5′-*N*-Ethylcarboxamidoadenosine, which binds to A_1 and A_2 receptors in rat striatal membranes, is inhibited

Table 1. Inhibition of specific [^{3}H]nitrobenzylthioinosine binding to rat cerebellar membranes

Compound	Concn (μM)	Inhibition of the specific binding* (%)
Dipyridamole	10	83
	30	93
	100	100
R-PIA	10	80
	100	100
MTA	10	0.6
	100	8

* Rat cerebellar membranes were incubated with [^{3}H]NBMPR (90 pM) in the absence and presence of various inhibitors. Mean values from two experiments, each in duplicate, are given.

biphasically by MTA [9]. Using a different ligand, *R*-(^{125}I)HPIA [11, 19], we have confirmed that MTA displaces adenosine A_1 receptor selective ligands from brain membranes (Fig. 3). MTA had approximately 50-fold lower affinity (K_i = 121 nM) than *R*-PIA (K_i = 2.4 nM) for the A_1 receptor in rat cerebellar membranes. A similar difference in the affinity of the two agonists for A_1 receptors has been demonstrated in guinea pig brain [18]. However, such binding studies with agonist radioligands may not indicate whether a compound acts as an agonist or an antagonist. Our direct studies of the effect of MTA on adenylate cyclase indicate that the nucleoside acts as an agonist of A_1 receptors in rat cerebellar membranes (Fig. 2). The adenylate cyclase in cerebellum has been shown previously to respond to *R*-PIA in an inhibitory fashion [20]. The IC_{50} of *R*-PIA reported in this study was 50 nM and the response was competitively antagonized by isobutylmethylxanthine. In a similar membrane preparation from rat cerebellum, MTA inhibited the basal adenylate cyclase activity with an IC_{50} of 90 nM, and the MTA concentration–response curve shifted to the right in the presence of 8-PST (Fig. 2). This effect of MTA on adenosine receptors was selective inasmuch as the nucleoside did not interact with other adenosine sensitive sites. MTA did not inhibit the adenylate cyclase activity through the P-site (since GTP was required and the effect was antagonized by 8-PST) nor did it antagonize the inhibition of adenylate cyclase activity induced by 2′,5′-dideoxyadenosine (a P-site agonist) in rat cerebellar membranes (data not shown). Similarly, MTA did not compete for the binding of [^{3}H]NBMPR to cerebellar membranes (Table 1).

Thus, MTA seems to be a nucleoside derivative that has opposing effects on adenylate cyclase coupled adenosine A_1 and A_2 receptors. It acts as an agonist of A_1 receptors and an antagonist of A_2 receptors. It, therefore, became of interest to use this compound as a probe to examine whether the relaxant effects of the agonists of adenosine receptors in smooth muscle are mediated by adenylate cyclase stimulation since it has been shown that elevation of cyclic AMP in smooth muscle (such as by β-adrenoceptor stimulation [21] or by forskolin [22]) forms a signal for relaxation. MTA proved to be an agonist of adenosine-mediated smooth muscle relaxation in the rabbit jejunum. The weak agonist effect of MTA observed in jejunum could be a result of metabolism of the nucleoside under the conditions of incubation. However, since MTA did not bind [^{3}H]NBMPR-binding sites in cerebellum (Table 1), we assume that MTA is probably not transported across the cells, unless there is an NBMPR-insensitive nucleoside transport in the jejunum. Furthermore, MTA was not subject to deamination by adenosine deaminase (unpublished data from this laboratory).

Since MTA had an agonist effect in the jejunum but acted as an antagonist of adenylate cyclase stimulating A_2 type receptors (Fig. 1), the jejunal smooth muscle receptor is probably different from A_2 receptors such as those in neuroblastoma cells. Furthermore, based on these data, it is difficult to support the hypothesis that adenylate cyclase stimulation and, therefore, cyclic AMP formation mediate the smooth muscle relaxant effects of adenosine and related nucleosides, unless a different type of receptor is involved. The possibility that A_1 receptors in the smooth muscle of jejunum mediate MTA- and NECA-induced relaxation cannot be excluded based on (1) an agonist effect of MTA both in jejunum and brain, and (2) the rank order potency of the agonists in the jejunum: *R*-PIA > NECA > *S*-PIA ([10]; Table 2). However, this A_1 receptor in jejunum may link to a different effector system such as guanylate cyclase [24, 25] and/or the metabolism of phosphoinositides [26] since inhibition of cyclic AMP would probably not form a signal for relaxation [21, 22].

Distinct interspecies differences in adenosine receptors have been documented recently [27]. However, if one were to compare the potencies of various nucleosides in cerebellum and jejunum, it would appear that, in spite of the same rank order potencies, the adenosine receptors in the two tissues are either different or coupled to two different effector systems. This is based on a 10- to 900-fold greater potency of various nucleosides in brain relative to that in jejunum (Table 2). Furthermore, since NECA produced only a maximum of about 80% of the relaxation produced by MTA, and since there was a greater than 25-fold difference in the antagonist potency of 8-PST versus NECA and MTA in jejunum

Table 2. Comparative potencies* of nucleosides in neuroblastoma, cerebellum and jejunum

Nucleoside	Adenylate cyclase (neublast.) (EC_{50})	Adenylate cyclase (cerbell.) (IC_{50})	*R*-(^{125}I)HPIA binding (K_i)	Relaxation (jejunum) (EC_{50})	Ratio (EC_{50}/K_i)
R-PIA		50†	2.4	23‡	9.6
NECA	800§		3.9‖	110	28.2
2-Chloroadenosine	1500§		4.1‖	510‡	124.4
MTA	8200¶	90	121.0	110,000	909.1

* Numbers represent concentrations in nM.
† Values obtained in mouse cerebellum [20].
‡ Values cited from Ref. 10.
§ Values cited from Ref. 23.
‖ Values obtained in whole brain [11].
¶ Represents potency as antagonist (K_b).

(Fig. 4), it is possible that the two agonists act through different receptors.

In summary, we have shown a receptor-selective agonist and antagonist activity of MTA on A_1 and A_2 adenosine receptors respectively. We also presented evidence to suggest that the adenosine receptor in rabbit jejunum is different from A_2 receptors in neuroblastoma cells. Our data also suggest that cyclic AMP may not form the signal for relaxation in smooth muscle in response to the nucleosides and, therefore, the adenosine receptor in jejunal-smooth muscle may be linked to a different effector system.

Acknowledgements—We would like to thank Dr. Joel Linden for helpful suggestions during the preparation of the manuscript. This project was supported by the Medical Research Council of Canada and the Alberta Heart and Stroke Foundation. Ravi Munshi was the recipient of a studentship from the Alberta Heritage Foundation for Medical Research.

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