Biochemical Pharmacology, Vol. 26, pp. 37–43. Pergamon Press, 1977. Printed in Great Britain.

ELEVATION OF CYCLIC AMP IN C-1300 MURINE NEUROBLASTOMA BY ADENOSINE AND RELATED COMPOUNDS AND THE ANTAGONISM OF THIS RESPONSE BY METHYLXANTHINES

RICHARD D. GREEN and LAWRENCE R. STANBERRY
Department of Pharmacology, University of Illinois at the Medical Center, Chicago, Ill., U.S.A.

(*Received* 24 *September* 1975; *accepted* 12 *May* 1976)

Abstract—Adenosine, in the presence of the phosphodiesterase inhibitor, Ro 20-1724, produces large increases in the intracellular content of cyclic adenosine 3′,5′-monophosphate of murine neuroblastoma cells (clone N2a). This response is not blocked by dipyridamole, an antagonist of adenosine uptake, but is antagonized by certain methylxanthines. The structure activity relationships for agonistic and antagonistic activity were determined and the following observations made: (1) the N^6 is necessary for the binding of adenosine to its receptor, but not for antagonist activity; (2) the substitution of a methyl group at the 1 position increases antagonistic activity of xanthines (from no activity to measurable activity) but greatly decreases the affinity of adenosine for its receptor; and (3) the sugar moiety is important for the binding of adenosine to its receptor, but not for antagonistic activity. The dissimilarities in the structure–activity relationships for agonistic activity and antagonist activity are interpreted to indicate that this antagonism is not competitive in nature.

The ability of adenosine to elevate the cyclic adenosine 3′,5′-monophosphate (cyclic AMP) content of slices of guinea pig cerebral cortex was first reported by Sattin and Rall in 1970 [1]. Similar findings have been reported for slices of cerebral cortex of the rat [2–4], mouse [4] and human [5]. Studies in tissue culture have shown that the intracellular content of human astrocytoma cells [6], but not that of rat glioma cells [7, 8], is elevated by adenosine.

Gilman and Nirenberg [9] reported that four clones of mouse neuroblastoma C-1300 were unresponsive to the cyclic AMP-elevating effect of adenosine. More recent work from other laboratories has shown that, while the cyclic AMP content of this cell line is minimally affected by adenosine alone, it is markedly elevated by adenosine in the presence of certain phosphodiesterase inhibitors [10, 11]. Blume *et al.* [10] showed that, in the clones they studied, the cyclic AMP elevation in response to adenosine in the presence of the phosphodiesterase inhibitor, Ro 20-1724, was antagonized by theophylline. The ability of theophylline to antagonize the cyclic AMP-elevating effect of adenosine was first reported by Sattin and Rall [1] for the guinea pig cerebral cortex, and has been reported in other systems [6, 12, 13]. While this antagonistic effect of theophylline is usually thought to be competitive in nature [6, 10, 13, 14], other workers have presented evidence that theophylline and other methylxanthines are not specific competitive antagonists of adenosine [15, 16]. The present report of studies on the N2a clone of murine neuroblastoma gives further evidence that the cAMP-elevating effect of adenosine is mediated by an extracellular receptor. In addition, a comparison between the structure–activity relationships of adenosine analogs (agonists) and xanthine derivatives (antagonists) gives a further indication that this antagonism is not via a competitive mechanism.

MATERIALS AND METHODS

Materials. Dipyridamole was a gift from Geigy Pharmaceuticals to Dr. Harold Feinberg in this department. Ro 20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] was kindly supplied by Dr. H. Sheppard of Hoffmann-LaRoche, Inc. Prostaglandins were provided by Dr. J. E. Pike of the Upjohn Co.; 1,3-di-*n*-propylxanthine (SC-2627) and 1,3-diallylxanthine (SC-4572) were provided by Dr. K. J. Rorig of G. D. Searle & Co. All other unlabeled chemicals and drugs were purchased from commercial sources (Sigma, P-L Biochemicals, Inc., Cyclo Chemicals, ICN Pharmaceuticals, Inc., Schwarz/Mann, and Gibco). Adenosine[8-^{3}H] and adenosine 3′,5′-monophosphoric acid[8-^{3}H] were purchased from Schwarz/Mann. Where indicated the compound was purified on cellulose thin-layer chromatography with 1.0 M ammonium acetate–95% ethanol (30:75, v/v) as the solvent [17].

Cell line and culture methods. The neuroblastoma cell line (clone Neuro-2a, N2a) was derived from the spontaneous mouse C-1300 neuroblastoma by Klebe and Ruddle [18] and was a gift from Dr. Alvin Tesler, Northwestern University. Cells were grown to monolayer in Falcon plastic culture dishes (100 × 20 mm) containing Dulbecco's Modified Eagle's Medium (DMEM) (4.5 g glucose/liter) supplemented with penicillin G, 50 units/ml; streptomycin sulfate, 10 μg/ml; and 10% gamma globulin-free newborn calf serum in a humidified 5% CO_2 atmosphere at 37°. Under these conditions, the cells had a doubling time of approximately 20 hr. Cultures were passaged weekly by incubation with 0.25% Viokase in Modified Eagle Medium (MEM). Fresh medium was added every 2–3 days.

Cultures used in these experiments were obtained from stock cultures by incubation with 0.25% Viokase in DMEM for 5 min at 37°, triturated, and centri-

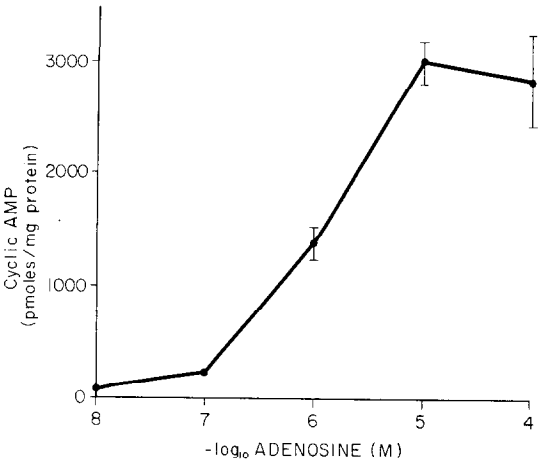

Fig. 1. Concentration:effect curve of adenosine on cyclic AMP. All cultures were pre-exposed to 0.7 mM Ro 20-1724. Adenosine incubation time was 15 min. The mean ± S.E.M. is shown for each point (n = 4) except where the S.E.M. is too small to show.

fuged with an equal volume of DMEM with 10% serum at 1000 rev/min for 2 min. The pellet was resuspended in DMEM with 10% serum and the cell number estimated with a hemocytometer. Dishes were inoculated with 5×10^5 cells (10 ml) and grown for 4–5 days; 5 ml of fresh medium was added 2 days prior to use. Most plates contained between 1 and 3 mg protein at the time of use.

Experimental protocol. Cell cultures were washed twice with serum-free DMEM and 5 ml of serum-free DMEM was added to each culture. Ro 20-1724 (0.7 mM) was added to the appropriate cultures at this time and the dishes were returned to the incubator. As it was necessary to dissolve some compounds, including Ro 20-1724, in ethanol, ethanol was added to other plates where necessary so that all plates within a given experiment were exposed to the same concentration of ethanol. The experiment was resumed approximately 30 min after adding the Ro 20-1724. The time of this initial preincubation period with Ro 20-1724 was not critical, as preincubation periods between 10 and 90 min gave similar results. The concentration of Ro 20-1724 used (0.7 mM) was determined in preliminary experiments to be at least twice that necessary to maximally potentiate the effect of adenosine on the N2a cells.

Most compounds studied were dissolved in distilled water and added to the cultures in microliter quantities. In a few cases, due to solubility limitations, the compound to be studied had to be directly dissolved in serum-free DMEM. Incubations were carried out in the tissue culture incubator and were terminated by removing the medium and adding 2 ml of 10% trichloroacetic acid (TCA). The samples were transferred to tubes, centrifuged, and the pellet was saved for a protein determination. The supernatants were made 0.1 N with HCl, extracted five times with water-saturated ether, purified on Dowex formate columns according to the method of Murad *et al.* [19], lyophylized and stored at −20° until assayed. Samples were redissolved in 50 mM sodium acetate buffer, pH 4, and assayed by a protein-binding assay similar to that described by Gilman [20]. The protein precipitates were redissolved in 1 N sodium hydroxide, and the protein content determined by the method of Lowry *et al.* [21]. Cyclic AMP levels are given in terms of pmoles/mg of protein. The recovery of cyclic AMP assessed by the addition of [^{3}H]cyclic AMP at the beginning of the extraction procedure averaged 80 per cent. Values given are not corrected for recoveries.

RESULTS

Concentration effect relationship for adenosine. Cultures were preincubated in serum-free DMEM containing 0.7 mM Ro 20-1724, and the intracellular cyclic AMP levels measured after 15-min incubations with various concentrations of adenosine (Fig. 1). The EC_{50} of adenosine was slightly greater than 10^{-6} M and the maximum elevation was produced by 10^{-5} M. Other experiments in which higher concentrations of adenosine were studied showed a clear descending limb of the adenosine concentration effect curve.

Time course of effect of adenosine. Both the intracellular and medium concentrations of cyclic AMP were determined at varying times after exposure to 10^{-5} M adenosine. In order to directly relate the relative increases in the cells and the medium, both values are expressed in terms of cell protein (Fig. 2). The intracellular concentration of cyclic AMP rose rapidly after exposure to adenosine and reached a peak at about 15 min. The cyclic AMP content of the medium rose slowly over a period of 30 min, but never approached that in the cells. Some of the cyclic AMP assayed as "medium" cyclic AMP may be due to cyclic AMP contained in the few cells that lost their attachment during the experiment. In any event, it is clear that the rapid rise in the intracellular cyclic AMP content does not lead to a rapid diffusion or transport of cyclic AMP out of the cells.

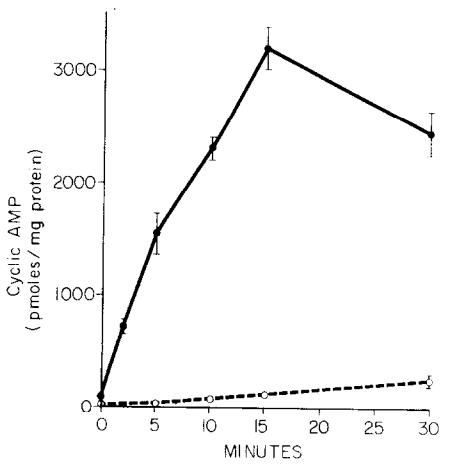

Fig. 2. Time course of effect of adenosine (10^{-5} M) on cyclic AMP. All cultures were pre-exposed to 0.7 mM Ro 20-1724. Key: (●——●) intracellular cyclic AMP; and (O---O) "media cyclic AMP." At the termination of the incubations, the media were collected into beakers containing 1.0 ml of 50% TCA and processed in the same manner as cell extracts. The mean ± S.E.M. is shown for each point except where the S.E.M. is too small to show.

Table 1. Effect of dipyridamole on adenosine uptake*

Treatment	Adenosine uptake† (pmoles/mg protein/15 min) Dipyridamole‡ Control	10^{-6} M	10^{-5} M	10^{-4} M
Adenosine, 10^{-6} M + Ro 20-1724§	63.7; 64.4	47.5 ± 4.5	12.6 ± 2.2	5 ± 1
Adenosine, 10^{-5} M + R0 20-1724§	430 ± 56	465; 333	89 ± 28	27 ± 3.4
Adenosine, 10^{-5} M	891 ± 83			

* Cells were washed with serum-free DMEM and incubated with [^{3}H]adenosine under conditions identical to those used when cyclic AMP was to be assayed. At the end of the incubation period, the cultures were rapidly washed five times with physiological saline solution and TCA was added. The TCA soluble radioactivity was determined after the TCA was extracted and the samples were lyophylized; it is expressed in terms of [^{3}H]adenosine.

† Individual values or mean ±S.E.M., N = 3.

‡ Fifteen min preincubation.

§ 0.7 mM.

Effect of dipyridamole on adenosine uptake and on the cyclic AMP-elevating effect of adenosine. Dipyridamole has been reported to inhibit adenosine uptake in several systems [22–24]. In rat astrocytoma cells [6] and guinea pig cerebral cortical slices [25], it inhibits adenosine uptake while it has no effect (astrocytoma) or a potentiating effect (cortical slices) on the cyclic AMP-elevating effect of adenosine. The effects of Ro 20-1724 and dipyridamole on adenosine uptake were therefore determined. The results of this experiment, along with those of a companion experiment in which cyclic AMP levels were determined, are summarized in Tables 1 and 2 respectively. It is clear that 10^{-4} M dipyridamole markedly inhibits adenosine uptake but does not inhibit the cyclic AMP-elevating effect of adenosine. Ro 20-1724 also decreases adenosine uptake.

Effects of analogs of adenosine and other related compounds on the intracellular cyclic AMP concentration and on the elevation of cyclic AMP in response to adenosine. The intracellular concentrations of cyclic AMP after 15 min of exposure to 10^{-4} M of a test compound or to the combination of the test compound and 10^{-6} M adenosine were determined. The purpose of these experiments was: (1) to define the structural requirements necessary for the cyclic AMP-elevating effect of adenosine, and (2) to determine if compounds devoid of agonist activity have any affinity for the "adenosine receptor," i.e. possess antagonist activity. The results of these experiments are summarized in Table 3. Many compounds devoid of significant agonist or antagonist activity are listed in the footnote to the table. [Some of the inactive compounds listed (adenosine-5′-propionate, adenosine-5′-monoacetate, 2′-AMP and 3′-AMP) appeared to have significant activity before purification by thin-layer chromatography.]

The agonist activity of adenylyl(3′→5′)adenosine was surprising in view of the lack of activity of the adenosine monophosphates. In an additional experiment 10^{-5} and 10^{-4} M adenylyl(3′→5′)adenosine elevated cyclic AMP from 63.3 ± 0.8 pmoles/mg of protein to 1071 ± 60 and 2662 ± 30 pmoles/mg of protein respectively. As this compound was chromatographically free of adenosine and the adenosine monophosphates are inactive, it must be concluded that this compound is a potent agonist at the "adenosine receptor."

The cultures exposed to exogenous cyclic AMP were rapidly washed five times with physiological saline at the termination of the incubation. In a similar experiment, the uptake of ^{3}H (expressed as cyclic AMP) after exposure to [^{3}H]cyclic AMP was 120 ± 52 pmoles/mg of protein. Further experiments with cyclic AMP purified by thin-layer and Dowex formate chromatography gave similar elevations in cyclic AMP. As the response to exogenous cyclic AMP could not have been due to the formation of 5′-AMP (phosphodiesterase was inhibited and this compound, even if formed, is inactive), it is likely that cyclic AMP can combine with the "adenosine receptor" to elicit an elevation in the intracellular concentration of cyclic AMP. Chasin *et al.* [26] have reported similar results in a preparation of guinea pig hippocampus.

5′-Deoxyadenosine produced a small elevation in cyclic AMP when added alone and inhibited the elevation in cyclic AMP in response to 10^{-6} M adenosine (Table 3). A complete dose–response curve (Fig. 3) showed this compound to produce a maximal effect which was much less than that of adenosine, i.e. it

Table 2. Effect of dipyridamole on cyclic AMP-elevating effect of adenosine

Treatment*	Cyclic AMP† (pmoles/mg protein)
Ro 20-1724	93; 40
Adenosine, 10^{-5} M	84 ± 14
Dipyridamole, 10^{-4} M	73 ± 6
Ro 20-1724 + adenosine, 10^{-6} M	523 ± 31
Ro 20-1724 + adenosine, 10^{-5} M	1467 ± 305
Ro 20-1724 + adenosine, 10^{-6} M + dipyridamole, 10^{-4} M	725 ± 124
Ro 20-1724 + adenosine, 10^{-5} M + dipyridamole, 10^{-4} M	2591 ± 106

* Cultures were pre-exposed to Ro 20-1724 (0.7 mM) and/or dipyridamole. Incubations with adenosine were for 15 min.

† Individual values or mean ±S.E.M., N = 3.

Table 3. Effects of adenosine analogs and related compounds on the cyclic AMP concentration in mouse neuroblastoma and on the elevation of cyclic AMP in response to 10^{-6} M adenosine

Test compounds*	Cyclic AMP (pmoles/mg protein)	
	Alone† (10^{-4} M)	Plus adenosine† (10^{-6} M)
None	40 ± 3.6 (26)‡	1200 ± 74 (25)‡
2-Chloroadenosine	2008, 1534	2500, 2672
Adenylyl-(3′→ 5′)adenosine	1538, 2068	
N^6-methyladenosine	677, 1192	1687, 1194
N^6-γγ-dimethyladenosine	950, 778	1125, 1569
Cyclic-AMP	475, 742	2601, 2515
1-Methyladenosine	145, 120	824, 877
5′-AMP	130, 163	816, 1406
Adenine	32, 38	1250, 1434
Inosine	56, 51	1133, 1364
5′-Deoxyadenosine	412, 299	414, 545

* The following compounds were devoid of clear agonist or antagonist activity when tested at 10^{-4} M: adenosine-5′-propionate, adenosine-5′-monoacetate, 1,N^6-ethenoadensine, guanosine, N^6,$O^{2'}$-dibutyrylcyclic-AMP, 6-mercaptopurine riboside, 5′-adenylyl imidodiphosphate, 5′-guanylylimidodiphosphate, puromycin, D(-)ribose, 2′,3′-isopropylidene adenosine, allopurinol, 2′-*O*-methyladenosine, 2′-AMP, and 3′-AMP.

† Fifteen min incubation.

‡ All individual values or mean ±S.E.M.; the number of determinations is given in parentheses.

is a partial agonist. Although the effect of 5′-deoxyadenosine on the dose–response curve of adenosine was characteristic for the interaction between a full and partial agonist (Fig. 4), a similar interaction was seen between PGE_2 and 5′-deoxyadenosine (Fig. 5). Experiments with 2′- and 3′-deoxyadenosine showed these compounds to have minimal agonist activity at 10^{-4} M but to antagonize both adenosine and PGE_1 at this concentration (data for 2′-deoxyadenosine is given in Table 4). It is clear that more work must be done in order to determine the nature of the inhibitory actions of the deoxyadenosines.

Effects of methylxanthines on the cyclic AMP-elevating effect of adenosine. 1,3-Dimethylxanthine, theophylline, has been reported to antagonize the cyclic AMP-elevating effect of adenosine in several systems [1, 6, 10, 12] including murine neuroblastoma [10]. The effects of 1,3-dimethylxanthine (theophylline), 3,7-dimethylxanthine (theobromine), and 1,3,7-trimethylxanthine (caffeine) on the cyclic AMP-elevating effect of adenosine were therefore determined. The results of these experiments are shown in Fig. 6. The concentration effect curve to adenosine was shifted to the right by theophylline and caffeine, but was unaffected by theobromine. These results indicated that the 1-methyl group is important for the antagonistic activity. Experiments with xanthine, 1-methylxanthine and 3-methylxanthine (Fig. 7) showed this to be the case. In addition, 1-methyl-3-isobutylxanthine was found to be equipotent with the other 1-methylxanthines in this respect (data not shown). Similar experiments with 1-methyladenine, 1-methyladenosine, 1-methylhypoxanthine, and 1-methylinosine (1-methylhypoxanthine riboside) showed these compounds to be completely devoid of antagonistic activity. Theophylline did not affect the cyclic AMP elevations in response to PGE_1 or PGE_2.

DISCUSSION

The cyclic AMP content of murine neuroblastoma clone N2a is rapidly elevated by adenosine in the presence of the phosphodiesterase inhibitor Ro 20-1724. The marked potentiation of the cyclic AMP-elevating effect of adenosine by the phosphodiesterase inhibitor strongly suggests that this effect of adenosine is primarily due to the activation of adenylate cyclase rather than the inhibition of phosphodiesterase. In this regard, two recent studies have shown that the adenylate cyclase activities of cell-free [27] or permeabilized cell preparations [16] of the NS 20 clone of murine neuroblastoma are sensitive to the stimulatory effect of adenosine. The finding that dipyridamole antagonizes the uptake of adeno-

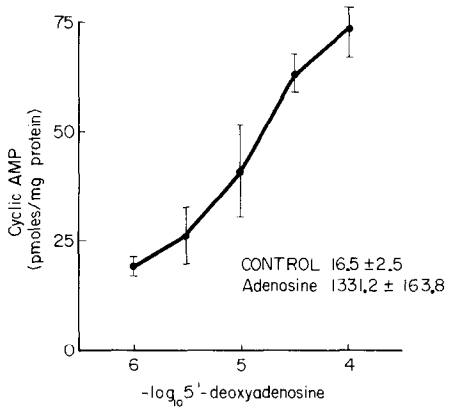

Fig. 3. Concentration: effect curve of 5′-deoxyadenosine on cyclic AMP. All cultures were pre-exposed to 0.7 mM Ro 20-1724. 5′-Deoxyadenosine incubation time was 15 min. The mean ±S.E.M. is shown for each point (n = 3). Adenosine response was to 10^{-5} M.

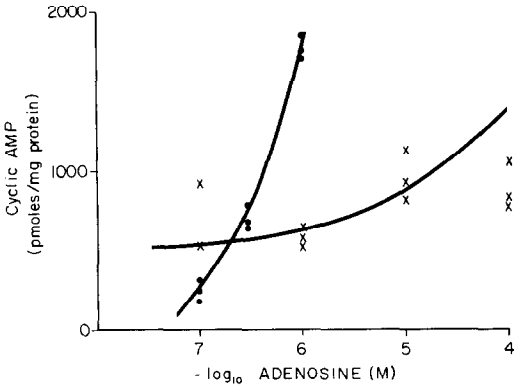

Fig. 4. Effect of 10^{-4} M/5'-deoxyadenosine on concentration:effect curve of adenosine. All cultures were pre-exposed to 0.7 mM Ro 20-1724. Incubations were for 15 min. Adenosine and 5'-deoxyadenosine were added simultaneously. Individual determinations are shown. Key: (●——●) adenosine; and (×——×) adenosine + 5'-deoxyadenosine.

sine but does not antagonize the elevation in cyclic AMP in response to adenosine indicates that the cyclic AMP-elevating effect of adenosine is mediated by an extracellular "adenosine receptor."

We have tested a fairly large number of adenosine analogs for their ability to elevate cyclic AMP, and also for their ability to antagonize the cyclic AMP-elevating effect of adenosine (see Table 3). Except where noted, our results are generally in agreement with studies on other systems [1, 6, 25, 28]. Inosine, guanosine and 6-mercaptopurine riboside all have little or no agonist activity and do not antagonize the effect of adenosine when present in a 100:1 molar ratio. In an additional experiment, inosine at 3×10^{-3} M did not elevate cyclic AMP or antagonize the effect of 10^{-6} M adenosine. Since these compounds are devoid of the N^6 nitrogen, it is clear that this group is of great importance for binding to the receptor and for agonist activity.

The importance of the sugar moiety is equally apparent. Adenine at 10^{-4} M is completely devoid of agonist and antagonist activity. At 3×10^{-3} M adenine does not elevate cyclic AMP but does slightly depress the elevation in cyclic AMP in response to 10^{-6} M adenosine (data not shown). Various modifications of the sugar moiety result in drastic reductions in activity; 2'-, 3'- and 5'-deoxyadenosine all have minimal agonist activity and antagonize the cyclic AMP-elevating effects of both adenosine and the E prostaglandins. Blume and Foster [27] have reported that 2'- and 5'-deoxyadenosine inhibit the stimulatory effects of both PGE_1 and 2-chloroadenosine on the adenylate cyclase activity in cell-free preparations of NS 20 neuroblastoma.

5'-AMP is largely, if not completely, devoid of activity. In an experiment (not shown) similar to those shown in Figs. 6 and 7, 10^{-4} M 5'-AMP produced very small elevations in cyclic AMP and did not antagonize the effect of adenosine. Similar results have been reported from studies on cell-free preparations of NS 20 neuroblastoma [27]. The adenylate cyclase activity of permeabilized cells of the same clone is inhibited by 5'-AMP. These results are in contrast to those guinea pig cerebral cortex slices [1] and human astrocytoma cells [6] where 5'-AMP is a potent agonist. Clark *et al.* [6] presented convincing evidence that the response to 5'-AMP in human astrocytoma cells cannot be accounted for by the conversion of 5'-AMP to adenosine. It would seem, therefore, that qualitative differences occur in the "adenosine receptors" present in different biological materials.

It is fairly well accepted that methylxanthines such as theophylline antagonize the cyclic AMP-elevating effect of adenosine which is seen in several preparations by a competitive mechanism. This conclusion is based on experiments which show the ability of increasing concentrations of adenosine to overcome the antagonism due to theophylline [1, 6, 10], and on Lineweaver–Burk plots of the relationships between the adenosine concentration and the cyclic AMP contents of whole cells [13], and of the stimulatory effects of adenosine on adenylate cyclase activity in broken-cell preparations [27]. It should be kept in mind that adenosine is a moderator of adenylate cyclase activity and not a substrate for the enzyme. Lineweaver–Burk plots which have been interpreted to show competitive antagonism merely show that the relationship

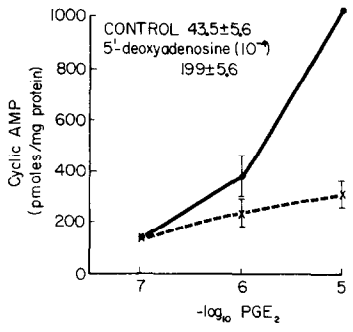

Fig. 5. Effect of 10^{-4} M 5'-deoxyadenosine on concentration: effect curve of PGE_2. All cultures were pre-exposed to 0.7 mM Ro 20-1724. PGE_2 and 5'-deoxyadenosine were added simultaneously and incubations were for 15 min. The mean ±S.E.M. is shown for each point (n = 3) except where S.E.M. is too small to show. Key: (●——●) PGE_2; and (×---×) PGE_2 + 5'-deoxyadenosine.

Table 4. Effect of 2'-deoxyadenosine on cyclic AMP-elevating effects of adenosine and PGE_1

Agonist	Cyclic AMP* (pmoles/mg protein)	
	Control	2'-Deoxyadenosine (2×10^{-4} M)
	44.1 ± 3.9† (2)	92.3 ± 15.9 (3)
Adenosine, 10^{-6} M	762.9 ± 33.7 (5)	466.0 ± 53.7‡ (3)
PGE_1, 3×10^{-8} M	860 ± 16.1 (5)	541.4 ± 98.5‡ (3)

* All incubations were for 15 min.

† Mean ±S.E.M.; the number of determinations is given in parentheses.

‡ Significantly different from control, $P < 0.01$.

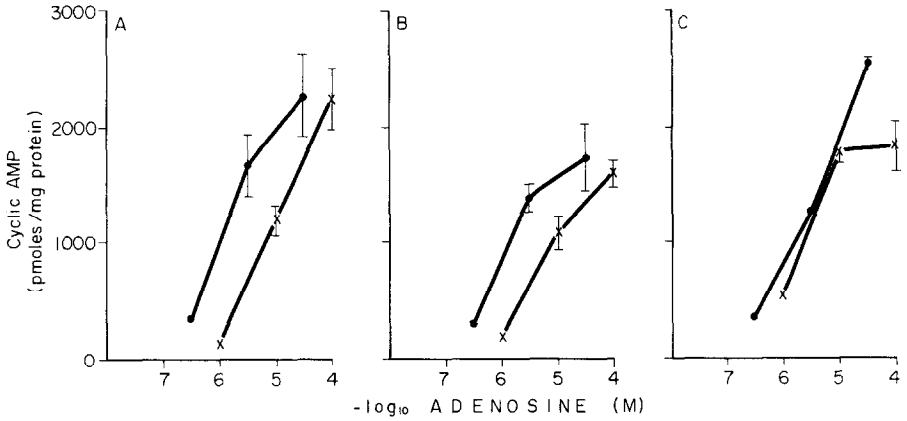

Fig. 6. Effects of xanthines on concentration: effect curve of adenosine (15-min exposure). All plates were pre-exposed to 0.7 mM Ro 20-1724 and the xanthine for a minimum of 15 min. The mean ±S.E.M. is shown for each point except where the S.E.M. is too small to show. Key: (●——●) control; and (×——×) xanthine, 3×10^{-4} M. Panel A, theophylline (1,3-dimethylxanthine); panel B, caffeine (1,3,7-trimethylxanthine); and panel C, theobromine (3,7-dimethylxanthine).

between adenosine concentration and adenylate cyclase activation (or cyclic AMP concentration) is roughly hyperbolic and that the dose–response curve to adenosine is shifted by methylxanthines in a roughly parallel manner. While more complete analyses such as the generation of "Schild Plots" could give support to the hypothesis that the antagonism is competitive in nature, even this procedure would not eliminate other types of antagonism based on other models [29].

There are several observations that do not fit well with the hypothesis that theophylline antagonizes adenosine competitively. Shimizu *et al.* [15] have reported that elevations in cyclic AMP in slices of guinea pig cortex in response to glutamate or adenosine are antagonized by methylxanthines. They also reported that 2'-deoxyadenosine specifically antagonized adenosine, while 2,3-diaminopropionic acid specifically antagonizes glutamate. Similar results have recently been reported for rat and mouse cerebellar cortex [30]. Schultz *et al.* [8] have reported studies on the N4GT3 clone of murine neuroblastoma in which 3-isobutyl-1-methylxanthine (IBMX) was used for its phosphodiesterase inhibitor action to potentiate adenosine. While they state that methylxanthines do not antagonize adenosine in this clone, it is possible that their dose–response curves to adenosine may have been shifted to the right due to the antagonistic action of the methylxanthine. The dose–response curve of the stimulatory effect of adenosine on the adenylate cyclase activity in permeabilized NS 20 cells is both shifted to the right and depressed by theophylline [16].

Theophylline antagonized the cyclic AMP-elevating effect of adenosine in the present experiments. The experiments summarized in Figs. 6 and 7 clearly show that the substitution in the 1 position is important for the antagonistic action of xanthines in N2a neuro-

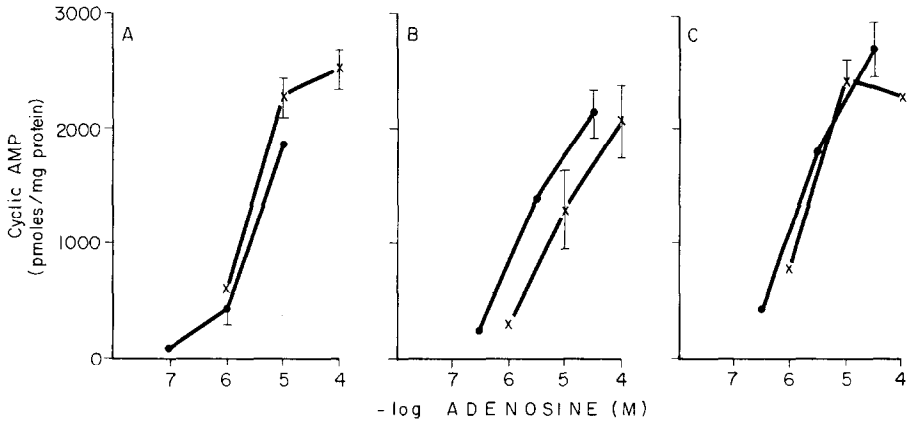

Fig. 7. Effects of xanthines on concentration:effect curve of adenosine (15-min exposure). All plates were pre-exposed to 0.7 mM Ro 20-1724 and the xanthine for a minimum of 15 min. The mean ±S.E.M. is shown for each point except where the S.E.M. is too small to show. Key: (●——●) control; and (×——×) xanthine, 3×10^{-4} M. Panel A, xanthine; panel B, 1-methylxanthine; and panel C, 3-methylxanthine.

blastoma (1-methylxanthine did not antagonize the cyclic AMP-elevating effect of adenosine in guinea pig cortical slices under the conditions studied [28]). Preliminary studies with 1,3-di-*n*-propylxanthine and 1,3-diallylxanthine indicate that these compounds are roughly equipotent with theophylline with regard to their ability to antagonize adenosine. 1-Methyladenine, 1-methyl-6-hydroxypurine (1-methylhypoxanthine) and 1-methylinosine (1-methylhypoxanthine riboside) had no agonist or antagonist action at 3×10^{-4} M. At this same concentration, 1-methyladenosine may have had a small amount of agonist activity (this compound was not purified and may have been contaminated with a trace amount of adenosine), but did not antagonize adenosine.

While there are many competitive antagonists that are structurally dissimilar to the substances they antagonize, there are many other competitive antagonists which are structurally similar to the substances they antagonize. As both agonist and antagonist activity depend on the affinity of the compound for the receptor site, chemically similar agonist:antagonist pairs often share common structure activity relationships. The importance of the *N*-isopropyl group in imparting selectivity to beta-receptor agonists and antagonists is a striking example of such a situation. It is, therefore, not unreasonable to assume that adenosine analog agonists and methylxanthine-type antagonists, both of which are purine derivatives, should share some common structure activity relationships if indeed they both compete for the same receptor site. In this regard, the following should be noted: (1) the N^6 is necessary for the binding of adenosine to its receptor and, thus, for agonist activity, but is not necessary for antagonist activity; (2) the sugar moiety is important for the binding of adenosine to its receptor, but is not necessary for antagonistic activity; and (3) the substitution of a methyl group at the 1 position increases the antagonistic activity of the xanthines, but greatly decreases the affinity of adenosine for its receptor. We interpret these dissimilarities in the structure activity relationships to indicate that the antagonism is allosteric rather than competitive in nature, i.e. to indicate that the xanthines act at a site distinct from adenosine. Obviously, more studies are necessary to determine the exact nature of the interactions between these agents.

Acknowledgement—The authors gratefully acknowledge the excellent technical assistance of Ms. Denise Duranczyk.

REFERENCES

1. A. Sattin and T. W. Rall, *Molec. Pharmac.* **6**, 13 (1970).
2. T. W. Rall and A. Sattin, *Adv. Biochem. Psychopharmac.* **3**, 113 (1970).
3. A. Kalisker, C. O. Rutledge and J. P. Perkins, *Molec. Pharmac.* **9**, 619 (1973).
4. J. Schultz and J. W. Daly, *J. Neurochem.* **21**, 1319 (1973).
5. T. Kodama, Y. Matsukado and H. Shimizu, *Brain Res.* **50**, 135 (1973).
6. R. B. Clark, R. Gross, Y-F. Su and J. P. Perkins, *J. biol. Chem.* **249**, 5296 (1974).
7. A. G. Gilman and M. Nirenberg, *Proc. natn. Acad. Sci. U.S.A.* **68**, 2165 (1971).
8. J. Schultz, B. Hamprecht and J. W. Daly, *Proc. natn. Acad. Sci. U.S.A.* **69**, 1266 (1972).
9. A. G. Gilman and M. Nirenberg, *Nature, Lond.* **234**, 356 (1971).
10. A. J. Blume, C. Dalton and H. Sheppard, *Proc. natn. Acad. Sci. U.S.A.* **70**, 3099 (1973).
11. J. Schultz and B. Hamprecht, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **278**, 215 (1973).
12. A. Sattin, *J. Neurochem.* **18**, 1087 (1971).
13. R. J. Haslam and G. M. Rossen, *Molec. Pharmac.* **11**, 528 (1975).
14. A. Sattin, T. W. Rall and J. Zanella, *J. Pharmac. exp. Ther.* **192**, 22 (1975).
15. H. Shimizu, I. Hiroko and I. Umeda, *Molec. Pharmac.* **11**, 866 (1975).
16. J. Penit, J. Huot and S. Jard, *J. Neurochem.* **26**, 265 (1976).
17. M. Tao, *Meth. Enzym.* **38**, 155 (1974).
18. R. J. Klebe and F. H. Ruddle, *J. Cell Biol.* **43** (2, pt. 2), 69a (1969).
19. F. Murad, V. Manganiello and M. Vaughan, *Proc. natn. Acad. Sci. U.S.A.* **68**, 736 (1971).
20. A. G. Gilman, *Proc. natn. Acad. Sci. U.S.A.* **67**, 305 (1970).
21. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
22. S. V. Hopkins, *Biochem. Pharmac.* **22**, 341 (1973).
23. M-S. Liu and H. Feinberg, *Biochem. Pharmac.* **22**, 1118 (1973).
24. P. G. W. Plagemann and M. R. Roth, *Biochemistry* **8**, 4782 (1969).
25. M. Huang and J. W. Daly, *Life Sci.* **14**, 489 (1974).
26. M. Chasin, F. Mamrak and S. G. Samaniego, *J. Neurochem.* **22**, 1031 (1974).
27. A. J. Blume and C. J. Foster, *J. biol. Chem.* **250**, 5003 (1975).
28. M. Huang, H. Shimizu and John W. Doly, *J. med. Chem.* **15**, 462 (1972).
29. D. Colquhoun, in *Drug Receptors* (Ed. H. P. Rang), pp. 149. University Park Press, London (1973).
30. M. J. Schmidt and J. F. Thornberry, *Fedn Proc.* **35**, 584 (1976).