Biochemical Pharmacology, Vol. 25, pp. 2713–2719. Pergamon Press. 1976. Printed in Great Britain.

EFFECT OF ADENOSINE ON CYCLIC AMP ACCUMULATION IN VENTRICULAR MYOCARDIUM

MINTA HUANG and GEORGE I. DRUMMOND
Biochemistry Group, Department of Chemistry, University of Calgary, Calgary, T2N 1N4, Canada

(*Received* 26 *February* 1976; *accepted* 6 *May* 1976)

Abstract—Adenosine stimulated cyclic AMP accumulation in guinea pig ventricular slice preparations. The response to adenosine was dose-dependent over the range 0.1 to 100 μM; half-maximal stimulation occurred at 25 μM. The response to the nucleoside was rapid; maximum levels of cyclic AMP were obtained in 3 min. Examination of a variety of adenosine analogues revealed that the 2′-, 3′-, and 5′-hydroxyl groups of the ribose moiety were important for activity. Agonist activity also required an amino group in the 6-position. Substitution of one hydrogen atom on the primary amino nitrogen did not alter activity, but substitution of both hydrogens abolished activity. Replacement of the N in position 7 of the purine ring with a C atom, or substitution of the hydrogen atom on position 8 with either an amino group or bromine atom abolished activity. Examination of the effect of several agents, papaverine, 5′-deoxyadenosine, 6-chloropurine riboside and 6-*N*[(ρ-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine, which inhibited adenosine uptake into cardiac cells, provided evidence which suggested that the action of the nucleoside was mediated via interaction with a receptor on the external cell surface. Several phosphodiesterase inhibitors (papaverine, SQ 20009, RO-20-1724 and 3-isobutyl-1-methylxanthine) potentiated the effect of adenosine; theophylline, on the other hand, antagonized adenosine stimulation of cyclic AMP levels. Hexobendine potentiated the stimulatory action of low (10 μM) concentrations of adenosine, and seemed to do so by preventing adenosine uptake. Lidoflazine potentiated the action of both low (10 μM) and high (100 μM) concentrations of adenosine and appeared to act primarily as a phosphodiesterase inhibitor. Dipyridamole potentiated the actions of both low and high concentrations of adenosine probably by blocking adenosine uptake and by inhibiting phosphodiesterase.

Adenosine has been shown to stimulate accumulation of cyclic AMP in brain slices [1, 2], platelets [3] and in a variety of cultured cells of both neuronal and non-neuronal origins [4–6]. This action seems to be effected by interaction of the nucleoside with an extracellular receptor [6–9]. Adenosine has a number of actions on the cardiovascular system. It relaxes smooth muscle resulting in dilation of coronary arteries, and much evidence suggests that the nucleoside is a physiological regulator of coronary blood flow during hypoxia or increased myocardial oxygen demand [10, 11]. The vasodilators, dipyridamole, hexobendine and lidoflazine, potentiate the effect of exogenously applied adenosine on coronary blood flow; this action is attributed to inhibition of nucleoside uptake into blood cells and tissues [12–14]. These coronary dilators are also potent inhibitors of cyclic nucleotide phosphodiesterases from a variety of tissues including heart and coronary arteries and could potentially affect cyclic AMP levels in these tissues [15, 16]. The negative chronotropic action of adenosine on the heart has been recognized for some time [17]; both rate and force of contraction of isolated atria are reduced by this nucleoside [18]. The above-mentioned coronary dilators potentiate these actions of adenosine on the intact heart [17], and in isolated atria [18, 19]. From these observations it seemed possible that adenosine might affect cyclic AMP levels in cardiovascular tissues. In this study, we report the effects of adenosine and agents which affect its metabolism on cyclic AMP levels in ventricular slice preparations.

MATERIALS AND METHODS

5′-Deoxyadenosine, ethyl adenosine 5′-carboxylate, dipyridamole, hexobendine and lidoflazine were gifts from Dr. J. W. Daly, National Institutes of Health, Bethesda, Md., U.S.A. 2-Fluoroadenosine, N^6-phenyladenosine, N^6-(3-methyl-2-butenyl)-adenosine, N^6-hydroxyadenosine, and ρNBTRP* were provided by Dr. A. R. P. Paterson, McEachern Laboratory for Cancer Research, University of Alberta; 6-mercapto, 6-methoxy- and 6-chloropurine riboside and N^6-dimethyladenosine were obtained from Terochem Laboratories Ltd., Edmonton, Canada. Other nucleosides were obtained from Sigma Chemical Co., St. Louis, Mo. 3-Isobutyl-1-methyl-xanthine was purchased from Aldrich Chemicals. RO-20-1724 was obtained through the courtesy of Dr. H. Sheppard, Hoffmann-La Roche Inc., Nutley, N.J., and SQ 20009 through Dr. S. Hess, Squibb & Sons, Inc., Princeton, N.J., U.S.A.

*The following abbreviations have been used: ρNBTRP, 6-*N*[(*p*-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine; SQ 20009, 1-ethyl-4-(iso-propylidenehydrazino-1*H*-pyrazolo-3,4-b)-pyridine-5-carboxylic acid ethyl ester HCl; RO-20-1724, DL-4-(3-butoxy-4-methoxy-benzyl)-2-imidazolidinone; IBMX, 3-isobutyl-1-methylxanthine; and AMP-PNP, 5′-adenylyl imidodiphosphate tetrasodium salt.

The medium used for perfusion and for all incubations was (concentrations in mM) NaCl, 122; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.3; KH_2PO_4, 0.4; $NaHCO_3$, 25; and glucose, 10, gassed continuously with 95% O_2–5% CO_2.

Preparation of tissue slices. Hearts were removed from female guinea pigs (350–500 g); each heart was perfused with 40 ml fluid at 37°, then placed on ice, and the atria and large vessels were removed. Slices (0.5 mm thick) were prepared from the ventricles using a Stadie–Riggs tissue slicer. Slices were further subdivided on a McIlwain tissue chopper set at 500 μm, giving tissue cubes of approximately $(0.5\ mm)^3$.

Incubation and measurement of cyclic AMP. All incubations were conducted at 37° in a Dubnoff metabolic shaker. Slices prepared from three ventricles were incubated in 30 ml medium for 15 min, washed three times with 30 ml of fresh medium by decantation, collected on fine nylon mesh and immediately dispersed in 24 ml medium. After 40 min of incubation,* slices were washed as described above, transferred to 60 ml medium and incubated for 10 min before being collected on a nylon mesh. Portions of the slice preparation, representing about 10 mg protein, were equilibrated for 3 min in 5 ml medium in 25-ml beakers before the test substances (in 50 μl solution) were added. Preparations were then incubated for 3–5 min unless otherwise indicated. The reaction was terminated by collecting the slices on nylon mesh, transferring them rapidly to a glass homogenizer tube containing 1 ml of ice-cold 8% trichloroacetic acid and homogenizing with a Teflon pestle. [^{14}C]cyclic AMP (2000 dis./min) was added to each extract to monitor recovery. After separation of denatured protein by centrifugation, the supernatant was extracted four times with 1.5 vol. ether and subjected to chromatography on Dowex 1-x8 (100–200 mesh, Cl^- form) as described by Schultz and Daly [20]. Cyclic AMP was determined in quadruplicate samples of each lyophyllized column effluent by the method of Gilman [21] using binding protein prepared from beef ventricle and inhibitor protein from rabbit leg muscle. Cyclic AMP content was expressed on the basis of tissue protein. Protein was determined from the trichloroacetic acid-precipitated residue after solubilization in 1 N NaOH using the method of Lowry *et al.* [22].

Measurement of uptake of [^{14}C]adenosine by ventricular slices. The procedure was similar to that used by Huang and Daly [7] for brain slices. Ventricular slices were incubated for 15 min and washed as described above. After incubation in fresh medium (20 ml/g wet wt) for 10 min, slices were collected on nylon mesh and were divided into portions containing 4–5 mg protein. Each portion was dispersed in 3.5 ml medium containing the test substance and 10 μM [^{14}C]adenosine (16 mCi/m-mole). After 20 or 40 min of incubation at 37°, the medium was decanted and 12 ml of fresh medium was added to quench further uptake. The slices were rapidly washed two more times, collected on nylon mesh and homogenized in 1 ml of 8% trichloroacetic acid. Denatured protein was separated by centrifugation, and 0.1-ml aliquots of the supernatant fluid were added to 10 ml Aquasol containing 2 ml of 50% ethanol and 15 μl of 10 N H_2SO_4, and radioactivity was determined by liquid scintillation spectrometry.

*This procedure was used because in some parallel experiments slices were prelabeled by incubation with [^{3}H]adenine for 40 min, and [^{3}H]cyclic AMP was measured chromatographically (see Ref. 2). Data from such experiments are not reported in this paper.

RESULTS

Effect of adenosine on cyclic AMP levels. The basal level of cyclic AMP in guinea pig ventricular slices was 1.4 ± 0.1 pmoles/mg of protein. When 0.1 mM adenosine was present in the medium, cyclic AMP levels rose rapidly to a maximum in 3 min and remained at this level for at least 12 min (Fig. 1A). Response to adenosine was dose-dependent between 0.1 and 100 μM (Fig. 1B). The effect of several phosphodiesterase inhibitors on the adenosine-elicited increase in cyclic AMP levels was examined (Table 1). Papaverine (0.5 mM), SQ 20009 (0.5 mM), RO-20-1724 (0.1 mM) and IBMX (1.0 mM) increased

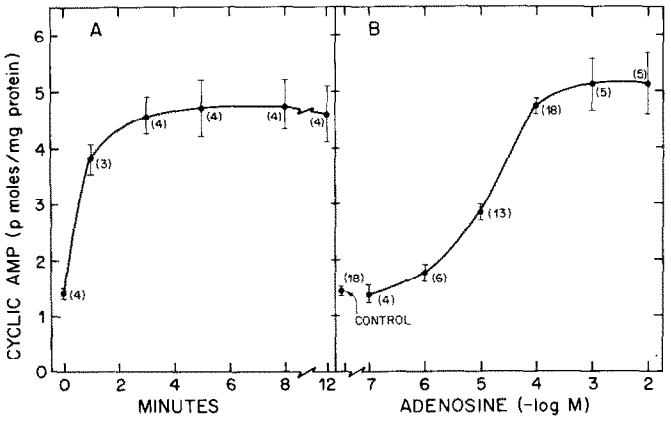

Fig. 1. Effect of adenosine on cyclic AMP accumulation in guinea pig ventricular slices. Conditions are as described in Materials and Methods. Panel A: Time course of action of 100 μM adenosine; panel B: dose dependency of adenosine-elicited stimulation of cyclic AMP levels. Vertical bars represent S.E.M. of the number of experiments shown in parentheses.

Table 1. Effect of phosphodiesterase inhibitors on adenosine-mediated accumulation of cyclic AMP in guinea pig ventricular slices*

Phosphodiesterase inhibitor	Concn (mM)	Adenosine (0.1 mM)	Cyclic AMP (pmoles/mg protein)
None		−	1.4 ± 0.1 (5)
None		+	4.5 ± 0.3 (5)
Papaverine	0.5	−	6.8 ± 0.6 (3)
		+	16.0 ± 0.7 (3)
SQ 20009	0.5	−	2.4 ± 0.1 (3)
		+	10.5 ± 1.9 (3)
RO-20-1724	0.1	−	3.0 ± 0.3 (6)
		+	13.3 ± 0.8 (5)
IBMX	1.0	−	12.3 ± 1.0 (3)
		+	22.8 ± 1.1 (3)
Theophylline	1.0	−	1.5 ± 0.2 (4)
		+	2.8 ± 0.3 (4)

* Slices were incubated in the presence of SQ 20009, IBMX or theophylline for 3 min before addition of adenosine; papaverine or RO-20-1724 was added together with adenosine to slices which had been pre-incubated for 3 min. Otherwise, conditions are as described in Materials and Methods. Values are means from the number of experiments shown in parentheses ±S.E.M.

basal levels of cyclic AMP by 5-, 2-, 2.5- and 10-fold respectively. When 0.1 mM adenosine was present, these compounds appeared to potentiate the effect of the nucleoside. In contrast, theophylline (1 mM) did not affect basal levels of cyclic AMP and partially inhibited the effect of adenosine.

Structure–activity relationships of adenosine analogues. Adenosine analogues modified in either the

Table 2. Structure–activity relationships of adenosine analogues*

Analogue	Concn (mM)	Cyclic AMP (pmoles/mg protein) −Adenosine	Cyclic AMP (pmoles/mg protein) +Adenosine (0.1 mM)
None		1.4 ± 0.10 (12)	4.2 ± 0.19 (14)
Analogues with modified ribose moiety			
2′-Deoxyadenosine	0.5	2.5 ± 0.3† (4)	5.4 ± 0.3‡ (3)
3′-Deoxyadenosine	0.5	1.6 ± 0.1§ (4)	4.4 ± 0.4§ (3)
Isopropylidine adenosine	0.1	2.0 ± 0.1‖ (4)	5.8 ± 0.7‖ (3)
5′-Deoxyadenosine	0.1	1.5 ± 0.1§ (4)	1.9 ± 0.2† (4)
Ethyl adenosine 5′-carboxylate	0.1	1.6 ± 0.2§ (5)	3.2 ± 0.3‡ (5)
AMP-PNP	0.1	2.8 ± 0.2† (4)	4.5 ± 0.5§ (4)
Analogues with modified purine moiety			
Adenosine N^1-oxide	0.1	3.6 ± 0.4† (4)	4.0 ± 0.3§ (3)
2-Chloroadenosine	0.5	4.5 ± 0.3† (3)	4.5 ± 0.3§ (3)
2-Fluoroadenosine	0.1	1.8 ± 0.2‡ (6)	2.6 ± 0.2† (5)
Isoadenosine	0.1	2.2 ± 0.1† (5)	3.4 ± 0.1‡ (5)
N^6-methyladenosine	0.1	4.1 ± 0.3† (3)	4.5 ± 0.6§ (3)
N^6-phenyladenosine	0.1	3.8, 4.6	4.2, 5.4
N^6-benzyladenosine	0.1	5.1 ± 0.4† (4)	5.0 ± 0.3¶ (3)
N^6-(3-methyl-2-butenyl)-adenosine	0.1	5.1 ± 0.5† (3)	5.9 ± 0.3‡ (3)
N^6-dimethyladenosine	0.1	1.8 ± 0.2¶ (5)	4.3 ± 0.4§ (4)
N^6-hydroxyadenosine	0.1	7.1 ± 0.5† (4)	10.5 ± 1.0† (3)
6-Methoxypurine riboside	0.1	2.3 ± 0.5‡ (3)	5.1 ± 0.6‡ (3)
6-Mercaptopurine riboside	0.1	1.6 ± 0.2§ (4)	4.1 ± 0.3§ (3)
6-Chloropurine riboside	0.5	1.7 ± 0.1§ (4)	3.7 ± 0.1§ (4)
Inosine	0.5	1.6 ± 0.1§ (3)	4.8 ± 0.1§ (4)
Tubercidin	0.1	1.5 ± 0.1§ (5)	3.2 ± 0.3‡ (5)
8-Aminoadenosine	0.1	2.3 ± 0.2† (4)	3.9 ± 0.4§ (3)
8-Bromoadenosine	0.1	1.8 ± 0.03‡ (3)	4.3 ± 0.2§ (3)

* Conditions are as described in Materials and Methods. Values are means ±S.E.M. for the number of experiments indicated in parentheses. The significance of differences from basal levels (+ or − adenosine as appropriate) was determined by Student's *t*-test and is indicated by the probabilities shown in the subsequent footnotes.

† $P < 0.001$.
‡ $P < 0.05$.
§ $P > 0.1$.
‖ $P < 0.01$.
¶ $P < 0.1$.

purine ring or sugar moiety were examined for possible effects on cyclic AMP accumulation (Table 2). Modification of the ribose moiety greatly reduced agonist activity; 3′-deoxyadenosine, 5′-deoxyadenosine and ethyl adenosine 5′-carboxylate were inactive at the concentrations used. 2′-Deoxyadenosine, AMP-PNP and isopropylidine adenosine were weak agonists. Of these, 5′-deoxyadenosine (100 μM) strongly antagonized the stimulatory action of 100 μM adenosine; ethyl adenosine 5′-carboxylate slightly decreased the effect of the nucleoside; the remainder were without significant effect. Modification of the purine moiety at N^1 did not significantly alter agonist activity; 100 μM adenosine N^1-oxide was almost as effective as 100 μM adenosine. When present together, the effect was not greater than that of adenosine alone. 2-Chloroadenosine was an effective agonist and when present with adenosine, cyclic AMP levels were equivalent to those produced by adenosine alone. In contrast, 2-fluoroadenosine (100 μM) had virtually no agonist activity and antagonized the effect of adenosine. Isoadenosine behaved similarly. Analogues in which a single H on the amino nitrogen was substituted retained agonist activity. Activity was not significantly different from that of adenosine whether the substituent was aliphatic (methyl- or 3-methyl-2-butenyl-) or aromatic (phenyl- or benzyl-), nor did these analogues affect the stimulation produced by 100 μM adenosine. In contrast, N^6-dimethyladenosine had no agonist action and did not affect the stimulatory action of adenosine. N^6-hydroxyadenosine at 100 μM was a more potent agonist than adenosine, and the effects of the two together were additive. With the exception of 6-methoxypurine riboside, which had marginal agonist activity, replacement of the 6-amino group with a mercapto-, chloro-, or hydroxyl group rendered the compound inactive at the concentrations examined and none of these affected the stimulatory action of adenosine. Similarly, at the concentration used, tubercidin (7-deazaadenosine) was devoid of agonist activity; 8-aminoadenosine had marginal agonist activity, 8-bromoadenosine was virtually inactive and neither of the two latter compounds affected the action of adenosine.

Uptake of [^{14}C]adenosine by ventricular slices. Evidence from studies on brain slices [7] and platelets [9] suggested that adenosine stimulates cyclic AMP formation by interaction with a receptor on the exterior cell surface. The effect of phosphodiesterase inhibitors, adenosine analogues and coronary dilators on uptake of 10 μM [^{14}C]adenosine into ventricular slices is shown in Table 3. Theophylline at 1 mM and IBMX at 100 μM had no significant effect on uptake; IBMX at 1 mM produced a small but significant inhibition (~25 per cent). SQ 20009 was more potent, producing 47 per cent inhibition of uptake at 0.5 mM; papaverine was the most potent agent in this category, producing 68 and 80 per cent inhibition at 0.1 and 0.5 mM respectively. The potent phosphodiesterase inhibitor, RO-20-1724, had no significant effect. The adenosine analogues, 5′-deoxyadenosine and 6-chloropurine riboside, at 100 μM pro-

Table 3. Effect of various agents on uptake of [^{14}C]adenosine by ventricular slices*

Compound	Concn (μM)	Per cent inhibition of adenosine uptake
Phosphodiesterase inhibitors		
Theophylline	1000	<4 (3)
IBMX	100	<5 (2)
	1000	24.6 ± 1.9 (5)
SQ 20009	500	47.2 ± 3.4 (3)
Papaverine	100	67.9 ± 1.2 (3)
	500	79.6 ± 2.8 (3)
RO-20-1724	100	<6 (4)
Adenosine analogues		
2-Chloroadenosine	500	24.3, 26.6
6-Chloropurine riboside	100	75.9 ± 3.8 (3)
2′-Deoxyadenosine	500	14.3, 15.4
3′-Deoxyadenosine	500	<6 (2)
5′-Deoxyadenosine	100	76.0 ± 0.7 (4)
Ethyl adenosine 5′-carboxylate	10	<9 (2)
	100	<10 (2)
ρNBTRP	1	67.1 ± 0.9 (3)
	10	86.5, 86.0
Coronary dilators		
Dipyridamole	1	36.3 ± 0.8 (3)
	10	80, 75.3
Hexobendine	1	53.6, 52.4
	10	88.0 ± 1.0 (3)
Lidoflazine	1	<12 (3)
	10	<14 (4)

* [^{14}C]adenosine (10 μM) (sp. act. 16 μCi/μmole) was used and incubations were for 20 or 40 min as described in Materials and Methods. Values are means ±S.E.M. from the number of experiments shown in parentheses; where only two experiments were performed, both values are given. Total incorporation of radioactivity under control conditions was 7 to 9 × 10^3 and 15 to 16 × 10^3 dis./min/mg of protein at 20 and 40 min respectively.

duced 76 per cent inhibition; 2-chloroadenosine and 2′-deoxyadenosine were less effective inhibitors while 3′-deoxyadenosine and ethyl adenosine 5′-carboxylate were virtually inactive. By far the most potent agent was ρNBTRP, which produced 67 per cent inhibition at 1 μM and 86 per cent at 10 μM. The coronary dilators dipyridamole and hexobendine at 100 μM strongly inhibited adenosine uptake (75 and 86 per cent respectively). Lidoflazine, on the other hand, had only marginal effects. When adenosine concentration was increased to 100 μM, dipyridamole (100 μM), hexobendine (10 μM), papaverine (0.5 mM), IBMX (1.0 mM), 6-chloropurine riboside (100 μM) and ρNBTRP (10 μM) did not significantly affect uptake of the radioactive nucleoside (data not shown).

Effects of vasodilators on adenosine-mediated accumulation of cyclic AMP. The effects of hexobendine, dipyridamole and lidoflazine on myocardial cyclic AMP levels were examined in the absence and presence of 10 and 100 μM adenosine. In additional experiments, RO-20-1724, a phosphodiesterase inhibitor which did not affect adenosine uptake, and/or ρNBTRP, a potent inhibitor of adenosine uptake (Table 3) which does not inhibit phosphodiesterase, were present in the incubation mixture. The results are shown in Table 4. Dipyridamole at 10 and 100 μM elevated cyclic AMP 2-fold and 4-fold, respectively, above the basal level. Hexobendine at 10 μM produced a smaller increase (1.5-fold). Dipyridamole (10 and 100 μM) potentiated the stimulatory effects of both 10 and 100 μM adenosine. At both concentrations of dipyridamole, the degree of potentiation was greater with 10 μM adenosine than with the higher concentration (100 μM). Hexobendine at both 10 and 100 μM stimulated the level of cyclic AMP elicited by 10 μM adenosine to that caused by 100 μM adenosine alone and did not increase cyclic AMP levels further in the presence of the higher concentration of the nucleoside. Lidoflazine potentiated the action of both concentrations of adenosine to a similar extent. This latter agent also potentiated the adenosine effect when present at 1 μM. ρNBTRP had little effect on basal levels of cyclic AMP; it increased the response elicited by 10 μM adenosine but had a lesser effect on the response produced by 100 μM nucleoside. RO-20-1724 increased the basal levels of cyclic AMP and potentiated the action of both concentrations of adenosine to a similar degree. A combination of ρNBTRP and RO-20-1724 elevated the basal levels of cyclic AMP as well as the responses of both 10 and 100 μM adenosine; the degree of potentiation was greater with 10 μM adenosine.

DISCUSSION

The data presented demonstrate that adenosine can augment intracellular cyclic AMP levels in ventricular myocardium. The phosphodiesterase inhibitors, papaverine, SQ 20009, RO-20-1724 and IBMX, potentiated the effect of adenosine, but theophylline produced inhibition. Inhibition by methylxanthines of either adenosine-mediated increases in cyclic AMP or adenosine-induced physiological responses has been observed in a number of systems [1, 23–26]. The studies with adenosine analogues provide some information on structural requirements for nucleoside action. In general, these requirements bear many similarities to those of brain preparations [7, 23]. Modifications of the ribosyl moiety caused partial or complete loss of agonist activity. Thus, 2′-deoxyadenosine had only low activity at 0.5 mM (it was without activity at 100 μM); the 3′-deoxy-, 5′-deoxy, and isopropylidine analogues and ethyl adenosine 5′-carboxylate were virtually inactive. Free hydroxyl groups in the 2′-, 3′- and 5′-positions, therefore, seem necessary for agonist activity; AMP-PNP (which is resistant to

Table 4. Effect of dipyridamole. hexobendine and lidoflazine on adenosine-mediated accumulation of cyclic AMP*

Agent(s)	Concn (μM)	Cyclic AMP (pmoles/mg protein)		
		−Adenosine	+Adenosine (10 μM)	+Adenosine (100 μM)
None		1.2 ± 0.1 (9)	2.3 ± 0.1† (11)	3.7 ± 0.2 (11)
Dipyridamole	10	2.6, 2.7	8.6, 7.0	8.9, 8.0
	100	5.1, 4.5	14.2	11.6, 15.5
Hexobendine	10	1.8 ± 0.2‡ (4)	3.2 ± 0.2§,‖ (3)	3.4 ± 0.4¶ (3)
	100	1.7 ± 0.1§ (4)	3.5 ± 0.5§,‖ (3)	3.7 ± 0.4¶ (3)
Lidoflazine	10	1.5 ± 0.1¶ (3)	5.1 ± 0.4**,†† (3)	8.2 ± 0.8** (3)
ρNBTRP	1	1.6 ± 0.1‡ (6)	3.8 ± 0.2**,†† (4)	4.3 ± 0.1§ (6)
RO-20-1724	100	3.1 ± 0.2** (8)	8.4 ± 0.5†,** (5)	12.9 ± 0.6** (7)
RO-20-1724 plus ρNBTRP	100 1	4.7, 4.9	10.3, 9.3	11.4, 10.8

* Values are means ±S.E.M. from the number of experiments indicated in parentheses. Where only two experiments were performed, both values are given. Significance of differences from basal levels (+ or − adenosine as appropriate) was determined by Student's *t*-test and is indicated by the probabilities shown in footnotes ‡, §, ¶ and **. Significance of differences from the levels in the presence of 0.1 mM adenosine is indicated by the probabilities shown in footnotes †, ‖ and ††.

† $P < 0.001$.
‡ $P < 0.05$.
§ $P < 0.01$.
‖ $P > 0.1$.
¶ $P > 0.1$.
** $P < 0.001$.
†† $P < 0.05$.

phosphatase), however, possessed activity. From the data presented, it is also possible to suggest that on the purine ring, a 6-amino group is important for activity. Thus, 6-methoxy-, 6-hydroxy-, 6-chloro-, and 6-mercaptopurine riboside possessed very low activity. One of the protons of the amino nitrogen could be substituted with either an aliphatic or an aromatic residue. However, N^6-dimethyladenosine, in which both protons are substituted, was virtually inactive. Thus, either one exchangeable proton on the 6-amino group, or the proper exposure of the N atom is important. The N atom at position 7 also appeared essential for activity; in addition, substitution at C-8 greatly impaired agonist activity. 2-Chloroadenosine and 2-fluoroadenosine, which have similar negative chronotropic actions on the beating heart [27], had opposite effects on cyclic AMP formation in the slice preparation. The 2-chloro analogue, a potent vasodilator [28] and stimulator of cyclic AMP formation in brain slices [7, 23] and human fibroblasts [8], possessed agonist activity in heart slices while 2-fluoroadenosine was inactive and actually inhibited adenosine-stimulated cyclic AMP formation. The two analogues, N^6-hydroxyadenosine and ethyl adenosine 5′-carboxylate, exhibited especially interesting actions. The former seemed to be a more potent agonist than adenosine and the combined actions of this analogue and adenosine were additive. This might imply different receptors for each. However, the action of the N^6-hydroxy compound was antagonized by theophylline (data not shown) as was adenosine (see Table 1). They might, of course, be acting on different cell types. Ethyl adenosine 5′-carboxylate has been reported to be a potent vasodilator [29]; however, unlike the other vasoactive agents examined, it did not stimulate cyclic AMP accumulation in ventricular slices. We considered it possible that the source of substrate for cyclic AMP formation by this analogue might be a discrete and minor component of the total adenine nucleotide pool, and thus small amounts of the cyclic nucleotide formed might escape detection when assaying for total tissue cyclic AMP. However, in experiments in which slices were prelabeled with [^{3}H]adenosine and the accumulation of labeled cyclic AMP measured by the chromatographic technique of Shimizu *et al.* [2], we were unable to detect radioactive cyclic nucleotide, although it was readily demonstrated using adenosine, 2-chloroadenosine or N^6-hydroxyadenosine. Recently it has been reported that the vasodilator action of ethyl adenosine 5′-carboxylate appears different from adenosine in that it is not potentiated by dipyridamole [30].

Uptake of 10 μM [^{14}C]adenosine was inhibited by dipyridamole, hexobendine, papaverine, 5′-deoxyadenosine, 6-chloropurine riboside and ρNBTRP while uptake of 100 μM adenosine was virtually unaffected by these agents. This could mean that different mechanisms might be involved in the uptake of high and low concentrations of this nucleoside analogous to those found in erythrocyte [31] and brain slices [7]. It could also mean that the uptake mechanism was saturated at 100 μM nucleoside so that the effect of these agents would not be apparent. ρNBTRP (1 μM), which inhibited adenosine uptake by 67 per cent, potentiated the accumulation of cyclic AMP in response to 10 μM adenosine but did not substantially augment the action of 100 μM nucleoside. 6-Chloropurine riboside acted similarly (data not shown). If inhibition of adenosine uptake increases the effective concentration of nucleoside at a surface membrane site, the expected response would be no greater than the maximum response of adenosine. The observations with ρNBTRP and 6-chloropurine riboside, therefore, are consistent with the idea that the site of adenosine action is at the external cell surface. The possibility can be considered, however, that these agents may be acting to inhibit the metabolism of adenosine intracellularly. In support of our feeling that their main action is to prevent entry of adenosine into the cell, it has been clearly shown that ρNBTRP specifically blocks nucleoside transport [32, 33]. It is unlikely that the action of any of these agents could be accounted for by inhibition of adenosine deaminase which might have leached from cells into the incubation medium, since this activity could not be detected in the media of control experiments after removal of the slices.

Different mechanisms seemed to be involved in the potentiative action of hexobendine, lidoflazine and dipyridamole on adenosine-elicited cyclic AMP accumulation in guinea pig ventricular slices, although all three compounds are known to inhibit phosphodiesterase [15, 16]. Hexobendine appeared to act by inhibiting adenosine uptake rather than by phosphodiesterase inhibition. Thus at 10 μM, this compound, which inhibited the uptake of 10 μM adenosine by 88 per cent (see Table 3), potentiated the accumulation of cyclic AMP in response to 10 μM adenosine to a level equivalent to that caused by 100 μM nucleoside alone (see Table 4). This action is unlikely to be due to inhibition of phosphodiesterase because the drug did not potentiate the effect of 100 μM adenosine. Indeed, the action of hexobendine is similar to that of ρNBTRP, which inhibited adenosine uptake but did not inhibit phosphodiesterase at the concentration used. Lidoflazine did not significantly affect the uptake of adenosine but potentiated the cyclic AMP-accumulating action of both 10 and 100 μM adenosine to the same extent. This would be consistent with an inhibitory action on phosphodiesterase. In agreement with this, the potent phosphodiesterase inhibitor, RO-20-1724, which had virtually no effect on adenosine uptake, potentiated the effects of both concentrations of adenosine to a similar extent. Dipyridamole seemed to exert its action by inhibiting both phosphodiesterase and adenosine uptake. Thus it potentiated the cyclic AMP-accumulating action of 100 μM adenosine but had no significant effect on uptake of the nucleoside at this concentration. This would be consistent with an inhibitory action on phosphodiesterase. However, the degree of potentiation of the effect of 10 μM adenosine was greater than that of 100 μM adenosine. This could result from inhibition of uptake since dipyridamole inhibited uptake of 10 μM adenosine by about 80 per cent. Indeed, the effects of dipyridamole could be mimicked by a combination of the phosphodiesterase inhibitor, RO-20-1724, and the nucleoside uptake inhibitor, ρNBTRP; the two potentiated the effect of 10 μM adenosine to a greater extent than that of 100 μM nucleoside. It has been shown [18] that the potentiative effects of dipyridamole and hexobendine on the

action of adenosine on guinea pig heart could be correlated with their actions on uptake of the nucleoside, but the action of lidoflazine could not be explained on the same basis. The present study confirms that lidoflazine is unlikely to inhibit adenosine uptake; however, all three vasodilators could potentiate the adenosine-elicited cyclic AMP accumulation in guinea pig heart slices but by apparently different mechanisms.

Whether the actions of adenosine and vasodilators described here are related to the cardiovascular actions of these agents cannot be determined from this study. Nor has it been determined what contribution to cyclic AMP levels was made by the smooth muscle cells of the coronary vasculature. In recent experiments we have shown that adenosine stimulates cyclic AMP formation in atrial slices, but we have not examined coronary vasculature as yet. Adenosine "receptors" in both coronary vasculature and myocardial cells seem to share certain basic features. Thus, both adenosine-induced vasodilation and negative chronotropism are potentiated by dipyridamole, hexobendine and lidoflazine and inhibited by methylxanthines. In a recent study, Kalsner [34] has shown that dipyridamole potentiated adenosine-induced relaxation of beef coronary artery strips. Uptake of radioactive adenosine into isolated arterial strips was inhibited by dipyridamole. Thus there may be similarities between the cardiac and vascular systems. Studies similar to those reported here on the coronary arterial system could be of some value.

In the presence of 0.1 mM IBMX or RO-20-1724, epinephrine and isoproterenol (10 μM) increased cyclic AMP levels in the ventricular slice preparation approximately 3-fold. Propranolol (10 μM) abolished the stimulatory action of the catecholamines. It will be of interest to determine what relationship, if any, exists between the catecholamine receptor and the stimulatory action of adenosine.

Acknowledgements—This study was supported by grants from the Medical Research Council and the Alberta Heart Foundation.

REFERENCES

1. A. Sattin and T. W. Rall, *Molec. Pharmac.* **6,** 13 (1970).
2. H. Shimizu, J. W. Daly and C. R. Creveling, *J. Neurochem.* **16,** 1609 (1969).
3. D. C. B. Mills and J. B. Smith, *Biochem. J.* **121,** 185 (1971).
4. A. G. Gilman and B. K. Schrier, *Molec. Pharmac.* **8,** 410 (1972).
5. A. J. Blume, C. Dalton and H. Sheppard, *Proc. natn. Acad. Sci. U.S.A.* **70,** 3099 (1973).
6. R. B. Clark, R. Gross, Y.-F Su and J. P. Perkins, *J. biol. Chem.* **249,** 5296 (1974).
7. M. Huang and J. W. Daly, *Life Sci.* **14,** 489 (1974).
8. T. W. Sturgill, B. K. Schrier and A. G. Gilman, *J. cyclic Nuc. Res.* **1,** 21 (1975).
9. R. J. Haslam and G. M. Rosson, *Molec. Pharmac.* **11,** 528 (1975).
10. R. M. Berne, *Am. J. Physiol.* **204,** 317 (1963).
11. R. Rubio and R. M. Berne, *Circulation Res.* **25,** 407 (1969).
12. O. Kraupp, E. Wolner, L. Adler-Kastner, B. Ploszczanski and E. Tuisl, *Arzneimittel-Forsch.* **15,** 1187 (1965).
13. S. Afonso, G. S. O'Brien and C. W. Crumpton, *Circulation Res.* **22,** 43 (1968).
14. S. Afonso and G. S. O'Brien, *Arch. int. Pharmacodyn. Thér.* **194,** 181 (1971).
15. W. R. Kukovetz and G. Pöch, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **267,** 189 (1970).
16. M. S. Amer and W. E. Kreighbaum, *J. pharm. Sci.* **64,** 1 (1975).
17. A. Stafford, *Br. J. Pharmac. Chemother.* **28,** 218 (1966).
18. S. V. Hopkins, *Biochem. Pharmac.* **22,** 341 (1973).
19. C. Kücükhüseyin and S. O. Kayaalp, *Arch. int Pharmacodyn. Thér.* **208,** 243 (1974).
20. J. Schultz and J. W. Daly, *J. biol. Chem.* **248,** 843 (1973).
21. A. G. Gilman, *Proc. natn. Acad. Sci. U.S.A.* **67,** 305 (1970).
22. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
23. M. Huang, H. Shimizu and J. W. Daly, *J. med. Chem.* **15,** 462 (1972).
24. J. Schultz and J. W. Daly, *J. biol. Chem.* **248,** 853 (1973).
25. S. Afonso, *Circulation Res.* **26,** 743 (1970).
26. J. Scholtholt, R. E. Nitz and E. Schraven, *Arzneimittel-Forsch.* **22,** 1255 (1972).
27. R. Einstein, J. A. Angus, L. B. Cobbin and M. H. Maguire, *Eur. J. Pharmac.* **19,** 246 (1972).
28. D. A. Clarke, J. Davoll, T. S. Philips and G. B. Brown, *J. Pharmac. exp. Ther.* **106,** 291 (1952).
29. H. H. Stein, *J. med. Chem.* **16,** 1306 (1973).
30. S. Afonso, R. R. Henderson, J. D. Folts and G. G. Rowe, *Bas. Res. Cardiol.* **70,** 390 (1975).
31. H. Roos and K. Pfleger, *Molec. Pharmac.* **8,** 417 (1972).
32. C. E. Cass and A. R. P. Paterson, *J. biol. Chem.* **247,** 3314 (1972).
33. C. E. Cass and A. R. P. Paterson, *Biochem. Pharmac.* **24,** 1989 (1975).
34. S. Kalsner, *Br. J. Pharmac.* **55,** 439 (1975).