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ADENOSINE REGULATION OF CANINE CARDIAC ADENYLATE CYCLASE*

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Abstract—Adenosine has a biphasic, [Mg^{2+}]-dependent effect on the catalytic activity of dog heart adenylate cyclase. In the presence of 0.5 mM Mg^{2+}, adenosine stimulated cyclic AMP formation, but when the cyclase was activated with 4 mM Mg^{2+} plus 0.5 mM Mn^{2+}, adenosine inhibited catalytic activity in a dose-dependent fashion. Adenine, 3′-deoxyadenosine, and selected purine-modified adenosine analogs stimulated the enzyme, whereas 2′-deoxyadenosine, 5′-deoxyadenosine and adenine-α-L-lyxofuranoside mimicked the inhibitory effect of adenosine on the Mg^{2+} plus Mn^{2+} stimulated enzyme. These results are consistent with the 'two receptor' model of Londos and Wolff [C. Londos and J. Wolff, *Proc. natn. Acad. Sci. U.S.A.* **74**, 5482 (1978)], but they raise the possibility of subtle organ and species differences in the chemical determinants of adenosine binding. Adenosine in both intracellular and extracellular compartments may modulate adenylate cyclase activity in the beating heart, in addition to its putative role in the regulation of coronary vascular resistance.

Adenosine exerts a biphasic, Mg^{2+}-dependent effect on the catalytic activity of adenylate cyclase* in several types of cells. At low [Mg^{2+}] this nucleoside stimulates the enzyme, but at higher [Mg^{2+}], which by itself enhances cyclase activity, adenosine inhibits the enzyme. Some base-modified ribosides mimic the stimulatory effect of adenosine, whereas several sugar-modified adenine nucleosides mimic the inhibitory effect of adenosine. This pattern is typical of the adenylate cyclase of platelets, Leydig cells and brain, whereas the adenylate cyclase of liver and adipocytes is only inhibited by adenosine. The chemical specificity, sensitivity to [Mg^{2+}], and tissue distribution of these responses are interpreted as evidence that adenylate cyclase has two adenosine binding sites. According to this hypothesis, the stimulatory site resides on the cell surface, while the inhibitory site is accessible to the cell interior [1].

Adenylate cyclase has an important influence on cardiac performance and metabolism. Cyclic AMP-dependent protein kinases appear to modulate the response of the heart to stimuli such as β-adrenergic activation by catalyzing protein phosphorylation at the level of the contractile apparatus (troponin I) [2], of the sarcoplasmic reticulum (phospholamban) [3], and also of the sarcolemma [4]. Cyclic AMP also enhances substrate availability—for example, by stimulating the activation of phosphorylase *b* kinase [5].

Since adenosine is continuously released into the cardiac extracellular space [6] and, according to recent evidence, may exist in an intracellular compartment as well [7, 8], this nucleoside is potentially able to both stimulate and inhibit cardiac adenylate cyclase. Studies employing a guinea pig heart microsomal fraction, however, suggest that the effect of adenosine on adenylate cyclase is solely inhibitory [9]. Because the evidence on this point is incomplete, we have examined the effects of [Mg^{2+}], adenosine, and certain adenosine analogs on the adenylate cyclase activity of dog cardiac cell fragments.

MATERIALS AND METHODS

Cardiac cell fragments. Hearts removed from pentobarbital-anesthetized dogs were rinsed and trimmed of fat and epicardial vessels. All subsequent steps were performed at 4°. Portions of muscle (10 g) were homogenized for 1 min in a Waring Blender with 200 ml of 50 mM Tris·HCl, pH 7.5. The homogenate was filtered through six layers of cheesecloth, and aliquots of the filtrate were further comminuted by five strokes in a Dounce homogenizer having a loose-fitting teflon pestle. The precipitate that collected during 15 min of centrifugation at 2000 *g* was washed once with, and finally resuspended in, 50 ml of 50 mM Tris·HCl, pH 7.5, per g original tissue weight.

Adenylate cyclase assay. The assay mixture consisted of 1 mM ATP, 60 mM KCl to activate pyruvate kinase, 1 mM MIX, 0.01 mM GMPPNHP, 0.02 mM EHNA, and 50 mM Tris·HCl, pH 7.5, in a total volume of 0.2 ml. The concentrations of adenosine, its analogs, and of Mg^{2+} and Mn^{2+} were adjusted as

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* Non-standard abbreviations and trivial names used in this report are: adenylate cyclase, ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1; cyclic AMP, adenosine-3′:5′-cyclic monophosphate; EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; GMPPNHP, guanosine-5′-(β,γ-imino)triphosphate; MIX, 1-methyl-3-isobutylxanthine; PEP, phosphoenolpyruvate; and PK, pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40).

described below. The suspension of washed cell fragments was diluted so that cAMP production was linear for at least 5 min and was proportional to protein concentration. The protein concentrations varied between 16 and 87 μg/ml. The assay began with the addition of 50 μl of membrane fragments and after 5 min at 37° the reaction was stopped by adding 50 μl of 0.1 M EDTA. The tubes were then placed in a boiling water bath for 3 min and cooled, and the cAMP formed was estimated by the method of Gilman [10] as modified by Beck *et al.* [11]. Protein was estimated by the method of Lowry *et al.* [12].

Adenosine deaminase assay. The extent of adenosine deamination under the conditions of the cyclase assay was assessed in reaction mixtures containing 0.1 mM [2,8,5′-^{3}H]adenosine (sp. act. 2.25 Ci/mole). The mixtures were incubated, treated with EDTA, and boiled exactly as in the cyclase assay. Coagulated protein was separated by centrifugation, and aliquots of the supernatant fraction were spotted on silica gel t.l.c. plates with adenosine, inosine, and hypoxanthine standards. These plates were developed in 1-butanol–methanol–ethyl acetate–NH_4OH (7:3:4:4, by vol.). The spots corresponding to each purine were cut out and counted in a mixture of 8 g Omnifluour and 60 g naphthalene per liter of *p*-dioxane.

cAMP phosphodiesterase assay. Reaction mixtures for the assessment of cAMP degradation under the conditions of the adenylate cyclase assay contained all the components of the cyclase assay plus 10^{-8} M [^{3}H]cAMP (28 Ci/mmole) and 0.1 unit of snake venom 5′-nucleotidase. The reaction was started by adding membrane fragments and was stopped with EDTA and boiling. Purines were separated by t.l.c. as in the adenosine deaminase assay, and were eluted from the silica gel with 0.5 ml glacial acetic acid; ^{3}H-activity in the cAMP and adenosine spots was counted. The percentage of cAMP degradation was calculated by the formula $100 \times$ adenosine (adenosine + cAMP).

The Sigma Chemical Co. (St. Louis, MO) supplied snake venom 5′-nucleotidase, PK, PEP and, except as noted, the purines used in this study. CalBiochem (La Jolla, CA) supplied 6-thioinosine; 5′-deoxyadenosine and adenine-α-L-lyxofuranoside were purchased from Terra Marine (La Jolla, CA). EHNA was a gift from Dr. James M. Downey, University of South Alabama, Mobile, AL, and RO-20,1724 was a gift from Hoffmann-LaRoche, Inc. (Nutley, NJ). All radiolabeled purines were purchased from the New England Nuclear Corp. (Boston, MA). J. T. Baker silica gel 1 B-F sheets were used for t.l.c.

Unless noted otherwise, data are presented as means ± S.E.M. The statistical significances of the differences were evaluated by the Student's *t*-test for paired samples or by analysis of variance.

RESULTS

In the absence of added divalent cation, adenylate cyclase activity in cell fragment suspensions from nine hearts averaged 4.1 ± 1.1 pmoles cAMP formed·min^{-1}·(mg protein)$^{-1}$. The adenosine deaminase activity of these preparations was high, but the addition of EHNA to the reaction mixtures reduced the degradation rate of 10 μM [^{3}H]adenosine from a high of 26 per cent per 5 min to <0.7 per cent per 5 min. These fragments also contained significant cAMP phosphodiesterase activity, having hydrolysed between 16 and 33 per cent of added 10 nM [^{3}H] cAMP under the conditions of the cyclase assay. Including 1 mM MIX reduced degradation to < 9 per cent. Although MIX significantly (though only modestly) antagonized adenosine activation of the cyclase (see below), this effect was balanced by an effect of MIX on the phosphodiesterase. For this

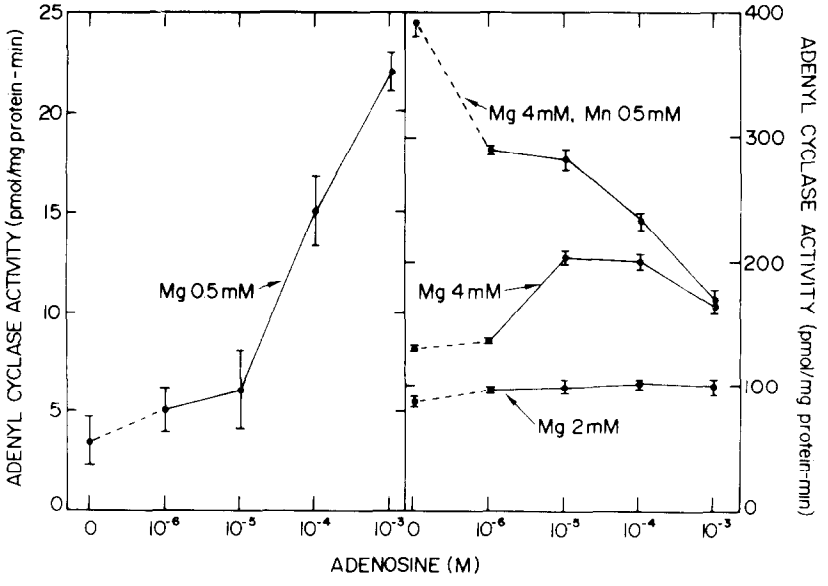

Fig. 1. Interaction of adenosine and divalent cations on adenylate cyclase activity of washed dog heart particles. See text for details. Note the different ordinate scales for the two panels.

Table 1. Effects of divalent cations and adenosine on cardiac adenylate cyclase*

Cation	Adenosine 0	0.001 mM	0.01 mM	0.1 mM	1 mM
Mg^{2+} (0.5 mM)	3.7 ± 0.4	3.5 ± 0.6	6.5 ± 1.3	9.9 ± 2.2	13.1 ± 3.3
Mg^{2+} (2 mM)	16.6 ± 1.2	19.2 ± 1.4	18.1 ± 1.4	18.5 ± 1.6	19.1 ± 0.4
Mg^{2+} (4 mM)	25.9 ± 1.4	23.1 ± 2.8	24.5 ± 6.3	16.4 ± 2.4	15.0 ± 0.7
Mg^{2+} (4 mM) plus Mn (0.5 mM)	244 ± 53	233 ± 53	245 ± 57	195 ± 43	108 ± 22

* Data represent adenylate cyclase activity [pmoles cAMP formed · min^{-1} · (mg protein)$^{-1}$] expressed as mean ± S.E.M. of preparations from nine hearts.

reason, and because the supply of RO-20,1724 was limited, MIX was used routinely.

In the presence of 0.5 mM Mg^{2+}, cAMP formation was stimulated 1.7- to 2.5-fold by 10 μM isoproterenol and 7- to 9-fold by 10 mM NaF.

Effects of divalent cations and adenosine on adenylate cyclase activity. Mg^{2+} produced a concentration-dependent enhancement of cyclase activity (Fig. 1 and Table 1). In the example shown in Fig. 1, a combination of 4 mM Mg^{2+} plus 0.5 mM Mn^{2+} raised catalytic activity an average of 29-fold, to 153 pmoles cAMP formed · min^{-1} · (mg protein)$^{-1}$. The requirement for the divalent cation was absolute; 25 mM EDTA completely inhibited cyclase activity.

Adenosine exerted a biphasic effect on cyclase activity which depended on divalent cation concentration. In the presence of 0.5 mM Mg^{2+}, adenosine (1 mM) caused an average 3.5-fold concentration-dependent enhancement of cyclase activity. Stimulation by this concentration of adenosine varied considerably from one preparation to another from levels as low as 1.3-fold to as high as 7.3-fold, probably reflecting the effects of endogenous adenosine, divalent cations, and perhaps degradation of the enzyme during preparation. In the presence of 2 mM Mg^{2+}, adenosine neither stimulated nor inhibited the cyclase, but at 4 mM Mg^{2+}, with or without 0.5 mM Mn^{2+}, 1 mM adenosine invariably produced concentration-dependent inhibition of cyclase activity.

To determine whether alkylxanthines antagonize the stimulatory and inhibitory effects of adenosine, additional experiments employing three cell fragment preparations were performed to compare the effects of adenosine on cyclase activity in the presence of 1 mM RO-20,1724, a non-xanthine cAMP phosphodiesterase inhibitor, with those in the presence of this inhibitor plus 1 mM MIX. These experiments showed that MIX significantly inhibited basal cyclase activity (probably reflecting antagonism of endogenous adenosine), as well as the stimulatory effect of 1–100 μM adenosine in the presence of 0.5 mM Mg^{2+} (Fig. 2). Although in each individual experiment the effect of MIX at each adenosine concentration was statistically significant ($P < 0.01$), it was not large. The reductions in cyclase activity in the presence of 0, 1, 10 and 100 μM exogenous adenosine averaged 24, 18, 13 and 7 per cent. MIX did not influence the inhibitory effect of adenosine elicited by 4 mM Mg^{2+} plus 0.5 mM Mn^{2+}.

Influence of adenosine analogs on adenylate cyclase. The effects of purine- and ribose-modified adenosine analogs on adenylate cyclase activity were examined at low and high divalent cation concentrations (Table 2). At 0.5 mM Mg^{2+}, N^6-methyladenosine and 2-chloradenosine produced dose-dependent activation of the cyclase, as did both adenine and 8-bromoadenosine. Because stimulation by adenine and 8-bromoadenosine was unexpected, the effects of these purines were tested in three additional membrane preparations; each time they stimulated cAMP production. At a purine concentration of 0.1 mM, the cyclase activity in the four experiments averaged 144 ± 31 per cent of control for adenine and 157 ± 48 per cent for 8-bromoadenosine. In one of these experiments, alkylxanthine inhibition also was evaluated by comparing catalytic activity in the presence of 1 mM MIX plus 0.1 mM RO-20,1724 with that in the presence of RO-20,1724 alone. MIX inhibited adenine stimulation by 17 per cent and 8-bromoadenosine stimulation by 24 per cent. Inosine, guanosine, and 2-aminoadenosine had no effect on cAMP accumulation, but 6-thioinosine significantly inhibited the enzyme. Of the ribose-modified analogs, 3'- and 5'-deoxyadenosine significantly stimulated the enzyme.

At 4 mM Mg^{2+} plus 0.5 mM Mn^{2+}, the inhibitory effect of adenosine was mimicked by 2'-deoxyadenosine, 5'-deoxyadenosine and adenine-α-L-lyxofuranoside, whereas 3'-deoxyadenosine slightly, though not significantly, stimulated activity. The stimulation

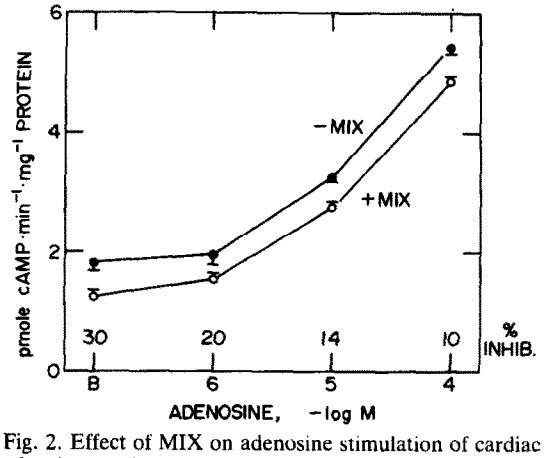

Fig. 2. Effect of MIX on adenosine stimulation of cardiac adenylate cyclase. Reaction mixtures contained 0.5 mM Mg^{2+} and 1 mM RO-20,1724. Curves represent cyclase activity in the presence (○) and the absence (●) of additional 1 mM MIX. B is the activity in the absence of exogenous adenosine. See text for additional discussion.

Table 2. Effect of adenosine analogs and metal ions on dog cardiac adenylate cyclase

Test compound, log M		Percentage of control activity* 0.5 mM Mg^{2+}	4 mM Mg^{2+} plus 0.5 mM Mn^{2+}
Adenosine	−5	131 ± 4	
	−4	158 ± 12	119 ± 12
	−3		77 ± 2
Adenine	−5	132 ± 16	
	−4	158 ± 5	146 ± 6
	−3		230 ± 43
2-Aminoadenosine	−5	98 ± 3	
	−4	111 ± 3	115 ± 7
	−3		123 ± 4
2-Chloroadenosine	−5	108 ± 3	
	−4	117 ± 1	134 ± 16
	−3		142 ± 3
N^6-Methyladenosine	−5	102 ± 6	
	−4	117 ± 6	111 ± 7
	−3		126 ± 13
Inosine	−5	97 ± 4	
	−4	101 ± 2	93 ± 2
	−3		101 ± 15
Guanosine	−5	93 ± 5	
Guanosine	−4	99 ± 1	101 ± 5
	−3		100 ± 5
6-Thioinosine	−5	91 ± 5	
	−4	85 ± 2	92 ± 5
	−3		92 ± 5
8-Bromoadenosine	−5	137 ± 7	
	−4	159 ± 26	148 ± 4
2′-Deoxyadenosine	−5	95 ± 4	
	−4	93 ± 5	41 ± 3
	−3		28 ± 4
3′-Deoxyadenosine	−5	137 ± 11	
	−4		118 ± 8
5′-Deoxyadenosine	−5	113 ± 1	
	−4	129 ± 9	69 ± 1
2′,3′-*O*-Isopropylideneadenosine	−4	93 ± 4	103 ± 3
	−3		85 ± 3
Adenine-α-L-lyxofuranoside	−5	106 ± 12	75 ± 4
	−4	105 ± 4	54 ± 26
	−3		26 ± 4

* Adenylate cyclase activity in the absence of adenosine analogs ranged between 6.2 ± 0.2 and 7.2 ± 0.4 pmoles cAMP/min per mg protein at 0.5 mM Mg^{2+} and 208 ± 15 to 260 ± 8 pmoles cAMP/min per mg protein at 4 mM Mg^{2+}, 0.5 mM Mn^{2+}.

of cyclase activity by adenine, 8-bromoadenosine, N^6-methyladenosine, and 2-chloroadenosine observed at 0.5 mM Mg^{2+} persisted, and inosine and 2-aminoadenosine, which were inactive at 0.5 mM Mg^{2+}, then significantly enhanced activity. Guanosine and 6-thioinosine were neither stimulatory nor inhibitory.

DISCUSSION

This study complements the observations of Huang and Drummond [13] on guinea pig heart slices, which show that adenosine and many of its analogs stimulate cAMP accummulation. They did not observe inhibition of cyclase activity, but several adenosine analogs did inhibit the extent of adenosine activation. The lack of an inhibitory effect on cyclase activity could have been due to poor penetration of the nucleoside into the tissue, to its metabolism after its entry into cells, or to an intracellular [Mg^{2+}] too low to support an inhibitory effect. Our results are also consistent with the observations that intracoronary adenosine infusions in open-chest dogs increase myocardial cAMP levels and the release of this nucleotide into the coronary venous effluent [14, 15].

The present study demonstrates in dog heart muscle the same Mg^{2+}-dependent biphasic effects of adenosine on adenylate cyclase that have been observed in a number of other tissues. Adenosine stimulated cyclase activity at low [Mg^{2+}] but inhibited the enzyme at high divalent cation concentrations. Moreover, a dialkylxanthine inhibited the stimulatory effect but had no influence on inhibition. These results are compatible with the 'two receptor' hypothesis advanced by Londos and Wolff [1] to explain the biphasic effect of adenosine. The most recent refinement of this hypotheses [16] describes two types of cell surface 'R' receptors that bind adenosine and similar ligands having an intact ribose

moiety and are relatively tolerant of base modifications. Stimulatory R_a receptors are found in tissues such as adrenal cells, erythrocytes, and Leydig and neural tumor cells. Inhibitory R_i receptors are found, e.g. in adipocytes. Alkylxanthines antagonize the effects of adenosine acting through these receptors. A second class of adenosine receptors, whose affinity for ligand tolerates ribose but not base modifications, mediates inhibition of the cyclase. The influence of this receptor is evident at higher $[Mg^{2+}]$ and is insensitive to alkylxanthine antagonism [1]. Since inhibition via the R_i receptor has an absolute requirement for GTP, which cannot be met by GMPPNHP [17, 18], the inhibitory effect of adenosine found in the present study, which employed GMPPNHP to activate the cyclase, is probably an expression of P receptors.

The pattern of chemical specificity of the R_a and P receptors observed in the present study and by Huang and Drummond [13] differs in some details from the general rule proposed by Londos and Wolff [1], i.e. that the ribose moiety determines a stimulatory effect, whereas inhibition depends on the adenine base. Several purine-modified ribosides failed to stimulate adenylate cyclase activity at low $[Mg^{2+}]$, whereas all but guanosine and 6-thioinosine stimulated the enzyme at high divalent cation concentrations. Although 3'-deoxyadenosine activates the Leydig cell cyclase and inhibits the platelet enzyme [1], it stimulated the dog heart enzyme at both low and high $[Mg^{2+}]$. Further, both adenine and 8-bromoadenosine, which are inactive in other cell types, stimulated the cardiac enzyme. These exceptions to general rules do not seriously challenge the 'two receptor' hypothesis. Rather, we interpret them as evidence of more subtle differences in the determinants of ligand affinity that may exist between tissues and perhaps also between species. A detailed test of this hypothesis is beyond the scope of the present study.

An earlier report that adenosine only inhibits the adenylate cyclase of guinea pig heart [9] is nonetheless consistent with our results. The experiment cited employed buffers containing 15 mM Mg^{2+}, conditions which our experiments show would elicit only an inhibitory response. Adenosine also antagonizes the beta adrenergic activation of adenylate cyclase in guinea pig hearts perfused by the Langendorf technique and in rat heart slices [9, 19]. Since these experiments concern the modulatory effect of adenosine on the action of a cyclase-stimulating hormone, rather than the direct effects of adenosine itself, they are not comparable to our study. It is not clear whether this antagonistic effect of adenosine on adrenergic activation of cardiac cyclase is mediated by P receptors or by R_i receptors, which seem to coexist with R_a receptors in cultured neuroglial cells [20]. Comparisons of the relative potencies of adenosine and its congeners may reveal the existence of all three types of receptors in heart muscle.

Our observations and those cited previously [13–15] support the notion that adenosine may regulate adenylate cyclase activity and thereby cardiac performance via both stimulatory and inhibitory receptors. Adenosine is released continuously into the cardiac interstitial space [6] and by gaining access to R_a receptors on the cell surface could support basal cyclase activity. Adenosine levels rise rapidly during cardiac ischemia [21]; the rate is rapid enough to explain the 3-fold rise in cardiac cAMP levels found after only 5 sec of ischemia [22], presumably reflecting activation of R_a receptors. Emerging evidence for an intracellular compartment of the adenosine pool [7, 8] admits the possibility that this adenosine, in concert with Mg^{2+}, may also modulate cyclase activity through the P receptor. The possibility that adenosine may attenuate beta adrenergic activation of the cardiac cyclase (? via R_i receptors) is yet a third way that this nucleoside could influence cardiac performance. Thus, adenosine may have cardiac metabolic effects in addition to its well-known effect on coronary vasomotion.

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