Mechanism of Adenosine Inhibition of Catecholamine-Induced Responses in Heart

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SUMMARY. The properties of adenosine inhibition of catecholamine-induced responses were investigated, using an isolated rat heart preparation. Perfusion of hearts with 0.1 μM isoproterenol increased myocardial cAMP content 2.8-fold, activation of cAMP-dependent protein kinase 4.4-fold, phosphorylase a formation 3.4-fold, left ventricular pressure 1.8-fold, rate of ventricular pressure development 2.1-fold, and rate of ventricular relaxation 2.2-fold within 1 minute. When perfused with the isoproterenol, 10 μM adenosine reduced the catecholamine-produced increase in cAMP, cAMP-dependent protein kinase, and phosphorylase by 30-40%, and the elevation in left ventricular pressure and rate of ventricular pressure development by 40-70% within 40 seconds. More than 2 minutes were required for the nucleoside to significantly reduce the isoproterenol-elicited increase in the rate of ventricular relaxation. Perfusion of adenosine alone at concentrations from 0.1 to 10 μM were without effect on the above parameters. Theophylline at 50 μM had no effect alone on the above parameters but blocked the inhibitory actions of adenosine on the isoproterenol-induced responses. In the presence of 15 mM Mg++ adenosine reduced by approximately 56% the 2-fold increase in myocardial membrane adenylate cyclase activity produced by 1 μM isoproterenol without affecting basal or fluoride-stimulated activity. Adenosine also reduced the isoproterenol-induced increase in enzyme activity assayed at 1-2 mM Mg++, a level that more closely approximates the intracellular activity of the ion. The results suggest that physiological concentrations of adenosine attenuate the catecholamine-induced increase in cAMP content, cAMP-dependent protein kinase activation, phosphorylase a formation, and contractile parameters in the working heart, via reducing the β-adrenergic activation of adenylate cyclase. (Circ Res 52:151-160, 1983)

ADENOSINE has been reported to inhibit the positive chronotropic (Sadavongvivad et al., 1977), inotropic (Schrader et al., 1977; Endoh and Yamashita, 1980; Rockoff and Dobson, 1980), and glycogenolytic (Schrader et al., 1977; Dobson, 1978b) effects of the catecholamines in isolated cardiac preparations. The nucleoside attenuates the catecholamine-induced formation of adenosine 3',5'-monophosphate (cAMP) in ventricular slices (Dobson, 1978b) and perfused hearts (Schrader et al., 1977). Adenosine also decreases catecholamine-induced activation of glycogen phosphorylase in ventricular slices (Dobson, 1978b). In this slice preparation, low concentrations (0.1-10 μM) of adenosine, while capable of reducing the catecholamine-produced increase in cAMP, have no effect alone on the cyclic nucleotide (Dobson, 1978b). However, higher concentrations (0.1-1 mM) of the nucleoside cause an increase in tissue cAMP in ventricular slice (Dobson, 1978b) and myocardial chopped cube (Haung and Drummond, 1976) preparations. Adenosine is also known to influence adenylate cyclase in a number of tissues (see review by Fain and Malbon, 1979).

Adenosine is known to be a smooth muscle relaxant (Herlihy et al., 1976) and has been postulated to be an important metabolite involved in the metabolic regulation of coronary blood flow (Rubio and Berne, 1969; Berne et al., 1971). Adenosine is normally present in micromolar amounts in the myocardium (Rubio et al., 1973; Schrader and Gerlach, 1976), and the production of the nucleoside is markedly augmented by myocardial hypoxic (Rubio et al., 1974) and ischemic conditions (Rubio and Berne, 1969; Schrader and Gerlach, 1976).

The β-adrenergic catecholamines are well known for their ability to increase cAMP, glycogenolysis, and contractility in the heart. The catecholamine-elicited activation of adenylate cyclase (Drummond and Duncan, 1970) which catalyzes the increase in cAMP (Dobson et al., 1976) and in turn leads to activation of cAMP-dependent protein kinase (Brown et al., 1978; Dobson, 1978a) appears to be important in both the activation of glycogen phosphorylase (Dobson, 1978a) and perhaps enhanced contractile state via mechanisms involving protein phosphorylation (Wollenberger and Will, 1978; Krebs and Beavo, 1979; Dobson, 1981).

The purpose of this investigation was to determine whether physiological concentrations of adenosine, known to attenuate the catecholamine-induced formation of myocardial cAMP, decrease the isoproterenol-elicited activation of cAMP-dependent protein kinase and phosphorylase in the isolated perfused rat heart. Since very little is known about the properties of adenosine attenuation of catecholamine-induced enhancement of contractile state in whole heart, the relationship between adenosine reduction of isoproterenol-induced cAMP formation and the increase in...
left ventricular pressure, rate of ventricular pressure development, and rate of ventricular relaxation was also determined in the isolated perfused hearts. In addition, the intent was to elucidate the mechanism by which adenosine attenuates the catecholamine-induced contractile and glycogenolytic responses by examining the effects of adenosine on catecholamine activation of adenylate cyclase activity in a crude myocardial membrane preparation.

Methods

Preparations

Perfused Hearts

The isolated perfused heart preparation of the rat used in this study was similar to one described previously (Dobson et al., 1974). Male Sprague-Dawley rats (250-300 g) were obtained from Charles River Breeding Laboratories and maintained on nonmedicated Purina rat chow ad libitum in rooms with a lighting sequence of 12 hours light and 12 hours dark. The rats were anesthetized with 40 mg/kg sodium pentobarbital (ip) and received 500 U of sodium heparin (ip). After 30-40 minutes, hearts were excised and their aortas immediately slipped onto perfusion cannulas that delivered physiological saline solution (PSS) at a constant rate of 10 ml/min. The hearts were paced at 300 contractions/min with a voltage 10% above threshold and pulse duration of 3-5 msec supplied by a Grass S88 stimulator via platinum wire electrodes that were inserted into the right atria. A degassed saline-filled cannula (polyethylene, 1.5 mm i.d., 8.0 cm long) attached to a Statham strain gauge manometer (P23Dd) was inserted into the chamber of the left ventricle through the left atrium and mitral valve for recording intraventricular pressure. The cannula was held in position by a snug tie of surgical silk around the base of the left atrium. In some experiments, a fluid-filled latex balloon was attached to the end of the cannula, which permitted the myocardium to develop force at a constant ventricular volume. The intraventricular balloon was initially inflated with fluid until left ventricular pressure development was maximal. The balloon volume was held constant during the course of an experiment. Similar mechanical results were obtained when a balloon cannula was used in place of the open-tip cannula. The rate of left ventricular pressure development (+dP/dt) and relaxation (−dP/dt) were derived from the left ventricular pressure signal by resistance-capacitance differentiation (Hewlett-Packard derivative preamplifier, model 8814A). All pressure, +dP/dt and −dP/dt data were recorded with a multichannel polygraph (Hewlett-Packard series 7700 recorder). In addition, all mechanical data were digitized and analyzed on a PDP11/40 (Digital) computer.

The hearts were equilibrated for 30 minutes and always perfused with nonrecirculated PSS at 37°C. The PSS was prepared fresh daily and contained in millimoles per liter: NaCl, 118.4; KCl, 4.69; CaCl2, 2.52; NaHCO3, 25.0; MgSO4, 1.18; KH2PO4, 1.18; glucose, 10.0. When gassed with 95% O2-5% CO2, the pH was adjusted before use to 7.4 at 37°C. After the equilibration period, isoproterenol, adenosine, 2-chloroadenosine, theophylline, or a combination of these compounds was suspended in 0.9% NaCl (containing 0.1% sodium metabsulphite) and administered (0.2-0.5 ml/min) into the aortic perfusion cannula just above the heart via a syringe infusion pump (Harvard, model 940). The resulting perfusion cannula PSS concentration of all agents is that which is reported herein. After administration of one or more of the compounds, as indicated in the Results, the hearts were frozen while still attached to the aortic cannula by compression into thin wafers (thickness 1-2 mm) with polished clamps precooled in liquid nitrogen.

Myocardial Membrane Preparation

Hearts were perfused for 20 minutes with oxygenated PSS and frozen as described above. Frozen myocardium (approximately 2.5 g) was broken into small pieces (1-4 mm2) and homogenized in 8 volumes (based on frozen tissue weight) in a buffer containing 10 mM Tris, pH 7.5, 0.5 mM KCl, 2 mM dithiothreitol (DTT), and 0.25 mM sucrose, for three 10-second periods 20 seconds apart with a Polytron (model PT-10) homogenizer. The homogenate was centrifuged at 10,000 g for 20 minutes. The pellet was resuspended in 10 volumes of 10 mM Tris, pH 7.5, 2 mM DTT, and 0.5 mM KCl, passed through four layers of cheesecloth, and centrifuged at 4,200 g for 20 minutes. The resulting pellet was resuspended in 10 mM Tris, pH 7.5, 2 mM DTT and centrifuged as described above. The final pellet was resuspended in 3 volumes of 10 mM Tris, pH 7.5, and 2 mM DTT and stored frozen for 1-4 weeks at −80°C until assayed. The membrane suspensions contained from 2.8 to 3.6 μg protein/ml and the adenylate cyclase activity did not diminish appreciably during 3 months of storage.

Analytical Procedures

Tissue Preparation

The frozen myocardial wafers were freed of ice and pulverized under liquid nitrogen as described previously (Dobson, 1978a). All frozen myocardial wafers and pulverized cardiac muscle samples were stored at −80 to −190°C in screw cap vials. In preparation for extraction procedures frozen cardiac muscle samples were handled at −25°C.

Tissue Extraction and Biochemical Determinations

cAMP. Approximately 25 mg of frozen cardiac muscle were transferred to Daul homogenization tubes (glass-glass size 20, Kontes Glass Co.), homogenized, extracted, and assayed by a method involving the activation of skeletal muscle cAMP-dependent protein kinase as described previously (Dobson, 1978a). The content of cAMP is expressed as pmol/mg protein.

ATP. For adenosine 5'-triphosphate, another 40-60 mg of frozen tissue was acid extracted, neutralized, and assayed enzymatically by a direct fluorometric method as described previously (Dobson, 1978b). The content of ATP is expressed as nmol/mg protein.

Protein Kinase. Myocardial extracts were prepared from approximately 30 mg of frozen myocardial tissue and assayed for cAMP-dependent protein kinase activity according to methods described previously (Dobson, 1978a). The activity of the kinase is based on the phosphorylation of histone and one unit (U) of the enzyme is defined as the amount of enzyme that catalyzes the transfer of 1 pmol of 32P from [γ-32P]ATP to histone in 1 minute at 30°C. Protein kinase is generally expressed as the ratio of the activity in the absence of cAMP to that in the presence of cAMP (2 μM). An increase in the activity ratio is assumed to reflect enzyme dissociation of the holoenzyme into its regulatory and catalytic subunits.

Phosphorylase. Glycogen phosphorylase activity was determined from an extract derived from approximately 10 mg of the frozen cardiac muscle by measuring the production of glucose-1-phosphate either in the absence or presence of adenosine 5'-monophosphate (AMP) according to
methods outlined previously (Dobson, 1978a). One unit of phosphorylase is defined as the amount of enzyme that produces 1 pmol of glucose-1-phosphate from glycogen per minute at 30°C. The results are expressed as the ratio of phosphorylase activity without AMP to phosphorylase activity with AMP (2 nm). An increase in the ratio indicates an increase in the conversion of phosphorylase b to phosphorylase a.

Adenylate Cyclase. The myocardial membrane preparation was assayed for adenylate cyclase after modification of the method of Salomon (1979). Briefly, 20 μg of membrane protein were incubated in 50 μl of buffer that contained 40 mM Tris acetate, pH 7.8, 0.5 to 15 mM MgAc₂, 1 mM DTT, 5.5 mM KCl, 1 mM cAMP, 0.6 mM ATP, 3 μM guanosine 5'-triphosphate (GTP), 2 × 10⁶ cpm [α-32P]ATP, 20 mM phosphoenolpyruvate, 7 μg (~2 μU) pyruvate kinase for 15 minutes at 30°C. The reaction was stopped by adding 50 μl containing 2% sodium dodecyl sulfate, 45 mM ATP, 1.3 mM cAMP, 5 H cAMP (3,000 cpm), and placing the mixture in a boiling water bath for 2 minutes. The formed 32 P cAMP was separated from the [α-32P]ATP by using cation exchange resin AG 50W-X4 (200–400 mesh) and neutral alumina AG 7 (100–200 mesh) columns after the methods of Salomon (1979). Recovery of 3 H cAMP ranged between 65 and 78%. Finally, all results are appropriately corrected for recovery. The activity of adenylate cyclase is expressed as the pmol of 32 P cAMP formed per min per mg membrane protein. All protein contents were determined as described previously (Dobson, 1978b).

Materials

All salts, acids, bases, dextrose, Norit-A (C-176, alkaline decolorizing carbon), ethylenediaminetetraacetate (EDTA), sodium heparin, and solvents were either reagent or certified grade from Fisher Scientific. Tris, I-isoproterenol hydrochloride, type II-A histone, phosphoenolpyruvate, β-glycerophosphate, β-mercaptoethanol, 2-chloroadenosine, theophylline, and dithiothreitol (DTT) were purchased from Sigma. Sodium dodecyl sulfate, cation exchange resin AG 50W-X4, and neutral alumina were obtained from Bio Rad. 3-Isobutyl-1-methylxanthine was purchased from Aldrich and was recrystallized from ethanol. Casein was purchased from Matheson, Coleman and Bell. All cyclic and non-cyclic nucleotides, adenosine, glucose-1-phosphate, adenosine deaminase (from calf intestine), pyruvate kinase (from rabbit muscle), and all enzymes used for phosphorylase and ATP assays and the synthesis (Glynn and Chappell, 1964) of [γ-32P]ATP (a substrate for the cAMP and protein kinase assays) were from Boehringer-Mannheim. Carrier-free (32P) inorganic phosphate, 3 H cAMP (40–60 Ci/mmol), and [α-32P]ATP (10–30 Ci/mmol) were obtained from New England Nuclear. Crystallized bovine albumin was obtained from Pentex Biochemicals.

Statistical Methods

Analysis of variance was performed for paired and unpaired observations (Sokal and Rohlf, 1969). A probability of <0.05 was accepted as indicating a significant difference.

Results

Effect of Adenosine on the Isoproterenol Produced Increase in cAMP, Contractility and Activities of Protein Kinase and Phosphorylase

Adenosine attenuated the isoproterenol produced dose-dependent increase in cAMP content, rate of ventricular pressure development, and activation of both cAMP-dependent protein kinase and phosphorylase in the isolated perfused rat heart (Fig. 1). While 10 nM isoproterenol was required to produce a significant increase in cAMP, cAMP-dependent protein kinase, and rate of ventricular pressure development in the presence of adenosine, only 1 nM isoproterenol was necessary to produce a significant increase in these three parameters in the absence of the nucleus. This indicated that adenosine decreased the apparent sensitivity of the myocardium to low concentrations of isoproterenol. With respect to all four parameters, adenosine decreased the maximum responsiveness to isoproterenol.

CAMP increased from a control of 2.5 to 6.3 pmol/mg protein with 10 nM isoproterenol in the absence of added adenosine but increased to only 5.1 pmol/mg protein when 10 μM adenosine was also present in the perfusing fluid. Although 10 μM adenosine alone had no effect on CAMP content, the nucleoside reduced the 1, 10 (half maximal), and 100 (maximal) nM isoproterenol-induced increase in CAMP by 32, 33, and 36%, respectively.

The rate of ventricular pressure development (+dP/dt, an index of contractility) increased from 2,900 to 5,800 mm Hg/sec with 10 nM isoproterenol and increased to only 3,900 mm Hg/sec with the isoproterenol in the presence of 10 μM adenosine. The adenosine caused a 51–62% reduction of the 10 nM to 1 μM (maximal) isoproterenol-induced enhancement of the rate of ventricular pressure development. The nucleoside alone did not influence this contractile parameter. Isoproterenol (0.1–100 nM) and/or adenosine (0.1–10 μM) had no effect on contraction frequency of the paced hearts (300 contractions/min).

Adenosine alone at 10 μM did not affect the basal activity ratios of both CAMP-dependent protein kinase and phosphorylase. Isoproterenol at 10 nM produced approximately half maximal increase in the activity ratios of CAMP-dependent protein kinase and phosphorylase. Adenosine at 10 μM reduced the isoproterenol-produced increase in activity ratios of CAMP-dependent protein kinase and phosphorylase by 37 and 35%, respectively. The maximal increase in the activity ratios of both CAMP-dependent protein kinase and phosphorylase elicited by 100 nM isoproterenol was reduced by 34–38% when the catecholamine was perfused in the presence of 10 μM adenosine.

CAMP-dependent protein kinase activity determined in the presence of 2 μM CAMP gave an estimate of total enzyme activity (maximal dissociation) which ranged from 36 to 42 U/mg tissue (wet weight). The total activity was not altered by perfusing the hearts with isoproterenol, adenosine, or a mixture of the two compounds at the concentrations employed. Total phosphorylase activity (a + b) determined in the presence of 2 mM AMP ranged from 10 to 13 U/g tissue (wet weight) and was not influenced by isoproterenol and/or adenosine perfusion. The myocardial content of ATP ranged from 43.4 to 48.2 nmol/mg protein and was not significantly altered by perfusion with 100 nM isoproterenol, 10 μM adenosine, or a combination of both compounds. The protein content
The effect of adenosine on the isoproterenol-produced increase in perfused heart cAMP concentration, rate of ventricular pressure development (+dP/dt), and the activity ratios of cAMP-dependent protein kinase (PK) and phosphorylase (PLASE). The hearts were exposed to isoproterenol at the concentrations indicated either in the absence (●) or presence of 10⁻⁶ M adenosine (○) for 2 minutes and the above data were obtained. All hearts that received adenosine were perfused with the nucleoside at 10⁻⁵ M for 5 minutes prior to and during the 2-minute exposure to isoproterenol. Each point presents the mean of five hearts. Asterisks denote a significant difference (P < 0.05) from zero isoproterenol. Daggers denote a significant difference from the value without adenosine at the same isoproterenol concentration. Daggers also denote a significant difference from both zero isoproterenol values. Bars represent ± 1 se.

Effect of Adenosine on the Time-Course of the Isoproterenol-Produced Increase in cAMP, Protein Kinase, and Phosphorylase

Adenosine reduced the isoproterenol-produced increase in cAMP content and activity ratios of cAMP-dependent protein kinase and phosphorylase produced by 10 nM isoproterenol (Fig. 2). Adenosine alone from 10 nM to 10 μM had no effect on these three parameters. However, at 100 μM, the nucleoside—when perfused alone—increased the content of CAMP and the activity ratio of cAMP-dependent protein kinase without influencing the phosphorylase activity ratio. Perfusion of the hearts with adenosine at concentrations that exceeded 50 μM generally caused the heart to become arrhythmic.

Effect of Adenosine on the Time-Course of the Isoproterenol-Produced Increase in cAMP, Protein Kinase, and Phosphorylase

Adenosine reduced the isoproterenol-produced increase in cAMP content and activity ratios of cAMP-dependent protein kinase and phosphorylase within 30 sec (Fig. 3). The attenuating effect of 10 μM adenosine on the 100 nM isoproterenol-produced increase in the activity ratios of cAMP-dependent protein ki-
Dobson/Adenosine-Catecholamine Interaction

**FIGURE 3.** Effect of adenosine on the time course of the isoproterenol-produced increase in cAMP concentration and activity ratios of cAMP-dependent protein kinase (PK) and phosphorylase (PLASE). Some hearts were perfused beginning at zero time with $10^{-8}$ M isoproterenol for the times indicated (○). Other hearts were perfused with the isoproterenol plus $10^{-5}$ M adenosine for the times indicated (□), but the adenosine perfusion was initiated 5 minutes prior to zero time. Each point represents the mean of six hearts. Asterisks denote a significant difference from zero time. Daggers denote a significant difference from the isoproterenol value at the same time. See legend of Figure 1 for further explanation.

nase and phosphorylase persisted for 10 minutes. However, the nucleoside reduced the catecholamine-produced increase in cAMP content for only 2 minutes. The time-course of the attenuation by adenosine was similar whether the nucleoside was administered 5 minutes prior to and during the isoproterenol perfusion (as above), or simultaneously with the isoproterenol starting at zero time.

To determine if the extraction procedure of cardiac tissue for cAMP-dependent protein kinase may have partially activated the enzyme by possibly liberating endogenous cAMP, some tissue samples were homogenized in the presence of charcoal (Norit-A, 10 mg/ml of homogenization solution). Total enzyme activity, as determined by assaying the kinase in the presence of $2 \mu$M cAMP, was not influenced by the presence of charcoal in the homogenization solution. When charcoal was used, the cAMP-dependent protein kinase activity ratio increased from $0.10 \pm 0.02$ to $0.42 \pm 0.04$ with 2 minutes of $100 \mu$M isoproterenol perfusion. If the isoproterenol was administered during the last 2 minutes of a 7-minute perfusion of $10 \mu$M adenosine, the kinase activity ratio increased from $0.11 \pm 0.02$ to $0.28 \pm 0.03$. Compared with the results obtained in Figure 3, these findings indicate that, whereas the charcoal lowered the isoproterenol-induced elevation of the kinase activity ratio, adenosine still attenuated the catecholamine response. This attenuation of cAMP-dependent protein kinase activity ratio persisted for the 10-minute perfusion of isoproterenol plus adenosine.

**Effect of Adenosine on the Isoproterenol-Produced Increase in Ventricular Mechanical Responses**

Since adenosine reduced the isoproterenol dose-dependent increase in the rate of left ventricular pressure development in the isolated rat heart (Fig. 1), it seemed imperative to investigate more closely the effects of the nucleoside on catecholamine-elicited mechanical responses. Adenosine decreased the isoproterenol-produced increase in left ventricular pressure, rate of ventricular pressure development, and rate of relaxation (Fig. 4). Left ventricular pressure increased from $84 \pm 8$ to $152 \pm 12$ mm Hg with $10 \mu$M isoproterenol. Isoproterenol was perfused during the last 2 minutes of a 7-minute perfusion of $10^{-5}$ M adenosine, as indicated, and the three contractile parameters were recorded for 120 seconds. The adenosine administrations were performed 5 minutes prior to and during the 120-second isoproterenol perfusion period. Each curve represents the mean of six hearts in which all three parameters were continuously recorded. Asterisks denote at what time point the $10^{-5}$, $10^{-6}$, or $10^{-7}$ M adenosine plus isoproterenol curves become significantly different from the curves that resulted from perfusing isoproterenol alone. See legend of Figure 1 for further explanation.
isoproterenol within 60 seconds. When administered 5 minutes prior to and during the isoproterenol perfusion, adenosine at 0.1, 1, and 10 μM attenuated the catecholamine response by 39, 62, and 74%, respectively. The isoproterenol increased the rate of ventricular pressure development from 2,650 ± 360 to 5,800 ± 480 mm Hg/sec within 60 seconds and the nucleoside at 1 and 10 μM reduced the response by 49 and 60%, respectively. The isoproterenol increased the rate of relaxation from −1,700 ± 320 to −3,750 ± 430 mm Hg/sec, and this increase was reduced by 48 and 65% with 1 and 10 μM adenosine. Adenosine perfused alone for 7 minutes from 0.1 to 10 μM had no effect on left ventricular pressure, rate of ventricular pressure development, or rate of relaxation.

The increase in left ventricular pressure, rate of ventricular pressure development and rate of relaxation became significantly different from control after 10–15 seconds of 10 nM isoproterenol perfusion (Fig. 4). Adenosine at 0.1 μM significantly reduced the isoproterenol-produced increase in left ventricular pressure and the rate of ventricular pressure development after approximately 40 and 70 seconds, respectively, but did not significantly reduce the increase in the rate of ventricular relaxation. Whereas only 20–32 seconds were required for 1 and 10 μM adenosine to significantly reduce the isoproterenol-produced increase in left ventricular pressure and rate of ventricular pressure development, 38–48 seconds were required to reduce the isoproterenol enhancement of the rate of relaxation.

When 10 μM adenosine perfusion was initiated at zero time simultaneously with 10 nM isoproterenol, rather than 5 minutes before and during the catecholamine administration, the time course and magnitude of the adenosine-induced reductions of the isoproterenol-produced increase in ventricular pressure and rate of ventricular pressure development were similar to that reported above. However, the nucleoside did not significantly reduce the isoproterenol-produced increase in the rate of relaxation under these conditions.

**Effect of 2-Chloroadenosine on Isoproterenol-Produced Increase in cAMP, Protein Kinase, Phosphorylase, and Contractility**

2-Chloroadenosine, an adenosine analogue relatively resistant to deamination by tissue adenosine deaminase (Clark et al., 1952), at a low concentration of 1 μM had no effect by itself on cAMP, cAMP-dependent protein kinase, phosphorylase, and rate of ventricular pressure development, but—like adenosine (10 μM)—decreased the isoproterenol (10 nM)-produced increase in these four parameters (Table 1). The adenosine analogue at 100 μM in the absence of isoproterenol increased cAMP, cAMP-dependent protein kinase, phosphorylase, and the rate of ventricular pressure development. However, the hearts were generally arrhythmic with this high concentration of 2-chloroadenosine. Perfusion of hearts with the high concentration of 2-chloroadenosine plus the isoproterenol further increased cAMP, cAMP-dependent protein kinase, and the rate of ventricular pressure development to levels higher than that observed with isoproterenol alone.

### Table 1

Effect of Adenosine, 2-Chloroadenosine and Theophylline on the Isoproterenol-Produced Increase in Myocardial cAMP, cAMP-Dependent Protein Kinase, Phosphorylase, and Contractility

<table>
<thead>
<tr>
<th>Isoproterenol (10^-6 M)</th>
<th>cAMP (pmol/mg prot)</th>
<th>Protein kinase (-cAMP/+cAMP)</th>
<th>Phosphorylase (-AMP/+AMP)</th>
<th>+dP/dt (mm Hg/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.6 ± 0.2</td>
<td>0.18 ± 0.02</td>
<td>0.18 ± 0.02</td>
<td>2,670 ± 360</td>
</tr>
<tr>
<td>+</td>
<td>6.5 ± 0.3*</td>
<td>0.41 ± 0.03*</td>
<td>0.48 ± 0.02*</td>
<td>5,720 ± 410*</td>
</tr>
<tr>
<td>Adenosine (10^-5 M)</td>
<td>2.7 ± 0.3</td>
<td>0.16 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>2,640 ± 330</td>
</tr>
<tr>
<td>+</td>
<td>5.1 ± 0.4*†</td>
<td>0.28 ± 0.03*†</td>
<td>0.35 ± 0.03*†</td>
<td>3,730 ± 350†</td>
</tr>
<tr>
<td>2-Chloroadenosine (10^-6 M)</td>
<td>2.9 ± 0.3</td>
<td>0.19 ± 0.02</td>
<td>0.20 ± 0.02</td>
<td>2,910 ± 360</td>
</tr>
<tr>
<td>+</td>
<td>5.2 ± 0.4*†</td>
<td>0.30 ± 0.03*†</td>
<td>0.37 ± 0.03*†</td>
<td>3,880 ± 440†</td>
</tr>
<tr>
<td>2-Chloroadenosine (10^-5 M)</td>
<td>4.3 ± 0.5*</td>
<td>0.29 ± 0.03*</td>
<td>0.31 ± 0.03*</td>
<td>4,120 ± 270*</td>
</tr>
<tr>
<td>+</td>
<td>9.4 ± 0.5*†</td>
<td>0.60 ± 0.02††</td>
<td>0.50 ± 0.04†</td>
<td>6,660 ± 360††</td>
</tr>
<tr>
<td>Theophylline (5 X 10^-5 M)</td>
<td>2.4 ± 0.3</td>
<td>0.21 ± 0.03</td>
<td>0.17 ± 0.02</td>
<td>2,750 ± 370</td>
</tr>
<tr>
<td>+</td>
<td>6.2 ± 0.4*‡</td>
<td>0.42 ± 0.02‡</td>
<td>0.50 ± 0.03‡</td>
<td>6,040 ± 420‡</td>
</tr>
<tr>
<td>Theophylline (5 X 10^-5 M) + adenosine (10^-5 M)</td>
<td>2.5 ± 0.4</td>
<td>0.20 ± 0.02</td>
<td>0.18 ± 0.02</td>
<td>2,878 ± 340</td>
</tr>
<tr>
<td>+</td>
<td>6.6 ± 0.4*‡</td>
<td>0.41 ± 0.03‡</td>
<td>0.47 ± 0.03‡</td>
<td>5,820 ± 280‡</td>
</tr>
</tbody>
</table>

All hearts were perfused with either adenosine, 2-chloroadenosine, or theophylline for 7 minutes. Isoproterenol was administered during the last 2 minutes of the perfusion, as indicated. At the end of the perfusion, the rate of left ventricular pressure development (+dP/dt) was recorded and the hearts were frozen for analysis of cAMP, cAMP-dependent protein kinase, and phosphorylase. Values represent the mean ± 1 se for five hearts.

* Significantly different from control minus isoproterenol value.
† Significantly different from control plus isoproterenol value.
‡ Significantly different from adenosine (10^-5 M) or 2-chloroadenosine (10^-6 M) plus isoproterenol value.
Effect of Theophylline on the Inhibition by Adenosine of the Isoproterenol-Produced Increase in cAMP, Protein Kinase, Phosphorylase, and Contractility

Since theophylline at high concentrations is known to be a phosphodiesterase inhibitor (Butcher and Sutherland, 1962) and an antagonist of the adenosine relaxation of vascular smooth muscle (Bunger et al., 1975), the methylxanthine was used in an attempt to block the inhibitory effects of \(10^{-6}\) M adenosine on the isoproterenol (10 nM)-produced enhancement of cAMP, cAMP-dependent protein kinase, phosphorylase, and the rate of ventricular pressure development (Table 1). Theophylline alone at 50 \(\mu\)M had no effect on the four parameters. However, the methylxanthine antagonized the adenosine attenuation of the isoproterenol-elicited increases in cAMP, cAMP-dependent protein kinase, phosphorylase, and contractility.

Effect of Adenosine on Isoproterenol-Sensitive Membrane Adenylate Cyclase

A membrane preparation of adenylate cyclase sensitive to isoproterenol stimulation was inhibited by adenosine when the enzyme was assayed in the presence of 15 mM Mg\(^{2+}\) (Fig. 5). Membrane adenylate cyclase increased from 27 \(\pm\) 2 to 37 \(\pm\) 3 pmol cAMP/min per mg protein with 0.1 \(\mu\)M isoproterenol. The cyclase activity increased maximally to 74 \(\pm\) 6 pmol cAMP/min per mg protein with 10 \(\mu\)M isoproterenol. Adenosine at 10 \(\mu\)M was without effect on basal adenylate cyclase activity but significantly reduced by approximately 56% the increase in adenylate cyclase activity produced by 1 \(\mu\)M isoproterenol. Theophylline at 50 \(\mu\)M did not inhibit the effect of the adenosine. The adenosine was without effect on the 14-fold increase in adenylate cyclase activity produced by 5 mM fluoride.

Whereas the total myocardial level of Mg may be approximated by 15 mM, the intracellular ionic activity of the ion appears to be in the range of 1 mM (Polimeri and Page, 1973). To determine whether adenosine would decrease the isoproterenol activation of membrane adenylate cyclase at a Mg\(^{2+}\) concentration that more closely approximates the presumed intracellular activity of the ion, the assay concentration of Mg\(^{2+}\) was varied between 0.5 and 15 mM (Fig. 6). Isoproterenol (1 \(\mu\)M) produced a marked increase in adenylate cyclase activity at Mg\(^{2+}\) concentrations from 1 to 15 mM. Whereas 10 \(\mu\)M adenosine decreased the isoproterenol-induced increase in adenylate cyclase activity, the attenuation caused by the nucleoside became statistically significant at a Mg\(^{2+}\) concentration of 2 mM or greater.

Discussion

The dose-dependent attenuation by adenosine of the isoproterenol-produced increase in cAMP con-
tent, left ventricular pressure, rate of pressure development, rate of relaxation, and activities of cAMP-dependent protein kinase and phosphorylase generally occurred within 30 seconds and persisted for 2 minutes in the isolated perfused rat heart (Figs. 1–4). It is interesting that the catecholamine-induced metabolic and contractile responses were reduced by physiological concentrations [approximately 1 μm (Rubio and Berne, 1969; Rubio et al., 1973)] of adenosine, whereas the nucleoside alone at these concentrations had no significant effect on these parameters. 2-Chloroadenosine at 1 μm mimicked the inhibitory action of 10 μm adenosine, presumably because this adenosine analogue is relatively resistant to adenosine deaminase, the enzyme that converts adenosine to inosine (Table 1). This suggests that the effective concentration of adenosine necessary to attenuate the catecholamine-induced responses might have been lower than that actually perfused into the coronary circulation. Many of the present results are in agreement with less complete studies reported previously. For example, the nucleoside has been shown to reduce catecholamine-produced increase in cAMP content and activity ratio of phosphorylase in ventricular slices of the rat heart (Dobson, 1978b) and contractility of isolated rat atria (Rockoff, 1980). Others have reported that adenosine decreases the catecholamine-induced elevation in cAMP, contractility, glucose-1-phosphate, and glucose-6-phosphate content in guinea pig hearts (Schrader et al., 1975) and tension development in guinea pig papillary muscles (Endoh and Yamashita, 1980). However, the present study clearly indicates that physiological concentrations may play an important role via the cAMP, cAMP-dependent protein kinase system to modulate catecholamine elicited contractile and glycogenolytic responses in the intact heart.

Since adenosine decreased both the apparent sensitivity and maximum responsiveness to isoproterenol stimulation, the ability of the nucleoside to attenuate appears to be more complex than simple competitive inhibition. Moreover, the half maximum responses to isoproterenol stimulation appeared to be unaffected in the presence of adenosine, suggesting noncompetitive inhibition. This would indicate that adenosine inhibition may not simply be explained by stating that both the nucleoside and catecholamine compete for the same receptor.

A high concentration of adenosine (>0.1 mM) caused an increase in cAMP content and cAMP-dependent protein kinase activity ratio without significantly increasing the activity ratio of phosphorylase (Fig. 2). The reason that phosphorylase was not activated under these conditions is not known. However, the apparent uncoupling may be due to the fact that the nucleoside, at high concentrations, may have elevated the intracellular content of the nucleoside. This in turn may exert an inhibitory effect at a point distal to the elevation of cAMP and activation of cAMP-dependent protein kinase, perhaps by inhibiting phosphorylase kinase and/or activating phosphorylase phosphatase. It has previously been observed that an increase in myocardial cAMP occurs with high concentrations of adenosine in ventricular slice (Dobson, 1978b) and chopped myocardial cubes (Huang and Drummond, 1976). It is difficult to make quantitative assessments of the influence of high concentration of adenosine on contractility per se, because these high concentrations of adenosine caused arrhythmias in the perfused hearts.

Simultaneous exposure of the perfused heart to isoproterenol and adenosine caused a reduction of left ventricular pressure and the rate of ventricular pressure development but not the rate of relaxation. Only when the adenosine was perfused 5 minutes before initiation of the isoproterenol was there a significant reduction of the elevated rate of relaxation (Fig. 4). It appears, from these results, that, whereas 20–38 seconds are required for adenosine to attenuate significantly the increase in left ventricular pressure and rate of ventricular pressure development caused by isoproterenol, 2–5 minutes are required for the nucleoside to reduce the catecholamine-produced increase in the rate of relaxation. A simple explanation for this difference is not apparent.

Generally, adenosine caused a greater reduction of the catecholamine-induced increase in left ventricular pressure and rate of ventricular pressure development than it did of the isoproterenol-elicited elevation of cAMP, cAMP-dependent protein kinase, and phosphorylase. A possible explanation of this difference is that adenosine may attenuate mechanism(s) independent of cAMP that are partially responsible for the catecholamine enhancement of contractile state. Noteworthy might be the fact that adenosine has been reported to reduce the catecholamine-induced slow action potentials seen in K-depolarized atria (Schrader et al., 1975; Belardinelli et al., 1979).

The attenuation of catecholamine responses caused by the nucleoside seems to be specific for adenosine because, as shown in the present study, theophylline antagonized the inhibitory action of adenosine on isoproterenol-produced increase in cAMP, cAMP-dependent protein kinase, and phosphorylase. Theophylline has been reported to block adenosine inhibition of catecholamine-produced enhancement of contractile state in the isolated guinea pig heart (Schrader et al., 1977) and the vasoactive actions of adenosine in vascular smooth muscle (Burger et al., 1975). In addition, other purines at physiological concentrations do not appreciably attenuate the catecholamine induced increase in cAMP (Dobson, 1978b) and contractility (Rockoff and Dobson, 1980). Since the concentration of ATP was not found to change in the present experiments, the availability of ATP for the catecholamine-induced formation of cAMP, enzyme activation, and contractile change was not limiting. Myocardial anoxia has been reported to inhibit completely the catecholamine-induced increase in cAMP in cardiac slices (Dobson, 1978b). However, the inhibition was partially reversed upon addition of adenosine deaminase.
to the bathing medium of the anoxic slices, presumably because the added deaminase degraded some of the endogenously formed adenosine known to accumulate under such conditions.

Adenosine attenuated the dose-dependent isoproterenol-produced increase in myocardial membrane adenylate cyclase activity when the enzyme was assayed in the presence of 15 mM Mg++ (Fig. 5). At this high Mg++ concentration, adenosine inhibition of membrane adenylate cyclase has been observed previously (Schroeder et al., 1977). Adenosine also inhibited the isoproterenol-produced increase in adenylate cyclase activity when the assay Mg++ concentration was lowered to 2 mM (Fig. 6). This concentration of magnesium in the assay more closely approximated the physiological activity level of the divalent cation to which the enzyme is normally exposed (~1 mM, Polimeni and Page, 1973). The findings indicate that adenylate cyclase may be an important point where adenosine exerts its inhibitory action on catecholamine effects in the myocardium. Since adenosine did not inhibit the fluoride-stimulated adenylate cyclase activity, it appears that the nucleoside does not exert its inhibitory effect on the enzyme in its activated form. Rather, it appears that adenosine interferes with the coupling between hormone receptor occupancy and enzyme activation (Fain and Malbon, 1979). It is also conceivable that the nucleoside may interfere with binding of β-adrenergic catecholamines to their presumed receptors in the myocardial cell membrane.

The present results support the idea that adenosine serves as a negative feedback regulator that modulates the catecholamine-induced inotropic and glycogenolytic responses in the heart. Previously, we have suggested that adenosine may serve as an important vasoactive factor involved in the metabolic regulation of coronary blood flow and as a modulator of catecholamine-induced responses in the myocardium (Dobson, 1978b; Rockoff and Dobson, 1980). This notion is based on the observation that the low (physiological) concentrations of adenosine required for its vasoactive actions (1-10 μM, Rubio and Berne, 1969; Berne et al., 1971) are similar, as shown in this study, to those necessary for attenuation of the catecholamine-produced increase in myocardial cAMP content, activation of cAMP-dependent protein kinase, phosphorylase α formation, and several contractile state parameters. The present study suggests that adenosine exerts its inhibitory action by reducing the β-adrenergic activation of adenylate cyclase.

Myocardial adenosine production is enhanced by either a decrease in oxygen supply, such as in the case of coronary artery obstruction (ischemia) or anoxia (Berne et al., 1971; Rubio et al., 1974), or by an increase in oxygen demand observed with increased cardiac performance, e.g., catecholamine stimulation (Wiedmeier and Spell, 1977). The augmented myocardial adenosine levels would in turn attenuate the catecholamine responses, thereby protecting the heart against excessive β-adrenergic stimulation, particularly when the myocardium is exposed to conditions of either decreased oxygen supply or increased oxygen demand. Since glycogen is depleted in a matter of several minutes in the anoxic heart (Mayer et al., 1967), perhaps attenuation by adenosine of catecholamine-elicited glycogenolysis and enhancement of contractile state is beneficial in prolonging survival of the myocardium under such conditions.
INDEX TERMS: Adenylate cyclase • cAMP-dependent protein kinase • Contractile state • Glycogen phosphorylase • Rate of ventricular pressure development
Mechanism of adenosine inhibition of catecholamine-induced responses in heart.
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