Myocardial adenosine A₁-receptor sensitivity during juvenile and adult stages of maturation

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Sawmiller, Darrell R., Richard A. Fenton, and James G. Dobson, Jr. Myocardial adenosine A₁-receptor sensitivity during juvenile and adult stages of maturation. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H627–H635, 1998.—In the heart, endogenous adenosine attenuates the β-adrenergic-elicited increase in contractile performance via activation of adenosine A₁ receptors. It has been recently reported that this function of adenosine becomes more pronounced with myocardial maturation. The purpose of the present study was to determine whether mature hearts possess a greater sensitivity than immature hearts to this antiadrenergic effect of adenosine. Isolated perfused hearts or atria from immature (ca. 23 days) and mature (ca. 80 days) rats were stimulated with isoproterenol (Iso), a β-adrenergic agonist, at 10⁻⁶ M and concomitantly exposed to increasing concentrations of 2-chloro-N⁶-6-cyclopentyladenosine (CCPA), a highly selective and potent adenosine A₁-receptor agonist, from 10⁻¹² to 10⁻⁶ M. CCPA at 10⁻¹⁰–10⁻⁶ M dose dependently reduced the Iso-elicited contractile response more in immature than in mature hearts or atria. At 10⁻⁶ M, CCPA reduced the Iso-elicited contractile response by 103% in immature hearts and by 55% in mature hearts. These effects of CCPA were attenuated by the adenosine A₁-receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine at 10⁻⁷ M. In additional experiments, CCPA exhibited similar effectiveness in reducing the spontaneous heart rate of immature and mature hearts, an effect also mediated by activation of adenosine A₁ receptors. Similar to CCPA, the adenosine A₁-receptor agonist R-N⁶-(2-phenylisopropyl)adenosine reduced the Iso-elicited contractile response more in immature than in mature hearts, albeit with less effectiveness than CCPA. In agreement with these results, CCPA reduced Iso-elicited adenylyl cyclase activity more in immature than in mature hearts. Overall, in contrast with our original hypothesis, these results indicate that immature hearts display greater sensitivity than mature hearts to the antiadrenergic effect of adenosine A₁-receptor activation.

perfused heart; β-adrenergic receptor; contractility; adenylyl cyclase

THE AUGMENTATION OF contractile and metabolic performance of the heart in response to β-adrenergic-receptor stimulation decreases progressively with maturation and aging (1, 10, 15). Adenosine has an antiadrenergic action that, via adenosine A₁-receptor stimulation, reduces β-adrenergic transduction and thereby reduces the contractile and metabolic responsiveness of the heart to β-adrenergic stimulation (7, 30). This action of adenosine is manifested primarily by reduced catecholamine-elicited activation of adenylyl cyclase and protein kinase A, resulting in the attenuation of catecholamine-elicited protein phosphorylation (13, 27). An enhanced antiadrenergic action of adenosine has been shown to play a role in the reduction of β-adrenergic-elicited metabolic and contractile responsiveness that occurs with aging during adulthood (10). In addition, the antiadrenergic effect of adenosine A₁-receptor stimulation is more pronounced in mature than in immature hearts (29).

Enhanced expression of the antiadrenergic action of adenosine in the mature compared with the immature heart could result from 1) greater levels of adenosine in the interstitial fluid or 2) greater adenosine A₁-receptor sensitivity. It was previously shown that venous adenosine concentration and release are greater in mature compared with immature hearts during β-adrenergic stimulation (29). Therefore, greater levels of interstitial adenosine in the mature heart could play a role in the greater expression of adenosine A₁-receptor activity at this stage of development. The purpose of the present study was to determine whether mature hearts display greater sensitivity than immature hearts to adenosine A₁-receptor stimulation. This was determined by assessing the effectiveness of the adenosine A₁-receptor agonists 2-chloro-N⁶-6-cyclopentyladenosine (CCPA) and R-N⁶-(2-phenylisopropyl)adenosine (R-PIA) in reducing the contractile response elicited by the β-adrenergic agonist isoproterenol in isolated immature and mature hearts. In addition, this study compared the effectiveness of CCPA in reducing the isoproterenol-elicited contractile response in isolated immature and mature atria. The effectiveness of this agonist in reducing the spontaneous heart rate, an action mediated by adenosine A₁ receptors, was also determined in immature and mature hearts. Finally, the effectiveness of this agonist in reducing isoproterenol-elicited adenylyl cyclase activity was determined in immature and mature ventricular membranes. In contrast with our original hypothesis, the results from this study indicate that immature hearts have greater sensitivity than mature hearts to the antiadrenergic effect of adenosine A₁-receptor stimulation.

MATERIALS AND METHODS

Preparation of isolated perfused hearts. Male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were used for these experiments. The rats were placed into two groups: immature (age 23 ± 0.3 days; wt 50 ± 1 g) and mature (age 80 ± 4 days; wt 321 ± 15 g). All animals were anesthetized intraperitoneally with 40 mg/kg pentobarbital. The hearts were excised, rinsed in ice-chilled physiological saline solution (PSS), and immediately perfused with nonrecirculated PSS at 37°C via an aortic cannula at constant perfusion pressures of 50 or 70 mmHg for the immature or mature hearts, respectively. These perfusion pressures correspond proportionately with the in vivo aortic pressures of rats in the age groups used in this study, which are 30% lower in immature than in mature rats (33). Therefore, the hearts...
were perfused at the flow rates that permit close approximation of the normal physiological condition. The coronary flows for the immature and mature hearts were 17 ± 1 and 16 ± 1 ml/min g⁻¹, respectively. The PSS contained (in mM) 120 NaCl, 4.7 KCl, 2.5 CaCl₂, 25 NaHCO₃, 2.1 MgSO₄, 1.2 K₂HPO₄, 10 glucose, and 0.57 ascorbic acid. The pH was maintained at 7.4 by gassing the PSS with 95% O₂-5% CO₂.

After an equilibration period of 20–30 min, the hearts were constant-flow perfused at their predetermined natural flow rates and paced at 10–20% above their unpaced rates of 277 ± 10 and 259 ± 19 contractions/min for the immature and mature hearts, respectively. Pacing was accomplished with a stimulator (model SD9, Grass Instruments, Quincy, MA) via platinum wire electrodes inserted into the right atrium and the superficial layer of the upper left ventricle. The voltage was set at 10% above threshold, and the pulse duration was set at 5 ms. Developed left ventricular pressure (LVP) was determined using a water-filled, latex balloon-tipped polyethylene cannula (1.5 mm ID) attached to a strain-gauge manometer (model P23 D3, Spectro Med/ Omeada, Rockaway, NJ). The balloon (Hugo Sachs, Hugstetten, Germany), appropriate in size for each age group, was inserted into the lumen of the left ventricle, and diastolic pressure was set at 5–10 mmHg. LVP was assessed throughout these infusions. Each atrium was mounted vertically in a drainable muscle bath as described previously (8). Briefly, each atrium was removed and discarded. Hearts from each heart were then each heart was perfused with PSS containing 10⁻⁸ M isoproterenol and CCPA. In the absence of CCPA, isoproterenol produced a contractile response that was sustained for at least 30 min (data not shown). In series 2, the effect of CCPA on isoproterenol-elicited contractile performance was determined in isolated immature and mature left atria. Each atrial preparation was stimulated with isoproterenol at 10⁻⁸, 10⁻⁶ M for 2–3 min and then treated with CCPA at sequentially increasing concentrations starting at 10⁻¹² M and ending at 10⁻⁶ M. To confirm that the effects of CCPA were mediated by stimulation of the adenosine A₁ receptor, some atria were pretreated with DPCPX at 10⁻⁷ M before treatment with isoproterenol and CCPA. The concentrations of isoproterenol (10⁻⁶, 10⁻⁴ M) utilized in these experiments were varied to produce between 50 and 100% of the maximal contractile response to isoproterenol (8). However, the effects of CCPA on the isoproterenol-elicited contractile response did not vary with the concentration of isoproterenol utilized, and therefore, the results utilizing these different doses of isoproterenol were pooled. In series 3, the effect of CCPA on spontaneous heart rate was determined in immature and mature hearts. In the absence of isoproterenol, CCPA was infused in a cumulative manner to achieve final PSS concentrations between 10⁻¹² and 10⁻⁶ M, and heart rate was determined throughout these infusions.

In series 4, the effect of adenosine A₁-receptor stimulation with R-PIA on isoproterenol-elicited ventricular contractile performance was determined in immature and mature hearts. Each heart was perfused with PSS containing 10⁻³ M isoproterenol, and then R-PIA was infused in a cumulative manner to achieve final PSS concentrations of 10⁻¹² to 10⁻⁶ M. Myocardial contractility was continuously recorded throughout these infusions. Series 5 experiments were performed to determine whether the ability of R-PIA to reduce the isoproterenol-elicited contractile response was limited by simultaneous activation of stimulatory adenosine A₂a receptors. This was accomplished with hearts initially stimulated with isoproterenol and then subsequently treated with R-PIA at 10⁻⁸ M and the adenosine A₁-receptor antagonist 8-(3-chlorostyryl)caffeine (CSC) at 10⁻⁸ and 10⁻⁷ M in a cumulative manner.

In series 6, the effect of CCPA on isoproterenol-elicited adenyl cyclase activity was determined in cellular membranes isolated from ventricles of immature and mature hearts. The system utilized for measurement of adenyl cyclase activity has been described previously (26). This system minimizes the formation of adenosine within the assay and reduces the presence of any adenosine inherent in the membrane preparation. The hearts from immature or mature rats were initially perfused with 3 or 10 ml of ice-cold 0.9% NaCl, respectively, to wash out any blood remaining in the heart. The atria were removed and discarded. Hearts were then frozen with aluminum clamps prechilled in liquid nitrogen and were stored in liquid nitrogen until assayed. On the day of the assay, ventricular membranes were prepared as follows. Each individual ventricle was thawed, minced in ice-cold saline, and transferred to a centrifuge tube containing 10 ml of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, which contained (in mM) 10.0 HEPES, 1.0 EDTA, 1.0 dithiothreitol (DTT), and 0.1 benzamidine and 10.0 μg/ml soybean trypsin inhibitor, pH 7.4. The suspension was homogenized twice for 15 s with a Polytron using a PT-10 generator at setting 6 with 15 s between homogenizations.
The homogenate received two strokes with a glass-Teflon Potter homogenizer and then was diluted with 4.7 ml of 1.25 M sucrose in HEPES buffer. This mixture was vortexed and centrifuged at 1,000 g for 15 min at 4°C. The supernatant was filtered through four layers of cheesecloth, and 14.5 ml of HEPES buffer without sucrose was added. The mixture was centrifuged at 45,000 g for 45 min at 4°C, and the pellet was resuspended in 40 mM HEPES (pH 7.4) to yield 3–5 mg protein/ml.

For determination of adenylyl cyclase activity, ventricular membranes (10–20 µg protein) were incubated for 10 min at 30°C in 50 µl of buffer containing (in mM) 40 HEPES, 100 NaCl, 5.0 MgCl₂, 5.5 KCl, 0.1 2',3'-dideoxycytidine 3',5'-cyclic monophosphate (dcAMP), 0.1 dATP, 0.01 GTP, 2.0 phosphoenolpyruvate, 1.0 DTT, 0.1 ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), and 1.0 ascorbic acid and 2 U pyruvate kinase, 0.25 U adenosine deaminase, and ∼2 × 10⁶ counts/min [α-32P]dATP, pH 7.4. Isoproterenol (10⁻⁷ M) and CCPA (10⁻⁸–10⁻⁶ M) were used as indicated. The reaction was stopped by adding 50 µl of a stop solution containing 2% sodium dodecyl sulfate (SDS), 45 mM ATP, 1.3 mM cAMP, and [³H]dATP (∼4,000 counts/min) and boiling for 2 min. The formed [α-32P]dcAMP was separated from the [α-32P]dATP by sequential chromatography using columns of cation exchange resin AG-50W-X4 (200–400 mesh) and neutral alumina AG-7 (100–200 mesh) after the methods of Salomon (28). All results were corrected for column recovery of [³H]dcAMP, which ranged between 60 and 90%. The protein levels were assessed by a bidichroic acid technique (Pierce, Rockford, IL) using bovine serum albumin as a standard. The activity of the adenylyl cyclase is expressed as picomoles of [α-32P]dcAMP formed per minute per milligram of protein.

Animals. The animals in this study were maintained and used in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals prepared by the National Research Council and the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Materials. Buffer salts, glucose, and ascorbic acid were certified grade from Fisher Scientific (Boston, MA) or J.T. Baker (Phillipsburg, NJ). CCPA, R-PIA, DPCPX, and GTP were purchased from Research Biochemicals International (Natick, MA). Phosphoenolpyruvate, pyruvate kinase, adenosine deaminase, adenosine, ATP, dATP, and GTP were purchased from Boehringer Mannheim (Indianapolis, IN). Isoproterenol, DTT, HEPES, cAMP, dCMP, EDTA, EGTA, dimethyl sulfoxide (DMSO), succrose, benzamidine, and soybean trypsin inhibitor were obtained from Sigma Chemical (St. Louis, MO). SDS, AG-50W-X4, and AG-7 were purchased from Bio-Rad (Richmond, CA). [α-32P]dATP (800 Ci/mmol) was obtained from Amersham (Arlington Heights, IL), and [³H]dcAMP (5.2 Ci/mmol) was purchased from ICN Pharmaceuticals (Irvine, CA). Pentobarbital was obtained from Abbott Laboratories (North Chicago, IL).

Stock solutions of isoproterenol were prepared in 0.1% (wt/vol) sodium metabisulfite. CCPA, R-PIA, DPCPX, and CSC were prepared in DMSO as stock solutions of 10⁻² M (CCPA, R-PIA, CSC) or 10⁻³ M (DPCPX). All of these solutions were diluted in PSS at the concentrations indicated and used immediately.

Data analysis and statistical treatments. Contractile performance was assessed as +dP/dtₐₘₙₓ and presented for each concentration of CCPA or R-PIA, as a percentage of the maximal isoproterenol-elicited contractile response determined in the absence of the adenosine receptor agonists. In particular, in the presence of CCPA or R-PIA, contractile performance was calculated utilizing the following formula:

\[(B – A)/(C – A) \times 100,\]

where A is the basal level of contractile performance determined before treatment with isoproterenol, B is the level of contractile performance in the presence of each concentration of CCPA or R-PIA plus isoproterenol, and C is the maximal level of contractile performance elicited by isoproterenol before treatment with CCPA or R-PIA. Likewise, adenylyl cyclase, in the presence of CCPA, was presented as a percentage of the maximal isoproterenol-elicited level of adenylyl cyclase activity determined in the absence of CCPA. All data are presented as means ± SE. Where SE bars cannot be seen in Figs. 1–7, the SE is confined within the area of the symbol. Data analysis was conducted by analysis of variance for multiple groups of samples or Student’s t-test for paired samples. P < 0.05 was accepted as indicating a statistically significant difference. The apparent concentrations of CCPA or R-PIA required to reduce the isoproterenol-elicited contractile response or heart rate by 50% of the maximal level (IC₅₀) were determined with GraphPad InPlot (GraphPad Software, San Diego, CA).

RESULTS

Adenosine A₁-receptor agonist CCPA was more effective in reducing isoproterenol-elicited contractile response in immature than in mature hearts. Isoproterenol at 10⁻⁸ M increased +dP/dtₐₘₙₓ 40% in the immature hearts and 53% in the mature hearts. Isoproterenol also increased −dP/dtₐₘₙₓ 74% in the immature hearts and 77% in the mature hearts (Table 1). In the immature heart, CCPA reduced the isoproterenol-elicited contractile response in a dose-dependent manner, starting between 10⁻¹¹ and 10⁻¹⁰ M and completely eliminating the contractile response at 10⁻⁶ M (Fig. 1). In the mature heart, CCPA reduced the isoproterenol-elicited contractile response, starting between 10⁻⁸ and 10⁻⁷ M and producing 55% reduction at 10⁻⁶ M. Overall, CCPA was ∼100-fold less effective in reducing the isoproterenol-elicited contractile response in the immature than in the mature heart. The apparent IC₅₀ values of CCPA to reduce the contractile response were 1.8 × 10⁻⁹ M for the immature heart and 4.0 × 10⁻⁷ M for the mature heart.

Adenosine A₁-receptor antagonist DPCPX attenuated the ability of CCPA to reduce the isoproterenol-elicited contractile response in immature and mature hearts. In the immature heart, isoproterenol at 10⁻⁶ M in the presence of DPCPX at 10⁻⁷ M increased +dP/dtₐₘₙₓ 61% (Table 1). CCPA at 10⁻⁶ M reduced this increase in contractility by 42% (Fig. 2), which is significantly less than the ability of 10⁻⁶ M CCPA to reduce the isoproterenol-elicited contractile response in the absence of DPCPX (103%). The apparent IC₅₀ value of CCPA to reduce the isoproterenol-elicited contractile response in the presence of DPCPX was 4.1 × 10⁻⁶ M, which is significantly greater than the IC₅₀ value for CCPA to reduce the contractile response in the absence of DPCPX (1.8 × 10⁻⁹ M).

In the mature heart, long infusion durations of isoproterenol elicited ventricular fibrillation in the presence of DPCPX. Therefore, it was not possible to determine the dose-dependent effect of CCPA on the isoproterenol-elicited contractile response in mature hearts pretreated with this antagonist. To circumvent this problem, the effect of a 3-s infusion of 10⁻⁷ M...
isoproterenol on ventricular contractility was determined in the absence or presence of CCPA at 10^{-8} M and then determined in the presence of both CCPA at 10^{-8} M and DPCPX at 10^{-7} M (Fig. 3). Isoproterenol at 10^{-7} M elicited a smaller contractile response in the presence than in the absence of CCPA, and DPCPX significantly reversed this depressant effect of CCPA. This infusion of isoproterenol increased contractility 60 and 62% in the absence of CCPA, 19% in the presence of CCPA, and 35% in the presence of both CCPA and DPCPX. The ability of two infusions of isoproterenol to produce similar increases in contractility in the absence of CCPA demonstrates the stability of the preparation.

Adenosine A1-receptor agonist CCPA was more effective in reducing the isoproterenol-elicited contractile response in immature than in mature left atria. In the absence of CCPA, isoproterenol increased peak contractile force by 191% in the immature left atria and by 218% in the mature left atria (Table 1). At 10^{-7} and 10^{-6} M, CCPA reduced the isoproterenol-elicited contractile response by 101 and 114%, respectively, in the immature.

Table 1. Left ventricular and atrial contractile performance of immature and mature hearts before and during treatment with isoproterenol and before treatment with CCPA or PIA

<table>
<thead>
<tr>
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<th>Immature Hearts</th>
<th>Mature Hearts</th>
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<tr>
<td></td>
<td>Ventricular contractility</td>
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<tr>
<td>Before CCPA, without DPCPX</td>
<td>+dP/dt_{max}</td>
<td>2,320 ± 190</td>
<td>8</td>
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<tr>
<td></td>
<td>dP/dt_{max}</td>
<td>1,170 ± 90</td>
<td></td>
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<tr>
<td>Before CCPA, with DPCPX</td>
<td>+dP/dt_{max}</td>
<td>2,500 ± 250</td>
<td>6</td>
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<tr>
<td></td>
<td>dP/dt_{max}</td>
<td>1,230 ± 120</td>
<td></td>
</tr>
<tr>
<td>Before PIA</td>
<td>+dP/dt_{max}</td>
<td>1,970 ± 320</td>
<td>5</td>
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<tr>
<td></td>
<td>dP/dt_{max}</td>
<td>1,020 ± 160</td>
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<tr>
<td></td>
<td>Atrial contractility</td>
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<tr>
<td>Before CCPA, without DPCPX</td>
<td>PCF</td>
<td>0.045 ± 0.014</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>PCF</td>
<td>0.031 ± 0.010</td>
<td>6</td>
</tr>
<tr>
<td>Before CCPA, with DPCPX</td>
<td>PCF</td>
<td>0.131 ± 0.018*</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>PCF</td>
<td>0.102 ± 0.026*</td>
<td>6</td>
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Values are means ± SE of 8 immature and 5 mature hearts. *Significant difference from corresponding value in absence of CCPA.
Adenosine A1-receptor agonist CCPA was equally effective in reducing heart rate in immature and mature hearts. The basal spontaneous heart rates of the immature and mature hearts in this series of experiments were 258 ± 11 and 236 ± 14 contractions/min, respectively. CCPA at 10^{-8}, 10^{-7}, and 10^{-6} M reduced heart rate by 34, 56, and 75%, respectively, in the immature hearts and by 28, 48, and 79%, respectively, in the mature hearts (Fig. 5). The apparent IC_{50} values for CCPA to reduce heart rate were 6.9 ± 0.3 M for the immature hearts and 8.2 ± 0.8 M for the mature hearts.

Adenosine A2a-receptor agonist R-PIA was more effective in reducing the isoproterenol-elicited contractile response in immature than in mature hearts. In the absence of R-PIA, isoproterenol (10^{-8} M) increased +dP/dt_{max} by 45% in the immature hearts and by 74% in the mature hearts (Table 1). R-PIA at 10^{-6}, 10^{-7}, and 10^{-8} M reduced the isoproterenol-elicited contractile response by 28, 61, and 84%, respectively, in the immature hearts and by 20, 38 and 55%, respectively, in the mature hearts (Fig. 6). Only the effect of R-PIA at 10^{-6} M was significantly greater in immature than in mature hearts. The apparent IC_{50} values for R-PIA to reduce the isoproterenol-elicited contractile response were 4.1 ± 0.8 M for the immature hearts and 5.4 ± 0.7 M for the mature hearts. These IC_{50} values were not statistically different.

Adenosine A_{2a}-receptor blockade did not uncover adenosine A1-receptor activity at a low dose of R-PIA in the immature heart. R-PIA was much less effective than CCPA in reducing the isoproterenol-elicited contractile response in the immature heart. For example, R-PIA at 10^{-8} M only subliminally reduced the contractile response in the immature heart (Fig. 6), whereas CCPA

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**Fig. 3.** Effect of CCPA on isoproterenol-elicited contractile response of mature hearts in absence (−) or presence (+) of DPCPX. Absolute values of +dP/dt_{max} are shown before (open bars) and during (hatched bars) isoproterenol treatment in absence or presence of CCPA at 10^{-6} M or during presence of both CCPA at 10^{-6} M and DPCPX at 10^{-7} M. In these experiments, isoproterenol-elicited contractile response was induced by 3-s infusions of 0.1 ml of 10^{-7} M isoproterenol. Values are means ± SE of 6 mature hearts. *Significant difference from immediately preceding value in absence of isoproterenol. †Significant difference from preceding isoproterenol-elicited level of contractility determined in absence of CCPA or DPCPX. ‡Significant difference from preceding isoproterenol-elicited level of contractility determined in presence of CCPA and absence of DPCPX.

**Fig. 4.** Effect of CCPA on isoproterenol-elicited contractile response of immature and mature left atria in absence (S) or presence (T) of DPCPX at 10^{-7} M. Contractile performance was monitored as peak contractile force and presented as a percentage of initial isoproterenol-elicited contractile response determined in absence of CCPA (C). Values are means ± SE of 5 immature and 7 mature atria in absence of DPCPX and 6 immature and 6 mature atria in presence of DPCPX. *Significant difference from corresponding value in mature atria. †Significant difference from corresponding value in absence of DPCPX.

**Fig. 5.** Effect of CCPA on spontaneous heart rate of immature and mature hearts. Heart rate is presented for each concentration of CCPA as a percentage of basal spontaneous heart rate determined in absence of CCPA (C). Values are means ± SE of 5 immature and 5 mature hearts. *Significant difference from corresponding value in absence of CCPA.
Fig. 6. Effect of R-N6-(2-phenylisopropyl)adenosine (PIA) on contractile response elicited by isoproterenol (10⁻⁸ M) in immature and mature hearts. Contractile performance was monitored as +dP/dt max and presented, for each concentration of PIA, as a percentage of initial isoproterenol-elicited contractile response determined in absence of PIA (C). Values are means ± SE of 5 immature and 4 mature hearts. * Significant difference from corresponding value in absence of PIA. † Significant difference from corresponding value in mature heart.

Table 2. Adenylyl cyclase activity in immature and mature myocardial membranes before and during treatment with Iso alone or in presence of CCPA

<table>
<thead>
<tr>
<th>Protein</th>
<th>Immature</th>
<th>Mature</th>
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<tr>
<td>Control</td>
<td>8.24 ± 1.19</td>
<td>4.42 ± 0.78</td>
</tr>
<tr>
<td>Iso 8 M</td>
<td>13.35 ± 2.08*</td>
<td>8.45 ± 1.58*</td>
</tr>
<tr>
<td>CCPA 10⁻⁸ M</td>
<td>9.70 ± 2.17</td>
<td>8.79 ± 1.75*</td>
</tr>
<tr>
<td>10⁻⁷ M</td>
<td>9.12 ± 1.80</td>
<td>7.28 ± 1.80*</td>
</tr>
<tr>
<td>10⁻⁶ M</td>
<td>9.08 ± 1.54</td>
<td>6.73 ± 1.50*</td>
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</table>

Values are means ± SE of 5 immature and 6 mature hearts. Adenylyl cyclase activity is expressed as picomoles of 2-deoxyadenosine 3',5'-cyclic monophosphate formed per minute per milligram of protein. Concentration of Iso utilized was 10⁻⁷ M. * Significant difference from control.

DISCUSSION

Adenosine A₁-receptor sensitivities in immature and mature myocardial contractile tissue. The present study indicates that immature hearts display greater sensitivity than mature hearts to the antiadrenergic effects of adenosine A₁-receptor stimulation. Previously, we reported that the antiadrenergic effect of endogenous adenosine becomes more pronounced as the heart matures (29). As a logical extension, the present study was performed to determine whether mature hearts exhibit greater sensitivity than immature hearts to exogenous adenosine A₁-receptor stimulation. It was a surprise to find that the reverse is true. The selective adenosine A₁-receptor agonist CCPA was more effective in reducing the β-adrenergic-elicited ventricular contractile response in immature than in mature hearts (Fig. 1). CCPA was also more effective in reducing the β-adrenergic-elicited contractile response in immature than in mature left atria (Fig. 4). In fact, in the immature atria, CCPA at 10⁻⁶ M abolished the isoproterenol-elicited contractile response and reduced the basal level of contractility, whereas, in the mature heart.

Fig. 7. Effect of CCPA on isoproterenol-elicited adenylyl cyclase activity in ventricular membranes from immature and mature hearts. Adenylyl cyclase activity, at each concentration of CCPA, is presented as a percentage of initial increase in adenylyl cyclase activity elicited by isoproterenol in absence of CCPA. Values are means ± SE of membrane preparations from 5 immature and 6 mature hearts. * Significant difference from corresponding value in absence of CCPA. † Significant difference from corresponding value in mature heart.

at 10⁻⁸ M reduced this contractile response by 63% (Fig. 1). It was considered that the ability of R-PIA to reduce the contractile response was limited by simultaneous activation of adenosine A₂a receptors, which may increase ventricular contractility (9, 31) and thereby counteract the antiadrenergic effect of adenosine A₁-receptor activation. Four immature hearts were stimulated with 10⁻⁸ M isoproterenol and then sequentially treated with R-PIA at 10⁻⁸ M together with the adenosine A₂a-receptor antagonist CSC at 10⁻⁸ M and then 10⁻⁷ M in a cumulative manner. R-PIA at 10⁻⁸ M did not reduce the isoproterenol-elicited contractile response in the absence or presence of CSC. Left ventricular +dP/dt max was 2,350 ± 320 mmHg/s before isoproterenol, 3,410 ± 423 mmHg/s during the treatment with isoproterenol, 3,550 ± 500 mmHg/s during the treatment with R-PIA plus isoproterenol, and 3,490 ± 500 and 3,720 ± 560 mmHg/s during the treatments with CSC at 10⁻⁸ and 10⁻⁷ M, respectively, plus R-PIA and isoproterenol. Thus adenosine A₂a-receptor antagonism with CSC did not uncover an inhibitory action of R-PIA to reduce the isoproterenol-elicited contractile response in the immature heart.

Adenosine A₂a-receptor agonist reduced isoproterenol-elicited adenylyl cyclase activity more in immature than in mature ventricles. Isoproterenol at 10⁻⁷ M increased adenylyl cyclase activity of isolated ventricular membranes by 62% in immature hearts and by 91% in mature hearts (Table 2). CCPA at 10⁻⁸, 10⁻⁷, and 10⁻⁶ M reduced the isoproterenol-elicited level of adenylyl cyclase activity by 72, 102, and 84%, respectively, in immature hearts and by 8.5, 29.1, and 42.8%, respectively, in mature hearts (Fig. 7). The apparent IC₅₀ values for CCPA to reduce the isoproterenol-elicited level of adenylyl cyclase activity were 7.9 × 10⁻⁹ M for the immature heart and 1.3 × 10⁻⁶ M for the mature heart.
atria, this same concentration of CCPA abolished the isoproterenol-elicited contractile response only. This high concentration of CCPA may exert direct as well as antiadrenergic effects to reduce contractility in the immature atria. In addition, the adenosine A<sub>1</sub>-receptor agonist R-PIA was more effective in reducing the β-adrenergic-elicited contractile response in immature than in mature hearts (Fig. 6). The enhanced expression of the antiadrenergic effect of endogenous adenosine in the mature heart, shown in our previous study (29), is therefore most likely due to greater levels of interstitial adenosine in mature compared with immature hearts during β-adrenergic stimulation. If this is the case, then myocardial adenosine receptors may possibly desensitize during maturation. This desensitization may be caused in turn by receptor uncoupling, phosphorylation or sequestration, or a decrease in the level of inhibitory G (G<sub>i</sub>) protein.

The antiadrenergic effects of CCPA in the isolated heart and atria were attenuated by the selective adenosine A<sub>1</sub>-receptor antagonist DPCPX, confirming that these effects of CCPA were mediated by activation of adenosine A<sub>1</sub> receptors (Figs. 2–4). Although DPCPX only attenuated the antiadrenergic response to CCPA, it is possible that the antagonism was incomplete because of the relatively low concentration of DPCPX utilized. A higher dose of DPCPX than that used in the present study, for example, 10<sup>-6</sup> M, might have blocked more of the response to CCPA, revealing greater activation of adenosine A<sub>1</sub> receptors by CCPA. However, doses of DPCPX higher than 10<sup>-7</sup> M could not be used in this study because this would have introduced levels of the vehicle DMSO > 0.01%. In preliminary studies, levels of DMSO > 0.01% produced ventricular depression (unpublished observations) and thereby might affect the contractile responses to isoproterenol or CCPA.

Effect of adenosine A<sub>1</sub>-receptor stimulation on β-adrenergic-elicited adenyl cyclase activity. The antiadrenergic effect of adenosine A<sub>1</sub>-receptor stimulation is known to be mediated by reduced β-adrenergic-elicited stimulation of adenyl cyclase activity, which reduces β-adrenergic-elicited cAMP formation, protein kinase A activation, and myocardial protein phosphorylation (13, 27). Because adenosine A<sub>1</sub>-receptor stimulation reduced β-adrenergic-elicited contractile performance more in immature than in mature hearts, it is expected that adenosine A<sub>1</sub>-receptor stimulation would also reduce β-adrenergic-elicited adenyl cyclase activity more in immature than in mature hearts. The present study supports this expectation (Table 2 and Fig. 7). Whereas CCPA at 10<sup>-8</sup>–10<sup>-6</sup> M reduced isoproterenol-elicited adenyl cyclase activity in a dose-dependent fashion in the mature heart, CCPA at 10<sup>-8</sup> to 10<sup>-6</sup> M completely abolished the isoproterenol-elicited adenyl cyclase activity in the immature heart. These findings are complemented by a recent report (6) that the density of adenosine A<sub>1</sub> receptors is greater in immature than in mature hearts.

Adenosine A<sub>1</sub>-receptor sensitivities in immature and mature myocardial pacemaking tissue. It is interesting to note that CCPA elicited a similar reduction of spontaneous heart rate in immature and mature hearts (Fig. 5). These findings agree with that of previous studies showing that R-PIA elicits a similar reduction of spontaneous heart rate in immature and mature rat hearts (29) and that adenosine elicits a similar reduction of atrioventricular nodal cycle length in newborn and adult rabbit hearts (32). Thus the atrial tissue controlling heart rate appears to have similar sensitivity to adenosine A<sub>1</sub>-receptor activation in immature and mature hearts. It is not known why adenosine A<sub>1</sub>-receptor sensitivity of the atrial tissue controlling heart rate does not decrease with maturation, similar to the adenosine A<sub>1</sub> receptors of the atrial or ventricular tissues controlling contractility. However, one possibility is that adenosine levels near the atrial pacemaking cells do not increase with maturation, and therefore, there is no cause for desensitization of the adenosine A<sub>1</sub> receptors in these cells. In addition, it is possible that the pacemaking cells of the atria are not as sensitive to increases in the endogenous adenosine level, resulting in reduced desensitization of the adenosine A<sub>1</sub> receptors.

Differences between CCPA and R-PIA as adenosine A<sub>1</sub>-receptor agonists. The adenosine A<sub>1</sub>-receptor agonist R-PIA was much less effective than CCPA in reducing the β-adrenergic-elicited contractile response in the immature heart (Figs. 1 and 6). In particular, at 10<sup>-8</sup> M, R-PIA was remarkably ineffective as an antiadrenergic agent compared with CCPA. The results may be explained by less selectivity of R-PIA compared with that of CCPA for binding to adenosine A<sub>1</sub> versus A<sub>2</sub> receptors. In rat brain membranes, R-PIA was only 100-fold selective for binding to adenosine A<sub>1</sub> versus A<sub>2</sub> receptors (5), compared with 10,000-fold selectivity for CCPA (21). In addition, R-PIA is known to bind to adenosine A<sub>3</sub> receptors (20), which may further distinguish R-PIA as having less selectivity than CCPA for adenosine A<sub>1</sub> receptors. Activation of myocardial adenosine A<sub>2</sub> receptors is known to increase contractility of isolated mammalian and avian cardiomyocytes (9, 31). This stimulation of contractility might overcome the antiadrenergic effect of adenosine A<sub>2a</sub>-receptor activation by R-PIA. In a recent study, the adenosine A<sub>2a</sub>-receptor antagonist CSC enhanced the ability of R-PIA to reduce the isoproterenol-elicited contractile response in chick ventricular myocytes (19). In the present study, however, R-PIA at 10<sup>-8</sup> M did not reduce the isoproterenol-elicited contractile response in the absence or presence of CSC in the immature rat heart. Therefore, the lack of effectiveness of R-PIA at 10<sup>-8</sup> M does not appear to be due to a counteractive effect of adenosine A<sub>2a</sub>-receptor activation. The ability of R-PIA to activate adenosine A<sub>2</sub> receptors, and thereby counteract the antiadrenergic effect of R-PIA, may differ between species or experimental model. In addition, concentrations of R-PIA > 10<sup>-8</sup> M might activate adenosine A<sub>1</sub> receptors in the immature heart, thereby limiting the antiadrenergic effect of R-PIA. It is also possible that CCPA exhibits a greater affinity than R-PIA for myocardial adenosine A<sub>1</sub> receptors or that CCPA elicits more efficient adenosine A<sub>1</sub>-receptor transduction in the immature heart. In
adult rat brain and myocardial membranes, CCPA and R-PIA bind to adenosine A1 receptors with affinity constants (Kd) of 0.4 (16, 21) and 1.2 nM (5, 22, 23), respectively.

Differences between maturation and aging. Recently, Romano and Dobson (26) compared the sensitivities of young adult and aged adult hearts to adenosine A1-receptor activation. In that study, R-PIA reduced β-adrenergic-elicited adenyl cyclase activity more in aged (18–20 mo) than in young adult (3–5 mo) myocardial membranes. In addition, by utilizing [3H]DPCPX, they found that aged hearts possess a greater density of adenosine A1 receptors than young adult hearts. Thus the sensitivity of the heart to adenosine A1-receptor activation appears to increase with age during adulthood, contrasting the results from the present study that indicate that adenosine A1-receptor sensitivity decreases with age before adulthood. It was not the original intention of the present study to compare and contrast the effects of maturation and aging on the sensitivity of the heart to adenosine A1-receptor activation. However, the present and previous studies from our laboratory indicate that adenosine A1-receptor sensitivity decreases with age before adulthood and then increases with adult aging. This pattern is paralleled by the effects of age on other myocardial functions. For example, the inhibitory effect of adenosine (17) or muscarinic receptor activation (25) on L-type calcium current is much more pronounced in neonatal than in adult rabbit ventricular myocytes. Cardiac myocytes from neonatal rats (2) or rabbits (18) express a greater level of inhibitory G protein (Gi) than myocytes from their more mature counterparts, whereas the level of stimulatory G protein (Gs) is unaltered during maturation (18). The myocardial level of Gi protein is also greater in senescent than in adult rats (3, 4) or guinea pigs (11). In support of these studies, adenosine A1-receptor activation with R-PIA has been reported to reduce contractility in the absence of β-adrenergic stimulation more in senescent than in young adult rabbit ventricular papillary muscle. However, other studies indicate that the dose-dependent inhibitory effect of adenosine on β-adrenergic-elicited contractility does not change with adult aging (11) and that the inhibitory effect of the adenosine A1-receptor analogs N6-cyclopentyladenosine or sulfophenyladenosine on β-adrenergic-stimulated adenyl cyclase activity decreases with adult aging (12). The animal species and techniques employed could account for these differences. Additional studies support the notion that the heart develops more immature features with adult aging. For example, the immature heart is highly dependent on transsarcolemmal calcium influx via reverse sodium-calcium exchange for increasing intracellular calcium levels during contraction (1). As the heart matures into adulthood, the heart becomes more dependent on the calcium stores of the sarcoplasmic reticulum for increasing intracellular calcium levels. During adult aging, however, the heart reverts toward a greater dependence on transsarcolemmal calcium influx (14). This is associated with a prolongation of the time course of the calcium transient and of the duration of the isometric twitch of aged compared with young adult cardiac muscle.

In summary, the results from the present study indicate that immature hearts express greater sensitivity than mature hearts to the antiadrenergic effects of adenosine A1-receptor activation. This was demonstrated by the finding that CCPA and R-PIA reduced isoproterenol-elicited contractile responsiveness to a greater degree in immature than in mature hearts. CCPA also reduced isoproterenol-elicited adenyl cyclase activity more in immature than in mature hearts. In contrast, CCPA elicited similar reduction of spontaneous heart rate in these two age groups of hearts. Thus the factors controlling adenosine A1-receptor sensitivity in the pacemaking cells of the atrial tissue act differently from those factors controlling adenosine A1-receptor sensitivity in contractile tissues of the atria or ventricles. These results also indicate that the effect of age on adenosine A1-receptor sensitivity is different before and during adulthood. The factors controlling adenosine receptor sensitivity in the atrial and ventricular tissues of the heart during myocardial maturation and aging should be an important and exciting area for future investigation.

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