Intrinsic A1 adenosine receptor activation during ischemia or reperfusion improves recovery in mouse hearts

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Peart, Jason, and John P. Headrick. Intrinsic A1 adenosine receptor activation during ischemia or reperfusion improves recovery in mouse hearts. Am J Physiol Heart Circ Physiol 279: H2166–H2175, 2000.—We assessed the role of A1 adenosine receptor (A1AR) activation by endogenous adenosine in the modulation of ischemic contracture and postischemic recovery in Langendorff-perfused mouse hearts subjected to 20 min of total ischemia and 30 min of reperfusion. In control hearts, the rate-pressure product (RPP) and first derivative of pressure development over time (+dP/dt) recovered to 57 ± 3 and 58 ± 3% of preischemia, respectively. Diastolic pressure remained elevated at 20 ± 2 mmHg (compared with 3 ± 1 mmHg preischemia). Interstitial adenosine, assessed by microdialysis, rose from ∼0.3 to 1.9 μM during ischemia compared with ∼15 μM in rat heart. Nonetheless, these levels will near maximally activate A1ARs on the basis of effects of exogenous adenosine and 2-chloroadenosine. Neither A1AR blockade with 200 nM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) during the ischemic period alone nor A1AR activation with 50 nM N6-cyclopentyladenosine altered rapidity or extent of ischemic contracture. However, ischemic DPCPX treatment significantly depressed postischemic recovery of RPP and +dP/dt (44 ± 3 and 40 ± 4% of preischemia, respectively). DPCPX treatment during the reperfusion period alone also reduced recovery of RPP and +dP/dt (to 44 ± 2 and 47 ± 2% of preischemia, respectively). These data indicate that 1) interstitial adenosine is lower in mouse versus rat myocardium during ischemia, 2) A1AR activation by endogenous adenosine or exogenous agonists does not modify ischemic contracture in murine myocardium, 3) A1AR activation by endogenous adenosine during ischemia attenuates postischemic stunning, and 4) A1AR activation by endogenous adenosine during the reperfusion period also improves postischemic contractile recovery.

There is evidence that adenosine may function as an endogenous cardioprotectant during ischemia-reperfusion. Activation of cardiovascular adenosine receptors by exogenously applied adenosine agonists protects from ischemic injury (4, 9, 31, 32), improves functional recovery during reperfusion (9, 11, 12, 30, 35, 39, 42, 43), and enhances metabolic recovery (49). There is also evidence that endogenous adenosine improves functional and metabolic tolerance to ischemia-reperfusion (2, 23, 24, 34, 47, 48). Although most data favor a key role for the A1 adenosine receptor (A1AR) in cardioprotection (11, 24, 30, 32, 33, 34, 48), some studies suggest that the recently characterized A2 receptor may mediate protection (40, 42, 44), and there is also evidence for beneficial effects of A2 receptor activation during ischemia-reperfusion (7, 35, 38, 43). The temporal characteristics of adenosine-mediated protection are also unclear. Some studies suggest that activation of adenosine receptors before and during ischemia is essential for reduction of stunning in vivo and in vitro (37, 39, 43, 46), whereas others demonstrate A1AR protection during early reperfusion (33).

Although such studies verify beneficial receptor-mediated protection with exogenous adenosine agonists, the role of endogenous adenosine in ameliorating injury during ischemia and/or reperfusion is much less clear. Evidence of A1-activated cardioprotection with exogenous agonists before and during ischemia and of A2 agonists during reperfusion does not demonstrate a role for intrinsic A1 or A2 receptor activation by endogenous adenosine at these times. The purpose of the present study was to delineate between cardioprotective effects of A1AR activation by endogenous adenosine during the ischemic episode itself versus during the postischemic period. Langendorff-perfused murine hearts were subjected to global ischemia-reperfusion in the absence and presence of a potent and selective A1AR antagonist [200 nM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX)]. To reduce endogenous activation of A1ARs during ischemia, DPCPX was applied during the ischemic episode alone. To reduce endogenous activation of A1ARs during reperfusion only, a second group received DPCPX throughout the postischemic period alone. In addition, we examined release of adenosine into the interstitial compartment and the potency of adenosine at A1ARs because there is presently no information regarding myocardial adenosine levels during ischemia in the increasingly studied murine heart.

MATERIALS AND METHODS

Langendorff-perfused murine heart model. Hearts (165 ± 36 mg wet heart wt, n = 70) were isolated from 7- to 12-wk-old C57BL/6 mice. Hearts were exposed to 20 min of ischemia (at 2, 300 ml/min flow rate, 37°C) followed by 30 min of reperfusion (1,000 ml/min). The hearts were perfused with nominally K+-free buffer under constant flow at 100 mmHg. Heart rate was maintained at 350 bpm throughout the protocol.

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old male C57/B16 mice (22–27 g body wt) anesthetized with 60 mg/kg pentobarbital sodium given intraperitoneally. The aorta was rapidly cannulated, and the coronary circulation was perfused at a constant pressure of 80 mmHg with modified Krebs-Henseleit buffer containing (in mM) 120 NaCl, 22 NaHCO₃, 4.7 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 KH₂PO₄, 15 glucose, and 0.5 EDTA. The buffer was equilibrated with 95% O₂-5% CO₂ at 37°C, giving a pH of 7.4 and a perfusion fluid PO₂ ≥ 640 mmHg. The buffer was initially passed through a 5-μ filter after preparation and was filtered through an in-line 0.45-μ Sterivex-HV filter cartridge (Millipore, Bedford, MA) within the perfusion apparatus. The left ventricle was vented with a polyethylene apical drain, and a fluid-filled balloon constructed of polyvinyl chloride plastic film was inserted into the left ventricle via the mitral valve. The balloon was connected to a P23 XL pressure transducer (Viggo-Spectramed, Oxnard, CA) by a fluid-filled polyethylene tube. The balloon was inflated using a 500-μl Hamilton threaded plunger syringe (Hamilton, Reno, NV) to give an end-diastolic pressure of 4 mmHg. Coronary flow was monitored using an anesthetizing Doppler flow probe (1N probe; Transonic Systems, Ithaca, NY) located in the aortic perfusion line and connected to a T206 flowmeter (Transonic Systems). Functional data were recorded at a sampling rate of 1 kHz on a four-channel MacLab data acquisition system (ADInstruments, Castle Hill, Australia) connected to an Apple computer. The ventricular pressure signal was digitally processed to yield peak systolic pressure, diastolic pressure, first derivative of pressure development or relaxation over time (±dP/dt), and heart rate. After the hearts were instrumented, they were immersed in perfusate inside a water-jacketed bath maintained at 37°C. The temperature of coronary perfusion fluid was continuously assessed by a needle thermistor at the top of the aortic cannula.

**Cardiac microdialysis.** To examine interstitial adenosine levels, microdialysis cannulas were implanted in the left ventricular wall of a subset of perfused mouse hearts (n = 6), as described by our laboratory in detail previously for rat heart (19–22). For the purposes of comparison, a group of rat hearts was also perfused as described for mouse hearts, and heart (19–22). For the purposes of comparison, a group of rat hearts (n = 5) was perfused under aerobic conditions for a period of 100 min. Function was monitored over this period, and the decline in developed pressure, heart rate, and coronary flow was assessed relative to function measured immediately after instrumentation of the hearts. To assess basal metabolic state, a group of hearts stabilized for 30 min under aerobic conditions was freeze-clamped and analyzed as described above (n = 6).

For ischemia studies, all hearts were initially stabilized for 30 min after which they were untreated or treated with drug for a period of 10 min. Global normothermic ischemia for 20 min was then initiated by cross clamping of the aortic cannula. This was followed by 30 min of aerobic reperfusion at a pressure of 80 mmHg. Hearts were permitted to beat at intrinsic rates throughout. Control hearts received no drug treatment at any time (n = 6). A parallel set of experiments was undertaken in a group of mouse (n = 6) and rat hearts (n = 8) with microdialysis probes in place to monitor interstitial purine levels during ischemia. To assess the possibility that A₁AR activation by endogenous adenosine during the ischemic period alone modifies functional recovery, a subset of hearts (n = 7) received the potent A₁ specific antagonist DPCPX during ischemia but not reperfusion. A 20 μM stock solution was infused at 1% of coronary flow to give a final perfusate concentration of 200 nM. DPCPX infusion was initiated 10 min before onset of the 20-min ischemic insult. Infusion was stopped at the onset of ischemia. To assess the possibility that A₁AR activation by endogenous adenosine during the reperfusion period alone modifies functional recovery, a subset of hearts (n = 6) was treated with 200 nM DPCPX throughout the 30-min reperfusion period. DPCPX infusion was initiated 30 s before reintroduction of coronary perfusion.

To assess the potential effects of selective A₁AR activation on ischemic contracture, a subset of hearts (n = 6) was subjected to ischemia alone and treated with 50 nM N⁶-cyclopentyladenosine (CPA) starting 10 min before onset of the 20-min ischemic insult. To examine the impact of a modest (10%) elevation in preischemic heart rate on functional responses to ischemia-reperfusion, a subset of hearts (n = 10) was switched from an intrinsic heart rate of 386 ± 23 to 423 ± 18 beats/min 10 min before the global ischemic insult. Pacing was stopped on initiation of ischemia, as described by our laboratory previously (34).

To determine whether levels of interstitial adenosine achieved during ischemia and reperfusion would exert significant functional effects at myocardial A₁ARs, a subset of hearts was treated with the endogenous agonist adenosine (n = 5) and the nonmetabolized and transported nonspecific A₁/A₂ agonist 2-chloroadenosine (n = 6). Agonists were applied at concentrations of 0.03–30 μM. Changes in heart
Table 1. Baseline functional parameters in Langendorff-perfused isovolumically contracting mouse and rat hearts.

<table>
<thead>
<tr>
<th></th>
<th>Control Hearts (n = 7)</th>
<th>Hearts With Dialysis Probe (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>372 ± 12</td>
<td>363 ± 17</td>
</tr>
<tr>
<td>Diastolic pressure, mmHg</td>
<td>3 ± 1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Systolic pressure, mmHg</td>
<td>164 ± 4</td>
<td>165 ± 10</td>
</tr>
<tr>
<td>Rate-pressure product, mmHg/min</td>
<td>61,010 ± 1,970</td>
<td>59,895 ± 2,692</td>
</tr>
<tr>
<td>+dp/dt, mmHg/s</td>
<td>5,070 ± 282</td>
<td>4,870 ± 384</td>
</tr>
<tr>
<td>-dp/dt, mmHg/s</td>
<td>4,210 ± 273</td>
<td>4,015 ± 250</td>
</tr>
<tr>
<td>Coronary flow, ml·min⁻¹·g⁻¹</td>
<td>20.1 ± 2.0</td>
<td>19.5 ± 1.8</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of hearts. Parameters were measured after 40 min of aerobic perfusion in hearts beating at intrinsic heart rates. ±dp/dt, first derivative of pressure development or relaxation over time. No significant differences existed between the 2 groups.

Baseline function and stability in perfused mouse hearts. Time-course studies revealed a progressive reduction in left ventricular pressure development equivalent to 74 ± 3% of the initial value over a 100-min period of aerobic perfusion. Heart rate remained stable throughout and was 99 ± 3% of baseline after 100 min of perfusion. Coronary flow was also stable, increasing very modestly with time to a value of 111 ± 5% at 100 min. As a result of the gradual fall in developed pressure, the rate-pressure product declined to 73 ± 2% after 100 min of perfusion. The baseline coronary flow rate (18–20 ml·min⁻¹·g⁻¹) was subsequently shown to be ~50% of maximal flows observed during A₁AR activation with 2-chloroadenosine. Baseline functional measurements from hearts with and without dialysis probes are shown in Table 1.

Metabolically, the perfused mouse hearts were relatively highly energized (Table 2). Under normoxic conditions, ATP, PCr, and Cr concentrations ([ATP], [PCr], and [Cr], respectively) were comparable in mouse and rat hearts. Cytosolic ATP, PCr, and Cr levels were ~8.5, 16.0, and 9.0 mM, respectively. The relatively high [PCr]/[ATP] ratio, low free cytosolic [ADP], and high [ATP]/[ADP] ratio are all indicative of highly energized myocardium. Employing free organic phosphate levels measured by our laboratory for the mouse (25), we calculated a Gibbs free energy of ATP hydrolysis (ΔG_ATP) of 61.3 kJ/mol for mouse heart, exceeding values measured in rat myocardium by our laboratory previously (e.g., Ref. 24) and reflecting efficient ATP maintenance at the expense of ADP (and P_i).

Response to ischemia-reperfusion in control hearts. Global normothermic ischemia rapidly abolished contractile function in all hearts within 120–180 s. The rate-pressure product and +dp/dt fell to ~3% of baseline after 60 s and to <1% after 120 s (Fig. 1A). A pronounced contracture developed very rapidly during ischemia, and significant diastolic dysfunction remained throughout the reperfusion period (Fig. 1A). On reperfusion, the diastolic pressure initially fell to ~30 mmHg during the first 1 min, rose to ~35 mmHg after 2 min, and then gradually fell to a value of 20 mmHg after 30 min of reperfusion (Fig. 1A). Recovery of the rate-pressure product displayed a similar pattern during reperfusion, with an early peak at 1–5 min before a gradual recovery throughout reperfusion (Fig. 1B). Recovery of +dp/dt paralleled the recovery of the rate-pressure product (Fig. 1C). Heart rate and coronary flow ultimately recovered to 98 ± 3 and 95 ± 7% of preischemic levels, respectively.

Effects of A₁AR antagonism during ischemia versus reperfusion. Preischemic contractile function was unaltered by DPCPX treatment with diastolic pressures of 3 ± 1 and 2 ± 1 mmHg in control and DPCPX-treated hearts, respectively, and left ventricular developed pressures of 161 ± 4 and 154 ± 7 mmHg in control and DPCPX-treated hearts, respectively (Fig. 1). Contractility assessed by +dp/dt was also unaltered in DPCPX-pretreated hearts (4,762 ± 284 mmHg/s) compared with control hearts (5,070 ± 282 mmHg/s).

Table 2. Baseline metabolic parameters in Langendorff-perfused hearts from C57/B16 mice.

<table>
<thead>
<tr>
<th>[ATP], mM</th>
<th>[PCr], mM</th>
<th>[Cr], mM</th>
<th>[PCr]/[ATP]</th>
<th>[ADP], μM</th>
<th>[ATP]/[ADP]</th>
</tr>
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<tbody>
<tr>
<td>8.4 ± 0.8</td>
<td>16.2 ± 1.5</td>
<td>9.2 ± 1.0</td>
<td>1.9 ± 0.2</td>
<td>56 ± 8</td>
<td>149 ± 16</td>
</tr>
</tbody>
</table>

Values are means ± SE. Baseline metabolic parameters were measured after 30 min of aerobic perfusion (n = 6). [ATP], ATP concentration; [PCr], phosphocreatine concentration; [Cr], creatine concentration; [ADP], ADP concentration. [PCr]/[ATP] and [ATP]/[ADP] are ratios.
However, preischemic DPCPX significantly elevated heart rate from 372 ± 6 ± 12 to 420 ± 6 ± 9 beats/min (P, 0.05). Treatment of hearts with 200 nM DPCPX before and during the ischemic episode itself resulted in significant depression of contractile recovery (rate-pressure product and +dP/dt) and produced a consistent elevation in postischemic diastolic pressure (Fig. 1). Treatment of hearts with DPCPX throughout the reperfusion period alone also significantly reduced recovery of the rate-pressure product and +dP/dt but failed to alter left ventricular diastolic pressure (Fig. 1). Final contractile recoveries in hearts receiving DPCPX pre-

ischemia or during reperfusion alone were similar at the end of the 30-min reperfusion period, whereas early recovery (between 10–20 min) was impaired to a greater extent in hearts receiving A,AR antagonist during ischemia (Fig. 1). Both heart rate and coronary flow recovered to between 95 and 100% of preischemic levels in all groups. Preischemic treatment with DPCPX failed to alter the initial decline in contractile function during ischemia (Fig. 1) and did not modify the rate and extent of contracture development during ischemia (Fig. 2). Similarly, treatment of hearts with 50 nM CPA failed to alter contracture development during ischemia (Fig. 2).

To examine the potential impact of the DPCPX-dependent elevation in preischemic heart rate (from 372 ± 12 to 420 ± 9 beats/min), we studied the effects of a direct increase in preischemic heart rate from 386 ± 23 to 423 ± 18 beats/min (i.e., a 10% elevation in heart rate). As shown in Figs. 1 and 2, this elevation in rate had no effect on initial changes in contractile function during ischemia, development of ischemic contracture, or postischemic functional recovery.
Changes in interstitial adenosine and effects of adenosine treatment. Microdialysis studies revealed that interstitial adenosine, inosine, and hypoxanthine levels rise dramatically during global ischemia in the mouse heart (Fig. 3). Adenosine initially rose to 580% of baseline levels between 5 and 10 min of ischemia before plateauing and then falling slightly in the final 5 min of ischemia. Adenosine then rapidly recovered to preischemic levels during reperfusion. Interestingly, both inosine and hypoxanthine peaked after adenosine (i.e., between 15 and 20 min). On the basis of 20 ± 3% recovery for microdialysis probes of the length used in mice in this study (i.e., reduced rate by 15–20% at 0.1–0.3 μM), with a pronounced bradydcardia at 1.0–3.0 μM (~50% reduction in rate), EC50 values for A1AR-mediated bradydcardia were 2.9 ± 0.4 μM for infused adenosine and 0.4 ± 0.2 μM for 2-chloroadenosine. Treatment with DPCPX fully blocked the negative chronotropic effects of 3 μM adenosine and 2-chloroadenosine (Fig. 4). Both agonists produced significant vasodilatation. Maximal coronary flows achieved during infusion of adenosine and 2-chloroadenosine were 33.5 ± 3.8 and 36.4 ± 3.9 ml·min⁻¹·g⁻¹, respectively.

DISCUSSION

The present study demonstrates that 20 min of global normothermic ischemia in murine heart produces 40–50% depression of contractile function after 30 min of reperfusion and results in a transient elevation in interstitial adenosine to physiologically active levels (~2 μM). Selective A1AR antagonism both during ischemia itself and during the reperfusion period alone impaired contractile recovery. Thus A1AR acti

Fig. 3. A: cardiac microdialysate adenosine, inosine, and hypoxanthine levels in control mouse hearts before, during, and after 20 min of global normothermic ischemia (n = 6). B: microdialysate purine levels for rat hearts (n = 8) subjected to the same ischemia-reperfusion protocol. Values shown are means ± SE.

Fig. 4. Concentration-response curves for adenosine- and 2-chloroadenosine-mediated bradydcardia (A1 adenosine receptor response) and vasodilatation (A2 adenosine receptor response) in mouse hearts. Values shown are means ± SE. Hatched columns reflect the estimated interstitial adenosine levels achieved during global ischemia and during early reperfusion. Also shown is the heart rate response to 3 μM adenosine (●) or 2-chloroadenosine (2-Cl-Ado; ▲) in the presence of DPCPX. Resist, resistance; [adenosine], adenosine concentration; [2-chloroadenosine], 2-chloroadenosine concentration.
vation by endogenous adenosine significantly improves postischemic function in murine myocardium, with beneficial effects mediated by receptor activation both during the preceding ischemic insult and during the postischemic period. We obtain no evidence for modification of ischemic contracture by exogenous or endogenous activation of A1ARs during ischemia in the mouse heart.

Interstitial adenosine levels and adenosine responses in murine heart. No data exist regarding interstitial adenosine in normoxic or ischemic murine heart. Our data (Fig. 3) indicate that basal interstitial adenosine levels are similar to those measured in isolated rat hearts (19, 20, 22) and in situ rat myocardium (21). These data are consistent with the recent observation that coronary venous adenosine in mouse heart approximates venous adenosine in the rat heart (15). Curiously, despite a higher basal metabolic rate and observations of profound reductions in cellular ATP in ischemic mouse heart (23), extracellular adenosine levels achieved during ischemia are much lower in mouse heart (i.e., ~2 μM) versus rat heart (i.e., ~15 μM). Reasons for this difference are not clear from the present study. However, Arch and Newsholme (3) have documented lower 5′-nucleotidase activity and higher adenosine kinase activity in mouse versus rat heart. Consequently, catabolism of 5′-AMP to adenosine may be reduced and recycling of adenosine to 5′-AMP enhanced in murine myocardium, leading to lower extracellular adenosine during increased ATP catabolism. Such a scheme would be consistent with our observation of reduced adenosine accumulation during ischemia (Fig. 3), despite marked hydrolysis of ATP in the mouse heart (23). An adaptation such as this may serve to protect adenine nucleotide levels in rapidly respiring murine myocardium.

Almost no data exist regarding responses to adenosine in murine heart. As shown in Fig. 4, adenosine and 2-chloroadenosine dose dependently dilate coronary vessels (A1 mediated) and inhibit intrinsic heart rate (A1 mediated) in mouse heart. On the basis of concentration-response profiles for adenosine and the stable analog 2-chloroadenosine, we predict that basal levels of interstitial adenosine (~0.3 μM) may modestly depress resting heart rate in mouse hearts. Validating this prediction, treatment of normoxic hearts with 200 nM DPCPX significantly elevated resting rate from ~370 to 420 beats/min. On the basis of sensitivities to adenosine and 2-chloroadenosine, we also predict that interstitial adenosine levels achieved during ischemia (~2 μM) will substantially activate myocardial A1ARs. Exogenously infused adenosine at 1–3 μM reduced rate by 35–55%, whereas the same levels of 2-chloroadenosine reduced rate by 70–90% (Fig. 4). Because adenosine is rapidly transported and catabolized, A1AR activation by 1–3 μM infused adenosine underestimates activation by 1–3 μM interstitial adenosine. On the other hand, 2-chloroadenosine is not catabolized, readily equilibrates across vascular and interstitial compartments, and is slightly more potent than adenosine at A1ARs. Thus A1AR-mediated effects of 1–3 μM interstitial adenosine will be greater than effects of 1–3 μM infused adenosine and less than those of 1–3 μM 2-chloroadenosine (Fig. 4). Collectively, data for interstitial adenosine levels (Fig. 3) and sensitivity to adenosine (Fig. 4) indicate that A1ARs will be substantially activated during ischemia and will also be activated during the reperfusion period. Treatment with a potent and selective A1AR antagonist should therefore unmask functional effects of intrinsic A1AR activation in ischemic and/or postischemic myocardium.

Lack of effect of A1AR blockade or activation on ischemic contracture. Although it is not known whether ischemic contracture is a direct indicator of irreversible ischemic damage, contracture is generally considered to reflect ischemic injury and may contribute to impaired recovery on reperfusion (29, 36). Studies employing exogenous adenosine agonists lend credence to the notion that A1ARs attenuate contracture development (12, 27, 31, 32). Moreover, we have recently shown that pronounced overexpression of myocardial A1ARs inhibits ischemic contracture (34). Contracture is thought to occur when anaerobic glycolytic flux and ATP generation fall to a threshold level (29, 36), and there is evidence that A1ARs modulate glycolysis, although there are observations of both accelerated (1, 10, 27) and reduced glycolysis (11, 12, 14). In the present study, selective A1AR antagonism with 200 nM DPCPX had no effect on rapidity or extent of ischemic contracture (Fig. 2). Additionally, 50 nM CPA (a level that almost maximally reduces heart rate) failed to alter contracture development. Thus A1AR activation by endogenous adenosine or exogenous agonists does not modify contracture in mouse, or, alternatively, DPCPX is unable to competitively block a supramaximal A1AR-mediated response. A near-maximal response is consistent with the levels of interstitial adenosine achieved (Fig. 3) and with the lack of effect of an A1AR agonist observed here (Figs. 1 and 2) and previously in mouse and rat (16, 34). In terms of antagonist treatment, DPCPX was applied at a concentration 200-fold higher than its inhibitor constant (Ki) for A1ARs, and we demonstrate practically that DPCPX effectively blocks A1AR responses to adenosine and 2-chloroadenosine when they are applied at levels (3 μM) approximating or exceeding adenosine concentrations attained during ischemia (~2 μM) (Fig. 4). Because 2-chloroadenosine is more potent than adenosine, our data verify that DPCPX will effectively antagonize A1AR effects mediated by lower levels of adenosine achieved during ischemia. The fact that functional recovery was depressed after ischemic DPCPX treatment (Fig. 1) also demonstrates antagonism of A1ARs during ischemia. We therefore conclude that the lack of effect of DPCPX (and CPA) indicates that A1AR activation by endogenous or exogenous adenosine during ischemia does not modify contracture in murine myocardium.

This conclusion appears to contrast with previous studies in which exogenous adenosine agonists and/or antagonists modify ischemic contracture (12, 27, 31,
However, effects of exogenous agonists do not reflect a role for endogenous adenosine, and many of these earlier studies employed agonists [adenosine, R-N\textsuperscript{-}phenylisopropyl adenosine (R-PIA)] that activate both A\textsubscript{1} and A\textsubscript{3} receptors at the levels used (e.g., Refs. 31 and 32). Recent evidence suggests that the A\textsubscript{3} receptor may be protective (40, 42, 44). Therefore, effects of exogenous agonists may not occur via A\textsubscript{1}ARs. Similarly, when antagonists have been employed, they are typically nonselective (e.g., 8-sulphonyltheophylline, BW-1433U) and are applied at levels sufficient to block A\textsubscript{1}, A\textsubscript{2}, and A\textsubscript{3} adenosine receptors (31, 32, 34). For example, levels of BW-1433U previously employed are 5- to 10-fold higher than the A\textsubscript{3} receptor K\textsubscript{b} (26), and Lasley and colleagues (32) report that this drug also blocked A\textsubscript{2}-mediated coronary vasodilation. Consequently, exaggeration of contracture with these antagonists may reflect A\textsubscript{3} rather than A\textsubscript{1}AR inhibition. Supporting this suggestion, selective A\textsubscript{2} activation reduces injury during (44) and after ischemia (42, 44). Surprisingly, we are aware of only one prior study of the effects of a selective A\textsubscript{1}AR antagonist during ischemia in the absence of exogenous adenosine agonists (16). In agreement with our observations, Grover et al. (16) found that A\textsubscript{1}AR-selective DPCPX failed to alter contracture in the rat heart in the absence of adenosine agonists. This is also consistent with the observation that A\textsubscript{1}AR antagonism with DPCPX fails to alter the effects of nucleoside transport blockade on ischemic contracture (17). Collectively, the lack of effect of A\textsubscript{1}AR blockade on contracture in mice (Figs. 1 and 2) and rat (16, 17), acceleration of contracture with nonselective A\textsubscript{1}/A\textsubscript{3} antagonists (31, 32, 34), delay of contracture with high levels of adenosine and R-PIA activating A\textsubscript{1} and A\textsubscript{3} receptors, blockade of the latter effect with nonselective A\textsubscript{1}/A\textsubscript{3} antagonists (32), and a lack of effect of A\textsubscript{2} activation on contracture (30) are all consistent with inhibition of ischemic contracture by an adenosine receptor other than the A\textsubscript{1} subtype.

A final possibility, consistent with levels of adenosine attained during ischemia, is that delayed contracture after nonselective adenosine agonist pretreatment (32) reflects activation of adenosine receptors before but not during ischemia (i.e., a “preconditioning-like” effect). It is difficult to reconcile cardioprotection by exogenous agonists with the supramaximal levels of endogenous adenosine attained during ischemia, as shown here (Fig. 3) and previously (19, 20). Supporting this possibility, transient activation of adenosine receptors before but not during ischemia does delay contracture (5), and there is evidence that this effect may be A\textsubscript{3} rather than A\textsubscript{1}AR dependent (4).

**Effects of intrinsic A\textsubscript{1}AR activation on postischemic function.** Some studies show that adenosine receptor activation during reperfusion affords cardioprotection (6, 7, 35, 47), largely via A\textsubscript{2}A-mediated mechanisms. The weight of evidence favors A\textsubscript{1}AR-mediated protection during ischemia rather than reperfusion (9, 27, 30, 32, 40, 48). However, this evidence has largely been accumulated via use of exogenous agonists rather than selective antagonists, and there is recent evidence of A\textsubscript{1}AR-mediated protection during reperfusion (34). We wished to test the hypothesis that A\textsubscript{1}AR activation during ischemia and/or reperfusion is cardioprotective. To address the hypothesis, cardiac A\textsubscript{1}ARs were antagonized during ischemia alone in one group and during reperfusion alone in another group. Functional recovery in hearts receiving DPCPX throughout ischemia alone differs from recovery in hearts not receiving DPCPX at any time (Fig. 1). Similarly, recovery in hearts receiving DPCPX throughout reperfusion alone differs from recovery in untreated hearts. Because DPCPX is a selective and potent A\textsubscript{1}AR antagonist, these observations show that intrinsic or endogenous A\textsubscript{1}AR activation during ischemia or reperfusion improves recovery. Preischemic treatment had a greater effect on early contractile recovery, yet ultimate effects of both treatment regimes were almost identical after 30 min of reperfusion.

The mechanism(s) by which ischemic A\textsubscript{1}AR activation modifies postischemic function is not known, although there are some similarities with preconditioning in that effects of receptor activation may not be realized until the recovery period. One possible mechanism contributing to effects of ischemic DPCPX treatment involves acceleration of cardiac activity before ischemia. We observed a small (∼10%) elevation in preischemic heart rate with A\textsubscript{1}AR blockade, and it is known that significant modification of preischemic metabolic rate can alter responses to ischemia. Although unlikely, we tested whether this change might contribute to altered posts ischemic recovery. As shown in Figs. 1 and 2, an equivalent rise in heart rate in untreated hearts had absolutely no effect on ischemic function or posts ischemic recovery. We conclude that detrimental effects of A\textsubscript{1}AR blockade before and during ischemia are unrelated to the modest 10% elevation in preischemic heart rate.

Reductions in postischemic contractile function reflect Ca\textsuperscript{2+} overload during reperfusion (18) with impaired myofibrillar sensitivity to Ca\textsuperscript{2+} (8, 13). A\textsubscript{1}AR activation by endogenous adenosine may modify sarcolemmal Ca\textsuperscript{2+} fluxes by activation of ATP-sensitive K\textsuperscript{+} (K\textsubscript{ATP}) channels (28, 46) and by more direct inhibition of Ca\textsuperscript{2+} channels. In support of these possibilities, Fralix et al. (12) have shown that adenosine-mediated protection is associated with reduced cellular Ca\textsuperscript{2+} accumulation and reduced acidosis, and our recent studies show that A\textsubscript{1}AR-mediated protection in mouse heart involves K\textsubscript{ATP} channel activation (24). We have also demonstrated that receptor activation by endogenous adenosine improves myocardial energy state during ischemia-reperfusion in the mouse (23). Although improved mitochondrial function and energy state may not modify function during ischemia itself, it will reduce Na\textsuperscript{+} and H\textsuperscript{+} accumulation, inhibiting Ca\textsuperscript{2+} overload during early reperfusion (41). Such a scheme could contribute to the temporal pattern observed here. The role of glycolysis in these effects remains obscure because A\textsubscript{1}AR activation can enhance (1, 10, 27) or inhibit (11, 12, 14) glycolytic metabolism, protecting by enhanced ATP generation in the former or reduced...
acidosis in the latter. Because contracture is most directly related to a fall in glycolytic ATP production (29, 36), the lack of effect of DPCPX and CPA on contracture (Fig. 2) indicates a lack of effect of endogenous or exogenous A1AR activation on glycolytic ATP formation during ischemia.

Consistent with a metabolically beneficial action of endogenous adenosine, we have documented worsening of postischemic energy metabolism in rat hearts subjected to low-flow ischemia in the presence of 8-phenyltheophylline (2). It is interesting to note, however, that no functional change was observed, in contrast to mouse data. Reasons for this difference include the different species studied, nonselectivity of the antagonist used, and the quite different types and severity of insult studied (low-flow versus zero-flow ischemia). Indeed, the level of deenergization in the rat model was modest (only 30–40% reductions in ATP and PCr) (2) compared with almost total loss of ATP and PCr in ischemic mouse heart (23). Furthermore, contracture in the low-flow rat model is much lower than in globally ischemic mouse heart, and rat hearts recovered to ~95% of preischemic function versus only 50–60% in the globally ischemic murine model. It is quite possible that the type and severity of insult, and the level of dysfunction that occurs, may determine whether A1AR activation enhances functional recovery.

In terms of the salutary effects of A1AR activation during reperfusion, previous studies verify that adenosine receptor activation at this time can reduce cell damage, although the major effect appears to be A2 receptor mediated with a minor effect of A1ARs at this time (7, 33, 47). However, we show that the ultimate degree of A1AR cardioprotection mediated by endogenous adenosine during either ischemia or reperfusion is similar. Only the rate of recovery differs, with ischemic A1AR activation apparently improving initial recovery to a greater extent than postischemic A1AR activation. The responses to DPCPX are consistent with a predominant effect on postischemic stunning rather than tissue necrosis, because recoveries for control and DPCPX-treated hearts are initially divergent but converge after 30 min of reperfusion and do not appear to plateau. We note that subsequent studies in our laboratory examining 60-min periods of reperfusion reveal that contractile recovery plateaus at ~40 min in the present murine model (data not shown). The postischemic recovery pattern observed in the present study (with and without DPCPX) is suggestive of modification of a reversible form of injury. Because there were no differences in postischemic coronary flow between groups, the observed alterations in contractile recovery do not result from differences in coronary flow.

Experimental limitations. Several experimental limitations deserve mention. The first relates to use of microdialysis in murine myocardium. No previous studies have measured extracellular adenosine in mouse heart during ischemia or applied microdialysis in murine myocardium. Owing to cell damage on probe implantation, we normally employ a 60- to 90-min stabilization period in rat hearts to allow for a decline in extracellular purines toward baseline levels (19–22). Here we employed a 40-min stabilization period before ischemia, potentially leading to overestimation of basal purine levels. Compounding this issue, it might be argued that the small size of the mouse heart will contribute to an increased percentage of left ventricular myocytes damaged by implantation of the 300-μm probe (relative to larger species). However, probe implantation did not alter left ventricular mechanics, heart rate, or coronary flow (Table 1). Indeed, we note that hearts in the present study possess levels of contractility generally exceeding those measured in previous perfused murine heart studies. Additionally, preliminary studies revealed no change in myocardial enzyme leakage after probe implantation. Thus the method does not appear to produce an excessive degree of damage in left ventricular myocardium. Importantly, although we recognize that basal levels of adenosine in the mouse may be overestimated by this technique, this does not alter our conclusion regarding lower interstitial adenosine in ischemic mouse versus rat heart. Moreover, because preischemic interstitial adenosine was estimated to be 0.3 μM, the value measured during ischemia (1.9 μM) will be overestimated (if at all) by ~15% due to potential lack of stabilization.

A second limitation is the finite period required for washout of interstitial DPCPX in hearts receiving the antagonist before and during ischemia. Thus there will be residual antagonist immediately on reperfusion in this experimental group. We have previously shown that 3 min of perfusion washes >95% of a large molecule, distributed throughout the extracellular compartment, from the perfused heart (25). The relatively small DPCPX should therefore be reduced to very low levels within the first 1–2 min of reperfusion, limiting its effects at this time. Nonetheless, given that these first minutes of reperfusion may be important in determining the extent of reperfusion injury, the presence of low levels of antagonist at this time may contribute to reduced recovery in the ischemic DPCPX treatment group. Importantly, effects of ischemic and postischemic DPCPX treatment on diastolic dysfunction differed markedly, and ischemic DPCPX produced a greater reduction in early recovery than postischemic DPCPX, supporting distinct A1AR-mediated effects during the two different phases of injury (ischemia versus reperfusion).

In conclusion, the results of the present study demonstrate that activation of A1ARs by endogenous adenosine significantly improves functional recovery from ischemia. Functional protection is mediated by intrinsic A1AR activation during either period is comparable. These effects are consistent with observed elevations of interstitial adenosine to active levels during ischemia and reperfusion. Our data also demonstrate that intrinsic A1AR activation during ischemia does not mod-
ify rate or extent of ischemic contracture development, consistent with previous observations in rat hearts treated with selective A1AR antagonists (16) but in contrast to previous studies employing less specific antagonists (32, 34). Data collectively support a role for other adenosine receptors (potentially the A2A subtype) in inhibition of ischemic contracture or suggest that preischemic adenosine receptor activation may be involved in reducing contracture in hearts pretreated with adenosine agonists. These latter issues deserve further investigation.

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