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Inhibition of glycolysis and enhanced mechanical function of working rat hearts as a result of adenosine A_1 receptor stimulation during reperfusion following ischaemia

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1 This study examined effects of adenosine and selective adenosine A_1 and A_2 receptor agonists on glucose metabolism in rat isolated working hearts perfused under aerobic conditions and during reperfusion after 35 min of global no-flow ischaemia.

2 Hearts were perfused with a modified Krebs-Henseleit buffer containing 1.25 mM Ca^{2+}, 11 mM glucose, 1.2 mM palmitate and insulin (100 μu ml^{-1}), and paced at 280 beats min^{-1}. Rates of glycolysis and glucose oxidation were measured from the quantitative production of ^3H_2O and $^{14}CO_2$, respectively, from [5-^{3}H/U-^{14}C]-glucose.

3 Under aerobic conditions, adenosine (100 μM) and the adenosine A_1 receptor agonist, N^6-cyclohexyladenosine (CHA, 0.05 μM), inhibited glycolysis but had no effect on either glucose oxidation or mechanical function (as assessed by heart rate systolic pressure product). The improved coupling of glycolysis to glucose oxidation reduced the calculated rate of proton production from glucose metabolism. The adenosine A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX 0.3 μM) did not alter glycolysis or glucose oxidation *per se* but completely antagonized the adenosine- and CHA-induced inhibition of glycolysis and proton production.

4 During aerobic reperfusion following ischaemia, CHA (0.05 μM) again inhibited glycolysis and proton production from glucose metabolism and had no effect on glucose oxidation. CHA also significantly enhanced the recovery of mechanical function. In contrast, the selective adenosine A_{2a} receptor agonist, CGS-21680 (1.0 μM), exerted no metabolic or mechanical effects. Similar profiles of action were seen if these agonists were present during ischaemia and throughout reperfusion or when they were present only during reperfusion.

5 DPCPX (0.3 μM), added at reperfusion, antagonized the CHA-induced improvement in mechanical function. It also significantly depressed the recovery of mechanical function *per se* during reperfusion. Both the metabolic and mechanical effects of adenosine (100 μM) were antagonized by the nonselective A_1/A_2 antagonist, 8-sulphophenyltheophylline (100 μM).

6 These data demonstrate that inhibition of glycolysis and improved recovery of mechanical function during reperfusion of rat isolated hearts are mediated by an adenosine A_1 receptor mechanism. Improved coupling of glycolysis and glucose oxidation during reperfusion may contribute to the enhanced recovery of mechanical function by decreasing proton production from glucose metabolism and the potential for intracellular Ca^{2+} accumulation, which if not corrected leads to mechanical dysfunction of the post-ischaemic myocardium.

Keywords: Adenosine; cyclohexyladenosine; CGS-21680; myocardial reperfusion; energy metabolism; glycolysis; glucose metabolism; cardioprotection

Introduction

Adenosine is an endogenous nucleoside that can exert a variety of effects throughout the body by interacting with several types of cell-surface receptors. In the cardiovascular system, adenosine can influence cardiac function either directly via stimulation of adenosine A_1 receptors located on cardiac myocytes (Martens *et al.*, 1987) or indirectly via stimulation of A_2 receptors located on the coronary and systemic vasculature (Collis, 1989) or by an inhibition of noradrenaline release (Richardt *et al.*, 1987). An adenosine A_3 receptor has also been described that, based on molecular biological evidence, possesses a wide tissue distribution (Tucker & Linden, 1993). Recent interest in adenosine has focused on it's ability to exert a cardioprotective action during ischaemia and reperfusion (Ely & Berne, 1992), particularly in its relationship to the phenomenon of ischaemic preconditioning (Mullane, 1992). Marked reductions in infarct size, (Toombs *et al.*, 1992; Zhao *et al.*, 1993; 1994), attenuation of dysrhythmias (Pantely & Bristow, 1990) and improved recovery of mechanical function during reperfusion have been noted following administration of adenosine (Lasley *et al.*, 1990; Lasley & Mentzer, 1992; Finegan *et al.*, 1993; Janier *et al.*, 1993; Nomura *et al.*, 1993). The mechanisms underlying these cardioprotective effects of adenosine remain unclear. Adenosine A_1 receptor stimulation has cardioprotective actions (Lasley *et al.*, 1990; Lasley & Mentzer, 1992), that may occur by a direct or indirect anti-adrenergic action (Richardt *et al.*, 1987). Adenosine A_2 receptor agonists can also attenuate reperfusion damage, potentially due to a relaxation of the coronary vasculature (King *et al.*, 1990), a reduction of neutrophil infiltration into damaged endothelium (Cronstein *et al.*, 1986) or by an inhibition of the release of superoxide anions from activated neutrophils (Cronstein *et al.*, 1990). Evidence for the involvement of an adenosine A_3 receptor in cardioprotection has also been presented (Liu *et al.*, 1994). The relative condition of each of the adenosine receptor sub-types to cardioprotection may depend on whether adenosine is acting during ischaemia or only during reperfusion. The finding that antagonism of endogenous

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adenosine only during reperfusion increases infarct size (Zhao *et al*,. 1993) indicates that substantial protective mechanisms of endogenous adenosine are operative in the reperfusion period.

In previous studies we demonstrated that adenosine inhibits glycolysis in rat isolated working hearts under aerobic conditions and so enhances the coupling between glycolysis and glucose oxidation (Finegan *et al*., 1992). In addition, we showed that, in hearts perfused in the presence of a high concentration of fatty acids (palmitate, 1.2 mM) which are known to be present after ischaemia in man (Lopaschuk *et al*., 1994) adenosine, given prior to a 60 min period of low-flow ischaemia decreases glycolysis and enhances the recovery of mechanical function during reperfusion (Finegan *et al*., 1993). We proposed that the adenosine-induced inhibition of glycolysis would slow the rate of proton production from glucose metabolism during ischaemia and during the critical early reperfusion period. The resulting reduction in H^+/Na^+ exchange would prevent Na^+ and ultimately Ca^{2+} overload, thereby allowing a more rapid and complete recovery of mechanical and metabolic function during reperfusion. Such a mechanism is supported by the observation that ischaemia-induced increases in intracellular H^+, Na^+ and Ca^{2+} (measured by n.m.r.) are attenuated by adenosine (Fralix *et al*., 1993). However, the role of proton production from glycolytic ATP hydrolysis in the cardioprotective effects of adenosine remains unclear as others have provided evidence, using glucose only perfused non-working hearts, that the beneficial effects of adenosine may arise from a stimulation of glycolysis, resulting in an increase in the main source of ATP production under ischaemic conditions (Wyatt *et al*., 1989; Janier *et al*., 1993). The role of adenosine-induced changes in glycolysis during ischaemia versus during reperfusion is also not clear.

This study tested the hypothesis that adenosine receptor stimulation only during reperfusion inhibits glycolysis and improves recovery of post-ischaemic mechanical function. Specifically, we determined the roles of adenosine receptor subtypes in the adenosine-mediated alterations in glucose metabolism and mechanical function, (1) during aerobic conditions and (2) during reperfusion post-ischaemia. Drugs were present either during both ischaemia and reperfusion or only during reperfusion. Working perfused rat hearts were used so that measurements of metabolic and mechanical function could be made under conditions of appropriate energy demand (workload) and energy substrate concentrations.

Methods

Heart perfusions

Hearts from sodium pentobarbitone-anaesthetized, male Sprague-Dawley rats (200 to 300 g), that had been fed *ad libitum*, were excised, the aortae were cannulated and a retrograde perfusion using Krebs Henseleit buffer (pH 7.4, gassed with a 95% O_2: 5% CO_2 mixture) initiated, as described previously (Finegan *et al*., 1992; 1993). During this initial 10 min Langendorff perfusion period, unpaced hearts were trimmed of excess tissue, the pulmonary artery was cut, and the point at which the pulmonary vein entered the left atria was cannulated. Hearts were then switched to the working mode by clamping the aortic inflow line from the Langendorff reservoir and opening the left atrial line. Working hearts were paced at 280 beats min^{-1} (Grass SD9 Stimulator) except during the period of global ischaemia. All working hearts were perfused in a close recirculating system at 34°C using an oxygenator with a large surface area in constant contact with 5% CO_2 and 95% O_2. The perfusion fluid (volume 100 ml) consisted of a modified Krebs Henseleit buffer containing 1.25 mM free Ca^{2+}, 11 mM glucose, 1.2 mM palmitate pre-bound to 3% bovine serum albumin (BSA, fraction V) and insulin (100 μu ml^{-1}). Perfusions were performed at a constant left atrial preload (11.5 mmHg) and the hydrostatic afterload was set to a column height equivalent to 80 mmHg. Heart rate and systolic and diastolic aortic pressures were measured with a Gould P21 pressure transducer connected to the aortic outflow line. Rate-pressure product (HR × PSP) served as a continuous index of left ventricular mechanical function. In some experiments, left atrial inflow (ml min^{-1}) and aortic flow (ml min^{-1}) were measured with ultrasonic flow probes (Transonic T206) placed in the left atrial inflow line and the aortic outflow lines, respectively. Coronary flow (ml min^{-1}) was calculated as the difference between cardiac output and aortic flow.

Measurement of steady state rates of glycolysis and glucose oxidation under aerobic conditions and during reperfusion following ischaemia

Glycolysis and glucose oxidation were measured simultaneously by the quantitative collection of ^3H_2O (liberated at the enolase step of glycolysis) and $^{14}CO_2$ (liberated at the level of pyruvate dehydrogenase complex (PDC) and in the citric acid cycle) from hearts perfused with buffer containing tracer amounts of [5-^{3}H]-glucose and [U-^{14}C]-glucose. Samples of perfusate for the determination of metabolic rates were collected at 10 min intervals. Steady-state rates of glycolysis and glucose oxidation were averaged over 30 min periods.

To measure glycolysis, the ^3H_2O in perfusate samples was separated from [^{3}H]-glucose and [^{14}C]-glucose on columns containing Dowex 1-X4 anion exchange resin, as previously described (Finegan *et al*., 1993). Steady state rates of glycolysis were expressed as μmol [5-^{3}H]-glucose metabolized min^{-1} g^{-1} dry wt. The closed perfusion system allowed collection of both gaseous $^{14}CO_2$ and $^{14}CO_2$ trapped as bicarbonate for the measurement of glucose oxidation, as described previously. Steady state rates of glucose oxidation were expressed as μmol [U-^{14}C]-glucose metabolized min^{-1} g^{-1} dry wt.

Calculation of the rate of proton production arising from glucose metabolism

When glucose (from exogenous or endogenous sources) is metabolized by glycolysis and subsequent glucose oxidation, with the associated synthesis and hydrolysis of ATP, the net production of protons is zero. However, if the rate of glycolysis exceeds that of glucose oxidation, there is a net production of two protons per molecule of exogenous glucose that passes through glycolysis that is not subsequently oxidized (Dennis *et al*., 1991).

Consequently, the rate of proton production attributable to the hydrolysis of ATP arising from exogenous glucose metabolism can be calculated as 2 × (rate of glycolysis − rate of glucose oxidation).

Experimental protocols

Hearts were randomly assigned to either the control of treated groups and measurements of mechanical function were made continuously throughout each perfusion protocol. At the end of perfusion, heart ventricles were frozen with Wollenberger clamps cooled to the temperature of liquid N_2 for the determination of their total dry weight.

Series 1 Aerobic perfusion Drug-induced alterations of glycolysis, glucose oxidation, proton production and mechanical function were examined in hearts perfused aerobically for a 60 min period. After a 30 min period of baseline aerobic perfusion, drug or drug combinations were added and their effects were examined during a subsequent 30 min treatment period. The direct effects of adenosine (100 μM), N^6-cyclohexyladenosine (CHA, 0.05 μM) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.3 μM) on glycolysis, glucose oxidation and proton production in the treatment periods were compared to their respective aerobic baseline values, as well as with an untreated parallel time control group. The effects of DPCPX (0.3 μM) when given during the treatment period in

combination with adenosine (100 μM) or CHA (0.05 μM) were compared to the direct effects of the corresponding agonists.

Series 2 Ischaemia and reperfusion Following 30 min of aerobic baseline perfusion, global (no flow) ischaemia was induced by cross-clamping the left atrial inflow and aortic outflow lines. After 35 min of global ischaemia, left atrial and aortic flows were restored and hearts were paced and reperfused under aerobic conditions for a further 30 min period. Total myocardial ^3H_2O and $^{14}CO_2$ production was determined at 10 min intervals during the aerobic baseline and reperfusion periods. Rates of glycolysis, glucose oxidation and proton production and recovery of mechanical function in drug-treated groups were compared with untreated control hearts.

Initially, in four separate groups of hearts, the effects of the selective adenosine A_1 and A_2 agonists, CHA (0.05 μM) and 2-[-4-(2-carboxyethyl) phenethylamino]-5'-N-ethylcarboxamido adenosine (CGS-21680, 1.0 μM), respectively, given either prior to ischaemia or only at reperfusion were examined. These experiments identified which agonist(s) enhanced recovery of mechanical function and whether the beneficial effect was elicited during ischaemia and/or only during reperfusion.

Receptor characterization To characterise the adenosine receptor subtypes involved, adenosine and the selective adenosine A_1 receptor agonist, CHA, in the absence or presence of adenosine receptor antagonists, DPCPX, 8-(*p*-sulphophenyl)theophylline (8-SPT) or BW-A1433 were added to the perfusion buffer 5 min prior to reperfusion to ensure that mixing in the recirculating system was complete before removal of the clamps from the inflow and outflow lines.

Drugs and reagents

D-[5-^{3}H]-glucose and D-[U-^{14}C]-glucose (5 mCi mmol^{-1}) were purchased from Dupont Canada Inc, Ontario, Canada and hyamine hydroxide was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Bovine serum albumin (BSA fraction V) was obtained from Boehringer Mannheim, IN, U.S.A. Insulin (Regular, CZI) was obtained from Connaught Novo, Ontario, Canada. Dowex 1-x4 anion exchange resin (200-400 mesh, chloride form) was obtained from Bio-Rad Laboratories, CA, U.S.A. Adenosine, N^6-cyclohexyladenosine (CHA), 2-[-4-(2-carboxyethyl)phenethylamino]-5'-*N*-ethylcarboxamido adenosine (CGS-21680), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 8-(*p*-sulphophenyl)theophylline (8-SPT) were obtained from Research Biochemicals International, MA, U.S.A. BW-A1433 was donated by Burroughs Wellcome CO., NC, U.S.A. All other chemicals were reagent grade.

Statistical analysis

Data are expressed as mean $\pm$ s.e.mean. Data have been compared by one-way analysis of variance supported by the Bonferroni Multiple Comparison test for between group comparisons. In the case of paired comparisons, Student's paired *t* test was used. Differences were considered significant when $P<0.05$.

Results

Series 1, Aerobic perfusion

Mechanical function was stable and similar in all groups during the 30 min period of baseline aerobic perfusion (Table 1). In unpaced hearts, adenosine (100 μM) and CHA (0.05 μM) exerted a marked negative chronotropic action. Consequently, hearts were paced at 280 beats min^{-1} to facilitate comparisons of the effects of the adenosine agonists in the absence of changes in heart rate. In paced heart, adenosine (100 μM), CHA (0.05 μM), DPCPX (0.3 μM) alone or in combination with the agonists did not affect mechanical function when present during the subsequent 30 min of aerobic perfusion. There was no significant differences in mechanical function among groups, relative to their aerobic baselines values or relative to the untreated time control. As reported in previous studies using buffer-perfused working hearts (Finegan *et al.*, 1992), coronary flow was not significantly affected by adenosine under our experimental conditions. Also coronary flow in the presence of 0.05 μM CHA (23.4$\pm$ 1.0 ml min^{-1}) was not significantly different from control (22.7$\pm$1.3 ml min^{-1}). Thus, this experimental preparation permitted an assessment of the direct effects of adenosine agonists on energy metabolism, independent of changes in heart rate, coronary flow and rate pressure product.

During baseline aerobic perfusion, the steady state rate of glycolysis was 1.65$\pm$0.35 μmol min^{-1} g^{-1} dry wt ($n=7$) and was similar to that described previously for rat hearts perfused with 1.25 mM Ca^{2+} and 1.2 mM palmitate (Finegan *et al.*, 1993). Baseline (pre-drug) rates of glycolysis in all treatment groups were comparable to that seen in the control group (Figure 1). During the subsequent 30 min period of aerobic perfusion, rates of glycolysis in untreated hearts were the same as during baseline perfusion. However, when present during the subsequent 30 min period of aerobic perfusion, adenosine (100 μM) decreased the rate of glycolysis by 44% (Figure 1). CHA (0.05 μM) also inhibited glycolysis (by 45%). The effects of DPCPX, at a concentration (0.3 μM) expected to antagonize selectively adenosine A_1-mediated effects, were examined when added to the perfusion alone or in combination with agonists. DPCPX *per se* had no effect on glycolysis but antagonized the adenosine- and CHA-induced inhibition of glycolysis. Rates of glycolysis in the adenosine + DPCPX-treated group and the CHA + DPCPX-treated group were significantly different from the rates in the presence of their respective agonists alone but were not different from the equivalent time control value (Figure 1).

Glucose oxidation (0.15$\pm$0.02 μmol min^{-1} g^{-1} dry wt,

Table 1 Mechanical function (HR $\times$ PSP $\times$ 10^{-3}) of working rat hearts during aerobic perfusion

Group	n	*After 30 min aerobic perfusion*	*After 60 min aerobic perfusion*	*% change*
Control	7	30.0$\pm$0.8	29.8$\pm$0.9	99.3$\pm$0.7
Adenosine 100 μM	10	32.1$\pm$1.0	31.8$\pm$0.7	99.2$\pm$1.8
CHA 0.05 μM	9	31.6$\pm$1.7	31.6$\pm$1.7	100.0$\pm$0.9
DPCPX 0.3 μM	6	32.1$\pm$0.7	30.4$\pm$1.1	94.8$\pm$2.1
Adenosine 100 μM + DPCPX 0.3 μM	6	32.1$\pm$2.2	31.5$\pm$2.3	98.0$\pm$1.1
CHA 0.05 μM + DPCPX 0.3 μM	8	29.5$\pm$1.1	29.2$\pm$1.3	98.9$\pm$1.6

Values (mean$\pm$s.e.mean for *n* hearts) are shown for mechanical function of working rat hearts perfused under baseline aerobic conditions at 30 min or 60 min. Between 30 and 60 min of aerobic perfusion, hearts either received no treatment (Control) or were exposed to the indicated agonist and agonist-antagonist combinations. Changes in mechanical function at the end of the treatment period are expressed as % of their respective baseline values at 30 min. No significant differences between control and treatment groups were observed.

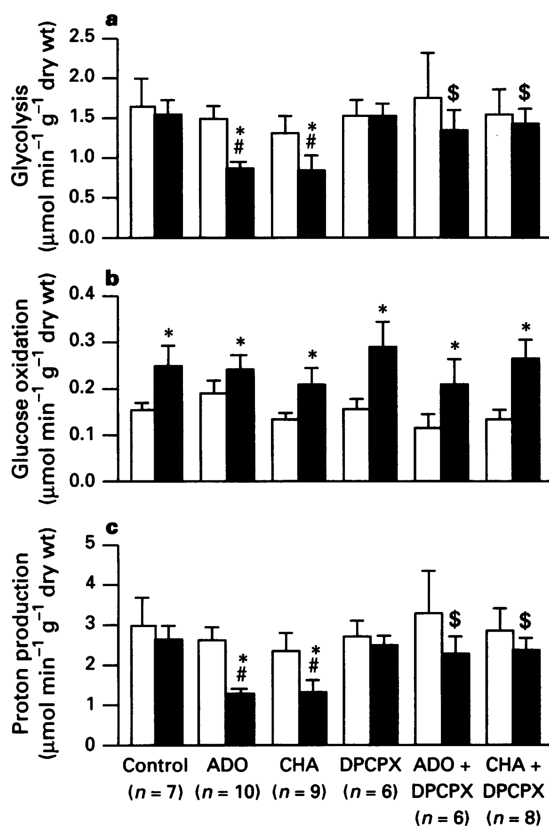

Figure 1 Effect of adenosine and CHA *per se* and in combination with DPCPX on rates of glycolysis (a), glucose oxidation (b) and proton production (c) from glucose metabolism during aerobic perfusion of working rat hearts (Series 1). Values are means±s.e.mean and are shown for hearts during 30 min of baseline aerobic perfusion (open columns) are during a subsequent 30 min period (solid columns) either in the absence (Control) or presence of the indicated drug or drug combination. A significant difference between a group during the subsequent period of perfusion and its own baseline period is indicated by *, while a significant difference during the subsequent period of perfusion between a treated group and the untreated time control group is indicated by #.

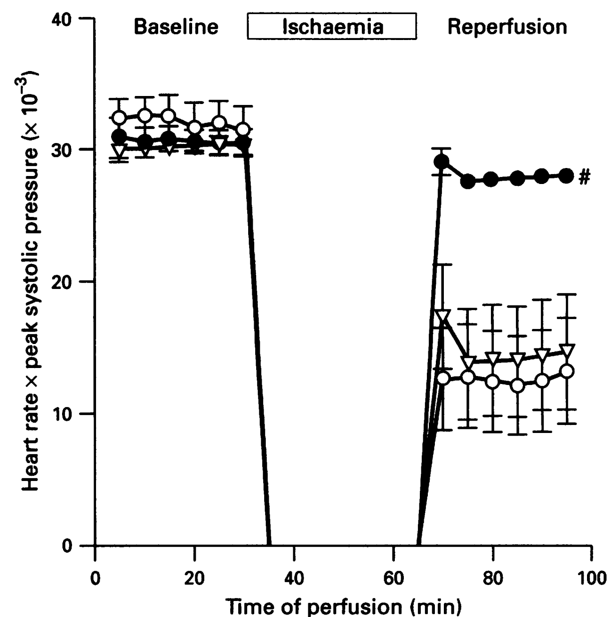

Figure 2 Recovery of mechanical function during reperfusion following 35 min of global ischaemia of working rat hearts (Series 2). Mechanical function was assessed as the product of heart rate and systolic pressure (HR x PSP). Values shown are means±s.e.mean measured during baseline aerobic perfusion (5 to 30 min), global ischaemia (30 to 65 min) and reperfusion (65 to 95 min) for control hearts (○, $n=16$) and for hearts that were exposed throughout reperfusion to either CHA (0.05 µM, ●, $n=7$) or CGS-21680 (1 µM, ▽, $n=11$). Groups that had a significantly different recovery of mechanical function from the untreated control group are indicated by #.

$n=7$) was similar to that described previously for rat hearts perfused with 1.25 mM Ca^{2+} and 1.2 mM palmitate (Finegan *et al.*, 1993). The approximately 10 fold lower rate of glucose oxidation, relative to glycolysis, reflected the inhibitory influence of the high concentration of free fatty acid (1.2 mM palmitate) in the perfusion fluid (Lopaschuk *et al.*, 1993). Baseline (pre-drug) rates of glucose oxidation in all treatment groups were comparable to that seen in the control group (Figure 1). Glucose oxidation, however, was high in all treatment groups during the subsequent period of aerobic perfusion relative to their respective baseline rates, but not significantly different from the corresponding time control group (Figure 1).

Calculated rates of proton production attributable to the metabolism of exogenous glucose (Figure 1) during baseline (pre-drug) aerobic perfusion in all treatment groups were similar to control (2.99±0.68 µmol min^{-1} g^{-1} dry wt, $n=7$). Proton production in the control untreated hearts was not altered during the subsequent 30 min period of aerobic perfusion (Figure 1). Adenosine (100 µM) and CHA (0.05 µM) significantly reduced proton production by 52% and 50%, respectively. DPCPX (0.3 µM) *per se* did not influence proton production, but significantly inhibited the adenosine- and CHA-induced inhibition of proton production. Rates of proton production in the adenosine+DPCPX group and the CHA+DPCPX group were significantly higher than in the presence of their respective agonists alone but were not different from the equivalent time control value (Figure 1).

Series 2, Ischaemia and reperfusion

LV function was similar in all groups during the period of aerobic baseline perfusion prior to the onset of no-flow ischaemia (Figures 2–5). During the 35 min of global ischaemia, all measurable mechanical work ceased. CHA, either when present during ischaemia and throughout reperfusions or only at reperfusion, significantly inhibited glycolysis and proton production. These changes were accompanied by an improved and almost complete recovery of mechanical function in both treated groups, relative to untreated control hearts (Figure 2, Table 2). The pre- or post-ischaemic additions of CGS-21680 (1.0 µM), were devoid of any metabolic or cardioprotective effect (Figure 2, Table 2).

Receptor characterization The ability of CHA to elicit equivalent effects when administered pre- or post-ischaemia indicated that receptor activation only during reperfusion was sufficient to exert beneficial metabolic and functional effects. Consequently, to clarify which receptor subtype was predominantly responsible for the effects, the actions of adenosine and CHA, in the absence or presence of adenosine receptor antagonists given only during reperfusion were examined in greater detail.

Adenosine (100 µM) given at the onset of reperfusion significantly enhanced the recovery of mechanical function (Figures 3 and 4). DPCPX *per se* (0.3 µM) significantly reduced the recovery of mechanical function. However, this concentration of DPCPX, when present in combination with adenosine (100 µM), did not inhibit the protective effects of adenosine (Figure 3). Similarly, BW-A1433 (30 µM), a combined adenosine A_1/A_3 receptor antagonist, did not antagonize the bene-

Table 2 Mechanical function and glucose metabolism of working rat hearts durin reperfusion after 35 in of global ischaemia

Group	*Treatment time*	*Glycolysis*	*Proton production* (μmol min^{-1} g^{-1} dry wt)	*Glucose oxidation*	*Mechanical function* (*% recovery*)
Control	None	1.57±0.26 (7)	2.80±0.51 (7)	0.17±0.01 (7)	41.4±12.3 (16)
CHA 0.05 μM	Pre-ischaemia	0.82±0.13 (6)†	1.28±0.30 (6)†	0.18±0.03 (6)	93.5±2.0 (6)†
CHA 0.05 μM	Reperfusion	0.76±0.15 (6)†	1.20±0.27 (6)†	0.17±0.02 (6)	91.8±3.0 (6)†
CGS-21680 1.0 μM	Pre-ischaemia	1.90±0.30 (6)	2.85±0.45 (5)	0.21±0.03 (6)	48.8±14.3 (11)
CGS-21680 1.0 μM	Reperfusion	1.82±0.39 (5)†	3.20±0.76 (5)	0.21±0.03 (6)	47.6±14.1 (11)

Changes in mechanical function (HR x PSP) at the end of reperfusion are expressed as % of their respective pre-ischaemic value. Rates of glucose metabolism were determined as described in Methods. Values are mean ± s.e.mean for (*n*) hearts; †significant difference from control.

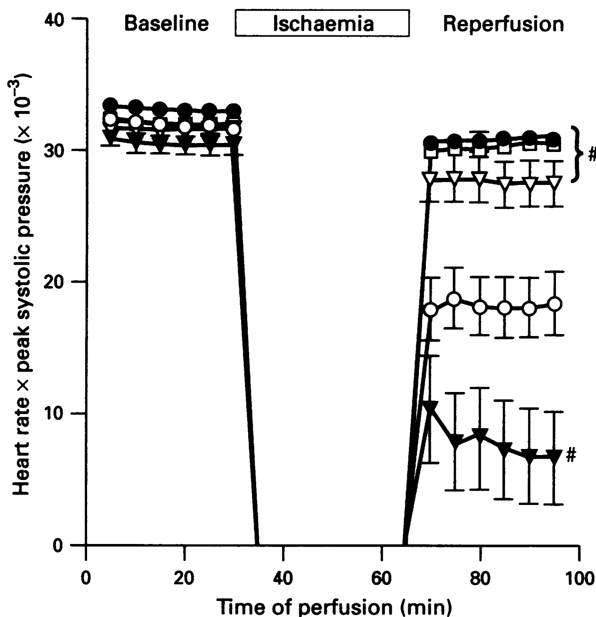

Figure 3 Recovery of mechanical function during reperfusion following 35 min of global ischaemia of working rat hearts (Series 2). Mechanical function was assessed as the product of heart rate and systolic pressure (HR x PSP). Values shown are means ± s.e.mean measured during baseline aerobic perfusion (5 to 30 min), global ischaemia (30 to 65 min) and reperfusion (65 to 95 min) for control hearts (○, $n=37$) and for hearts that were exposed throughout reperfusion to either adenosine (100 μM, ●, $n=13$), DPCPX (0.3 μM, ▼, $n=10$), adenosine (100 μM) and BW-A1433 (30 μM) in combination (□, $n=8$) or adenosine (100 μM) and DPCPX (0.3 μM) in combination (▽, $n=23$). Groups that had a significantly different recovery of mechanical function from the untreated control group are indicated by #.

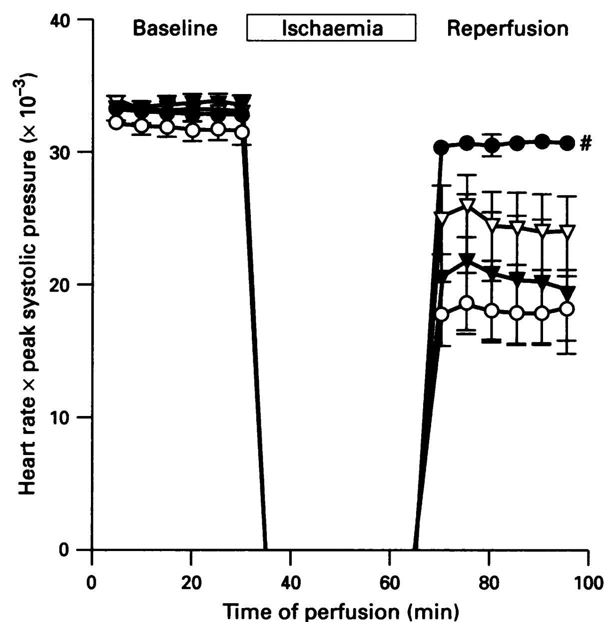

Figure 4 Recovery of mechanical function during reperfusion following 35 min of global ischaemia of working rat hearts (Series 2). Mechanical function was assessed as the product of heart rate and systolic pressure (HR x PSP). Values shown are means ± s.e.mean measured during baseline aerobic perfusion (5 to 30 min), global ischaemia (30 to 65 min) and reperfusion (65 to 95 min) for control hearts (○, $n=37$) and for hearts that were exposed throughout reperfusion to either adenosine (100 μM, ●, $n=13$) or 8-SPT (100 μM, $n=8$) or to adenosine (100 μM) and 8-SPT (100 μM) in combination (▽, $n=19$). A significant difference between the adenosine-treated group and the untreated control group is indicated by #.

ficial effects of adenosine (100 μM) on the recovery of mechanical function (Figure 3). Interestingly, a relatively non-selective adenosine receptor antagonist, 8-(*p*-sulphophenyl)theophylline (8-SPT, 100 μM) when added in combination with adenosine (100 μM) significantly antagonized the adenosine-induced enhancement of the recovery of mechanical function (Figure 4), but had no significant effect (nonparametric ANOVA followed by Dunn's post-tests) on coronary flow during reperfusion (control, 24.3±4.3; adenosine, 36.1±2.0; 8-SPT, 12.8±4.2; and adenosine plus 8-SPT, 27.6±3.8 ml min^{-1}). In contrast to DPCPX, 8-SPT (100 μM) *per se* had no detrimental effect on recovery of function compared to controls (Figure 4).

CHA (0.05 μM) markedly improved the recovery of mechanical function (Table 2, Figures 2 and 5). DPCPX (0.3 μM) antagonized the beneficial effects of CHA (0.05 μM) on the recovery of mechanical function (Figure 5). In the series of hearts that were subjected to ischaemia and reperfusion in the presence of DPCPX, either alone or in combination with CHA, insufficient hearts recovered from the period of global ischaemia to allow measurements of glycolysis and glucose oxidation during reperfusion.

As observed in the hearts of Series 1, the values for baseline rates of glycolysis were similar in all groups during the 30 min period of baseline aerobic perfusion (data not shown). During the 30 min period of reperfusion after 35 min of global ischaemia (Figure 6), the rate of glycolysis in untreated hearts was 1.49±0.17 μmol min^{-1} g^{-1} dry wt ($n=12$). Adenosine (100 μM) reduced glycolysis relative to the time control value by 37% confirming our previous data (Finegan *et al.*, 1992; 1993). CHA (0.05 μM) inhibited glucolysis by 49%. Interestingly, the adenosine-induced inhibition of glycolysis was not altered by DPCPX (0.3 μM), but was prevented by 8-SPT (100 μM). The rate of glycolysis in the presence of adenosine and SPT was significantly higher than that in the presence of adenosine alone but not significantly different from the corrsponding time control value (Figure 6).

During the 30 min period of baseline aerobic perfusion, the rate of glucose oxidation was similar among all groups and to those measured in Series 1. During reperfusion of untreated

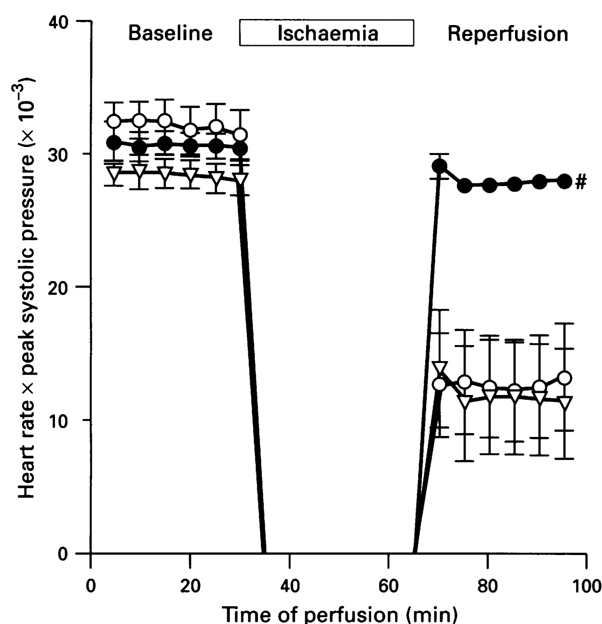

Figure 5 Recovery of mechanical function during reperfusion following 35 min of global ischaemia of working rat hearts (Series 2). Mechanical function was assessed as the product of heart rate and systolic pressure (HR × PSP). Values shown are means ± s.e.mean measured during baseline aerobic perfusion (5 to 30 min), global ischaemia (30 to 65 min) and reperfusion (65 to 95 min) for control hearts (○, $n=16$) and for hearts that were exposed throughout reperfusion to either CHA (0.05 μM, ●, $n=7$) or with CHA (0.05 μM) and DPCPX (0.3 μM) in combination (▽, $n=10$). A significant difference between the CHA-treated group and the untreated control group is indicated by #.

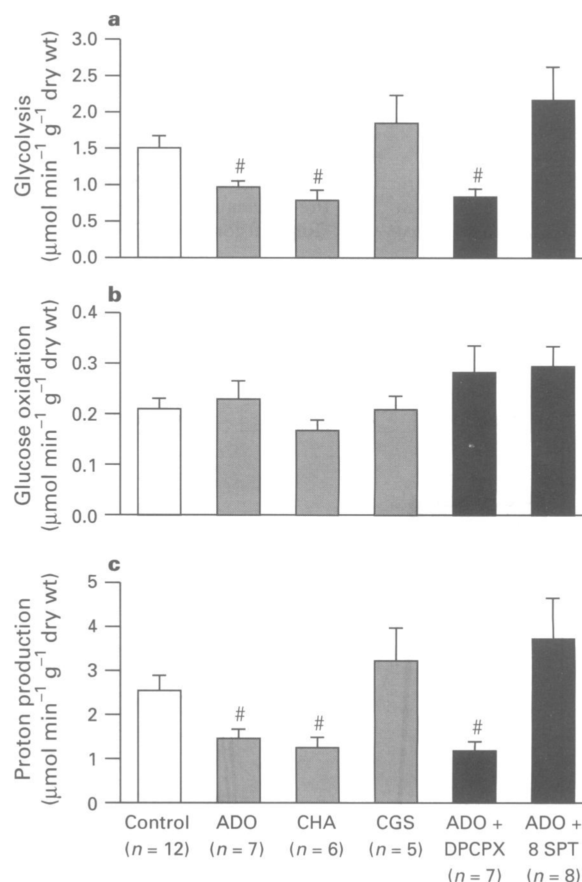

Figure 6 Rates of glycolysis (a), glucose oxidation (b) and proton production (c) from glucose metabolism during reperfusion following 35 min global ischaemia in working rat hearts (Series 2). Values are means ± s.e.mean and are shown for hearts during 30 min of reperfusion of untreated control hearts (open columns) and for hearts that were exposed throughout reperfusion to the indicated drug or drug combination (solid columns). A significant difference between a group during reperfusion and the untreated control group is indicated by #.

control hearts, the rate of glucose oxidation was not different from its aerobic baseline value despite the significantly lower level of mechanical function. Adenosine (100 μM) and CHA (0.05 μM) did not have any effect on glucose oxidation rates during reperfusion.

Calculated rates of proton production attributable to the metabolism of exogenous glucose during baseline aerobic perfusion prior to global ischaemia was similar to that observed in Series 1. During reperfusion of untreated hearts, the rate of proton production was similar to that observed during aerobic baseline perfusion (Figure 6). Adenosine (100 μM) and CHA (0.05 μM) significantly inhibited proton production by 46% and 55%, respectively. DPCPX (0.3 μM) was unable to antagonize the adenosine-induced inhibition of proton production. In contrast, 8-SPT (100 μM) significantly antagonized the adenosine-induced inhibition of proton production so that the rate in the presence of adenosine + 8-SPT was not different from control (Figure 6).

Discussion

This study showed that adenosine and the selective adenosine A_1 receptor agonist, CHA, directly inhibited glycolysis in rat working rat hearts perfused under aerobic conditions and that the effects of these CHA agonists were antagonized by the selective adenosine A_1 receptor antagonist, DPCPX. These data indicate that under aerobic conditions the inhibition of glycolysis by adenosine is by an A_1 receptor-mediated mechanism. In hearts subjected to reperfusion following global ischaemia, adenosine inhibited glycolysis and enhanced recovery of post-ischaemic mechanical function. CHA mimicked these effects of adenosine on mechanical function in a DPCPX-sensitive manner suggesting that an adenosine A_1 cardioprotective mechanism was operative during reperfusion. Antagonism of the metabolic and mechanical effects of adenosine during reperfusion was observed in the presence of the non-selective adenosine A_1/A_2 antagonist, 8-SPT, but not with DPCPX or the non-selective adenosine A_1/A_3 antagonist, BW-A1433. It is unlikely that adenosine A_{2a} receptor stimulation was responsible for the beneficial effects of adenosine during reperfusion following ischaemia as the A_{2a} selective agonist, CGS-21860, given either prior to ischaemia or only at reperfusion, had no beneficial effect. Overall, these data indicate that adenosine A_1 receptor stimulation inhibits glycolysis and causes myocardial protection during reperfusion.

Comparison of results obtained in hearts that were exposed to CHA during ischaemia and throughout reperfusion with those that were treated with CHA only at reperfusion indicates that no greater benefit arose from the presence of the drug during ischaemia. Thus, significant cardioprotection could be elicited by mechanisms that were operative primarily during reperfusion. These data also suggest that an improved coupling between glycolysis and glucose oxidation, thereby reducing proton production during the critical early period of reperfusion, may be of importance in contributing to the cardioprotective actions of adenosine A_1 receptor agonists.

We have shown previously that adenosine, in hearts perfused under aerobic conditions and with relevant concentrations of fatty acids, decreases glycolysis and increases glucose oxidation (Finegan *et al.*, 1992). We have also demonstrated that adenosine, given either prior to low-flow ischaemia and throughout reperfusion or only at the onset of reperfusion after low-flow ischaemia, improves the recovery of mechanical function and that this improvement in function is accompanied by a decrease in glycolysis and lactate accumulation (Finegan

et al., 1993). An important feature of the experimental condition used in our previous studies (Finegan *et al.*, 1992; 1993) and in the current study is the presence in the perfusion fluid of a relevant concentration of free fatty acid (1.2 mM palmitate), in addition to 11 mM glucose, as an energy substrate. Perfusions in the presence of high fat are important, because similar concentrations of fatty acids are seen in the clinical setting of acute myocardial ischaemia (Lopaschuk *et al.*, 1994). Also fatty acids *per se* are detrimental to the recovery of mechanical function due to a preferential inhibition of myocardial glucose oxidation relative to glycolysis, causing an uncoupling that results in elevated proton production from glucose metabolism during reperfusion (Lopaschuk *et al.*, 1993). Thus, this working rat heart model is particularly suited to the study of drug-induced alterations in myocardial energy supply and demand under these post-ischaemic metabolic conditions that directly influence the recovery of mechanical function.

Numerous studies have noted that the pre-ischaemic administration of adenosine and adenosine agonists attenuates reperfusion dysrhythmias (Pantely & Bristow, 1990), enhances mechanical recovery during reperfusion (Lasley *et al.*, 1990; Lasley & Mentzer, 1992; Janier *et al.*, 1993) and reduces infarct size (Toombs *et al.*, 1992; Zhao *et al.*, 1993). The presence of adenosine during both ischaemia and reperfusion does not permit separation between beneficial effects arising either during ischaemia or only during reperfusion. Other studies have discovered that infarct reduction elicited by endogenous adenosine may occur during the early phases of reperfusion (Zhao *et al.*, 1993). Our demonstration of the marked efficacy arising from adenosine A_1 stimulation in enhancing metabolic and mechanical recovery from ischaemic damage when given after the global ischaemic insult indicates that appropriate interventions during reperfusion are sufficient to enhance recovery. Similarly, cardioprotection by adenosine given only at reperfusion has been noted in several species *in vivo* (Olafsson *et al.*, 1987; Babbitt *et al.*, 1989; Pitarys *et al.*, 1991; Norton *et al.*, 1992; Zhao *et al.*, 1993). The data, therefore, imply that adenosine-mimetics may have a wide clinical application in the treatment of myocardial reperfusion injury, rather than being limited to situations allowing only the pre-ischaemic administration in the management of elective ischaemia and reperfusion.

The demonstration that both adenosine and CHA were antagonized by the selective adenosine A_1 receptor antagonist, DPCPX, strongly suggest that under aerobic conditions the adenosine-induced inhibition of glycolysis was mediated by an A_1 receptor mechanism. In addition, the effect of CHA on mechanical function during reperfusion was antagonized by DPCPX. In contrast, DPCPX, at a concentration equivalent to 670 fold its K_d value (0.46 mM) for the adenosine A_1 receptor (Jacobson *et al.*, 1992), failed to block the beneficial effects of adenosine, despite its direct impairment of mechanical recovery. Higher concentrations of DPCPX could not be investigated due to the loss of adenosine A_1 receptor selectivity and to the direct detrimental effects of this antagonist on mechanical function at reperfusion.

Interestingly, 8-SPT *per se*, at a concentration of 100 μM had no direct effect on the recovery of mechanical function during reperfusion, but antagonized the metabolic and mechanical effects of adenosine. While this antagonism may suggest that adenosine could have improved post-ischaemic recovery via an adenosine A_2 mechanism such as coronary vasodilatation (King *et al.*, 1990), this is unlikely for several reasons. First, the selective adenosine A_{2a} receptor agonist, CGS-21680, did not influence rates of glycolysis or glucose oxidation and also did not enhance the recovery of mechanical function during reperfusion. Second, coronary flow in aerobic hearts was not increased by adenosine or by CHA. Also, coronary flows during reperfusion were not significantly affected by adenosine. Coronary flow rates during reperfusion must be interpreted with caution because the actual flow may be a consequence of the extent of recovery rather than being the cause of improved function. If hearts with poor function are excluded from both the adenosine and control groups, coronary flows are similar (36 ± 2 and 33 ± 3 ml min^{-1}, respectively). Thus, the apparent higher flow in the adenosine-treated group relative to the control group, that did not reach statistical significance due to the large and skewed variance in the control group, may be a reflection of the improved functional recovery rather than an adenosine A_2 receptor-mediated vasodilatation. In addition, other studies have shown that a second selective adenosine A_2 receptor agonist, phenylaminoadenosine, when added prior to ischaemia (Lasley & Mentzer, 1992), does not improve post-ischaemic mechanical function in glucose perfused Langendorff rat hearts. Moreover, coronary vasodilatation has been shown to be insufficient by itself to account for the beneficial mechanical effects of adenosine receptor agonists at reperfusion (Nomura *et al.*, 1993). Although it has been demonstrated that adenosine A_2 receptor stimulation during reperfusion *in vivo* reduces infarct size (Norton *et al.*, 1992), suggested mechanisms, that included attenuation of neutrophil- and platelet-mediated damage (Cronstein *et al.*, 1990; Kitakaze *et al.*, 1991), were not operative in our buffer perfused system.

A possible explanation for the inability of DPCPX to antagonize adenosine relates to its poor aqueous solubility. The ratio of solubility to adenosine A_1 receptor affinity, which is an important determinant of antagonist effectiveness (Bruns & Fergus, 1989), is only 3.7 fold higher for DPCPX (37000) relative to 8-SPT (10000). As 8-SPT was present at a much higher concentration (100 μM) than DPCPX (0.3 μM), it functioned as the more effective adenosine A_1 antagonist. Stimulation of adenosine A_3 receptors, initially characterized by Linden (Tucker & Linden, 1993), also elicits an anti-ischaemic action (Lu *et al.*, 1994). No selective adenosine A_3 antagonists have been described. Our failure to block adenosine responses with BW-A1433, a non-selective A_1/A_3 antagonist, suggests that adenosine A_3 receptors were not involved. Alternatively, the concentration of BW-A1433 used (30 μM), which is greater than that required to block A_1-mediated cardioprotection (Lasley & Mentzer, 1993), may have been insufficient to antagonize rat adenosine A_3 receptors. Adenosine A_{2b} receptors may also be involved in the cardioprotective effects of adenosine, but further investigation awaits the development of A_{2b} selective agents. Thus, while the role of adenosine receptor subtypes in the cardioprotective actions of adenosine have not been clearly defined in this study, the ability of CHA to enhance mechanical function in a DPCPX-sensitive manner indicates the cardioprotective role of an A_1 receptor mechanism.

Functional glycolysis has been considered essential to provide ATP and to delay the onset of ischaemic damage and facilitate the return to near normal function during reperfusion (Owen *et al.*, 1990). However, during prolonged global ischaemia, the products of the hydrolysis of ATP produced by glycolysis, in particular protons, are not benign. Intracellular proton accumulation causes an increase in intracellular Na^+, which, via the Na^+-Ca^{2+} exchange mechanism, increases intracellular Ca^{2+} accumulation (Tani & Neely, 1989) and the associated consequences of Ca^{2+} overload. During the critical early period of reperfusion, clearance of the acid load may be impaired by a continued production of protons arising from the uncoupling in glucose metabolism. A lower rate of glycolysis and an improvement in the coupling of glucose metabolism during reperfusion limits proton production, and reduces the potential for further Na^+ and Ca^{2+} overload. This will permit a more rapid restoration of normal ionic gradients during the critical early period of reperfusion and improve the recovery of mechenical function. The degree of inhibition of glycolysis that was observed (approximately 50%) may represent a maximal response to adenosine receptor stimulation as higher concentrations of CHA had similar beneficial effects (data not shown). Nevertheless, this level of efficacy seems adequate to cause significant improvement in mechanical function. The apparent low potency of adenosine in inhibiting glycolysis appears due to its rapid metabolism in the recirculating perfusion system as a considerably lower con-

centration (50 nM) of CHA, that is a poor substrate for adenosine metabolic pathways, was able to mimic the metabolic and mechanical effects of adenosine.

The precise mechanism responsible for the reduction in glycolysis by adenosine A_1 receptor stimulation is unclear. Adenosine, and some adenosine receptor agonists, influence glucose uptake by the heart. Adenosine stimulates glucose uptake in isolated hearts (Angello *et al.*, 1993) and both adenosine and *R*-phenylisopropyladenosine (*R*-PIA) have been reported to increase glycolysis both in normoxic and hypoxic hearts (Wyatt *et al.*, 1989). In contrast, Dale *et al.* (1991) found that *R*-PIA, but not adenosine, decreased glycolysis in hypoxic hearts and in hearts perfused with glucose as the sole substrate. The inhibitory effect of *R*-PIA on glucose transport appeared to be nonspecific and unrelated to its adenosine A_1 receptor agonists properties. Our results with CHA are unlikely to arise from a similar nonspecific mechanism as DPCPX was able to antagonise the effects of CHA on the recovery of mechanical function in reperfused hearts in Series 2. Further confirmation comes from the observation that DPCPX could antagonize the CHA-induced inhibition of glycolysis and proton production under aerobic conditions in Series 1.

A possible transduction mechanism for the inhibitory effect of adenosine on glycolysis may involve reduced intracellular Ca^{2+} levels, possibly by an action on K^+_{ATP} channels that when opened cause membrane hyperpolarization and lessen Ca^{2+} entry. In support, K^+_{ATP} channels are functionally coupled to adenosine A_1 receptors in rat myocytes (Kirsch *et al.*, 1990). Also, K^+_{ATP} channel openers posses direct cardioprotective effects (Grover, 1994) and adenosine-mediated effects are antagonized by inhibition of K^+_{ATP} channel opening (Toombs *et al.*, 1993). Whether this mechanism explains the effects of adenosine on glycolysis when given only at reperfusion requires further investigation because most studies have examined only the cardioprotective efficacy of K^+_{ATP} channel openers when given prior to ischaemia.

In conclusion, adenosine and CHA, when administered at the onset of reperfusion after global no-flow ischaemia, inhibited glycolysis and this was associated with an enhanced recovery of mechanical function. The adenosine A_1 receptor antagonist, DPCPX, antagonized the metabolic effects of CHA in aerobic hearts and prevented the beneficial effects of CHA on mechanical function during reperfusion. These results suggest that the cardioprotective effects of adenosine A_1 receptor stimulation are not a consequence of stimulation of glycolysis but rather support the hypothesis that inhibition of glycolysis and proton production from glucose metabolism, that decrease the potential for Ca^{2+} accumulation, may be important in mediating the beneficial effects of adenosine A_1 receptor stimulation.

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