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Protection from myocardial stunning by ischaemia and hypoxia with the adenosine A_3 receptor agonist, IB-MECA

Helen L. Maddock[a,1], Neil M. Gardner[a], Nassirah Khandoudi[b,2], Antoine Bril[b,3], Kenneth J. Broadley[a,*]

[a] *Department of Pharmacology, Welsh School of Pharmacy, Cardiff University, King Edward VII Avenue, Cathays Park, Cardiff, Wales CF10 3XF, UK*
[b] *GlaxoSmithKline Laboratoires Pharmaceutiques, 4 rue du Chesnay-Beauregard, 35762 Saint-Grégoire, France*

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Abstract

Guinea pig isolated working hearts were exposed to 30-min ischaemia by reducing coronary flow to 10%, followed by reperfusion. Aortic output fell to 4.5 ± 4.5% of the pre-ischaemic value at reperfusion, recovering to 48.2 ± 14.6% at 20-min post-reperfusion; the index of myocardial stunning. IB-MECA (N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide, 3×10^{-7} M), infused from 10 min into ischaemia, did not affect recovery of aortic output 20 min after reperfusion (41.9 ± 1.9%). IB-MECA infused at reperfusion, however, significantly protected against stunning, aortic output recovering to 79.6 ± 3.9% at 20-min post-reperfusion. Hypoxic gassing (5% CO_2 in nitrogen, 30 min) of guinea pig isolated paced left atria and papillary muscles reduced the developed tension, recovering to 75% 5 min after re-oxygenation. This myocardial stunning was unaffected by IB-MECA (3×10^{-7} M) added 10 min into hypoxia. IB-MECA added at reoxygenation significantly improved recovery, which was prevented by the adenosine A_3 receptor antagonist, 1-propyl-3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)phenylxanthine (I-ABOPX, 1×10^{-5} M). Thus, stimulation of adenosine A_3 receptors at reperfusion/reoxygenation in guinea pig cardiac preparations protects against myocardial stunning.

Keywords: Adenosine A_3 receptor; Ischaemia; Hypoxia; Stunning; Heart, isolated, working; Papillary muscle; Atrium; Cardioprotection

1. Introduction

Myocardial ischaemia and subsequent restoration of normal coronary blood flow causes profound changes in contractile function and electrical activity of the heart (Opie, 1992; Heusch, 1992). The ventricular dysfunction following a period of cardiac ischaemia and reperfusion is known as myocardial stunning (Braunwald and Kloner, 1982). Providing there is no major irreversible damage, including tissue necrosis, e.g. infarction, the myocardial contractile function gradually improves when coronary flow is restored (Hess and Kukreja, 1994). Stunning may be important clinically in high-risk conditions such as reperfusion after thrombolysis following acute myocardial infarction, unstable angina, cardiac surgery with cycloplegic arrest and cardiac transplantation (Hess and Kukreja, 1994).

Endogenous adenosine is released during hypoxia and ischaemia and there is an accumulating evidence that it can exert cardioprotection against myocardial stunning (Mubagwa and Flameng, 2001). Enhancement of endogenous adenosine levels by inhibition of adenosine deaminase with deoxycoformycin (pentostatin) (McClanahan et al., 1995) or by inhibition of adenosine uptake with *p*-nitrobenzylthioinosine (Abd-Elfattah et al., 1993) improves the recovery of the stunned myocardium following coronary artery occlusion. Exogenous adenosine has been shown to augment post-ischaemic recovery in dog (Randhawa et al., 1993, 1995) and rabbit (Ogawa et al., 1996) hearts in situ. Adenosine also attenuates myocardial stunning in isolated perfused hearts (Lasley and Mentzer, 1995). Ischaemic contracture of isolated hearts is an index of Ca^{2+} overload

* Corresponding author. Tel.: +44-29-20-875832; fax: +44-29-20-874149.
E-mail address: BroadleyKJ@Cardiff.ac.uk (K.J. Broadley).
[1] School of Science and the Environment, Coventry University, Priory Street, Coventry CV1 5FB, UK.
[2] Research Centre, Za de Courtaboeuf, 25 Avenue de Quebec, Les Ulis 91940, France.
[3] LPPCE, Faculty of Medicine, 7 Bd Jeanne d'Arc, BP 87900, 21033 Dijon cedex, France.

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and exogenous adenosine has also been shown to protect against this contracture (Randhawa et al., 1993).

Endogenous adenosine also reduces the size of infarction following ischaemia and reperfusion by receptor-mediated mechanisms and this cardioprotection is most pronounced during the early phase of reperfusion (Zhao et al., 1993). Adenosine and adenosine analogues have been shown in several models to limit the size of the infarct induced by ischaemia/reperfusion (Toombs et al., 1992).

Adenosine receptors have been divided into A_1, A_{2A}, A_{2B} and A_3 subtypes (Fredholm et al., 2001; Dalziel and Westfall, 1994). The cardioprotective effects of endogenous adenosine are thought to be mediated via adenosine A_1 receptors and are mimicked by administration of adenosine A_1 receptor agonists prior to ischaemia (Randhawa et al., 1993; Lasley and Mentzer, 1995). The adenosine A_{2A} receptor-selective agonist, CGS21680 (2-*p*-((carboxyethyl)-phenethylamino)-5′-carboxyamidoadenosine), administered 5 min before reperfusion has also been shown to reduce infarct size after coronary artery ligation (Jordan et al., 1997). It also improved recovery of contractility of guinea pig isolated working hearts when administered from 10 min into a period of low-flow ischaemia followed by reperfusion (Maddock et al., 2001). Pretreatment of rabbit (Tracey et al., 1997; Hill et al., 1998) or rat (Thourani et al., 1999) isolated perfused hearts with an adenosine A_3 receptor agonist protects the heart against experimental infarction or contractile dysfunction, respectively. The adenosine A_3 receptor-selective agonist, IB-MECA (N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide), has also been shown to attenuate stunning and infarction after coronary occlusion in conscious rabbits (Auchampach et al., 1997b). Preconditioning is a phenomenon whereby a brief period of ischaemia protects the heart against a subsequent more substantial ischaemic insult (Parratt, 1994). This has been mimicked by pretreatment of human isolated atrial trabeculae with IB-MECA (Carr et al., 1997) and with N^6-(3-chlorobenzyl)-5′-*N*-methylcarboxamidoadenosine (CB-MECA) in rabbit isolated hearts through a mechanism involving K_{ATP} channels (Tracey et al., 1998).

In the present study, we examine the effects of the adenosine A_3 receptor agonist, IB-MECA, upon myocardial stunning of guinea pig isolated cardiac tissues induced by low-flow ischaemia or hypoxia followed by reperfusion/reoxygenation. In contrast to the previous studies cited above (Tracey et al., 1997, 1998; Thourani et al., 1999; Auchampach et al., 1997b; Carr et al., 1997), which examined the cardioprotective effects of adenosine A_3 agonists added prior to the ischaemic exposure as a preconditioning stimulus, in the present study, IB-MECA was introduced either during ischaemia or at reperfusion/reoxygenation. We consider that this is more clinically relevant since a myocardial infarction is rarely foreseen. Only in one other recent study has protection from infarct by an adenosine A_3 receptor agonist been demonstrated when administered at reperfusion, but cardiac contractility was not monitored (Maddock et al., 2002a). Since, the coronary vasculature and its endothelium may have a role in any cardioprotection by adenosine analogues, we removed the coronary endothelium from working hearts. Our previous studies have shown that this process does not affect the response to ischaemia and reperfusion of the working heart (Maddock et al., 2002b). To examine the effects of adenosine A_3 receptor activation without the complication of any coronary vascular components, IB-MECA was also examined in guinea pig isolated atria and papillary muscles in which myocardial stunning was induced by hypoxia.

2. Methods

2.1. Animals

Male Dunkin–Hartley guinea pigs were maintained in accordance with NIH guidelines (publication 85-23) and the Animals (Scientific Procedures) Act 1986 for animal care, and procedures were approved by the local ethics committees.

2.2. Isolated working hearts

Guinea pigs (300–380 g) were anaesthetized by intraperitoneal injection of sodium thiopental (50 mg kg^{-1} i.p.) and the heart quickly excised and immersed into heparinized ice cold saline solution. The aorta was dissected free and the endothelium was denuded by applying a short blast of O_2 via the aorta. This was achieved by cannulating the aorta with a tube attached to a small oxygen cannister and delivering dry oxygen at 2–5 psi for 15 s at room temperature. Previous experiments have established that this method is effective for removal of the vascular endothelium, as demonstrated by the substantial reduction of the coronary vasodilator response to acetylcholine (10^{-6} M) (Maddock et al., 2002b). Hearts de-endothelialized by this method also show an identical profile of responses to ischaemia and reperfusion (Maddock et al., 2001). The aorta was then cannulated for perfusion in the Langendorff retrograde mode with a filtered modified Krebs–Henseleit buffer containing (mM): NaCl 118; KCl 4.7; $NaHCO_3$ 25; $MgCl_2$ 1.2; KH_2PO_4 1.2; glucose 11; pyruvate 0.5 and $CaCl_2$ 1.25. The perfusion solution was continuously gassed with an O_2/CO_2 (95/5%) mixture (pH 7.4) and maintained at 37 °C throughout the experiment.

Retrograde perfusion of the heart started immediately at a perfusion pressure of 80 cm H_2O for 10 min and then switched to perfusion according to the working-heart technique (Neely et al., 1967). Preload was held at 10 cm H_2O and afterload was maintained at 80 cm H_2O. The perfusate was not recirculated and the hearts were allowed to equilibrate for 15 min before the beginning of the different interventions.

Global mechanical function was continuously recorded by a catheter-tipped manometer (Millar Instruments, Houston, TX, USA) placed into the left ventricle. Left ventricular pressure; the maximum rate of ventricular pressure rise (dP/dt_{max}) and heart rate were acquired directly on a recorder (Gould Electronics, Cleveland, OH, USA). Aortic output and coronary flow were determined volumetrically and cardiac output was calculated as the sum of aortic output and coronary flow.

2.3. Isolated left atria and papillary muscles

Guinea pigs (250–300 g) were killed by a Home Office approved Schedule 1 method using concussion of the brain by a blow to the head followed by exsanguinated under running water. The rib cage was opened and the left atrium was removed with cotton threads attached to the tip of the left atrial appendage and atrioventricular junction. The latter thread attached the atrium to the electrode tips of a Harvard bipolar platinum electrode (Harvard, Edenbridge, Kent, UK) and the first thread was attached to an isometric transducer (Devices, Welwyn Garden City, UK, type UFI, 57 g sensitivity range). A discrete papillary muscle was exposed in the left ventricle by cutting along the interventricular septum. One thread of cotton was placed around the chordae tendinaeae of the papillary muscle and another through its apical end. The papillary muscle was then dissected free of the ventricle. One of the threads was attached to an isometric transducer while the other allowed the muscle to be held in close contact with bipolar platinum electrodes. The tissues were immersed in 50-ml organ baths containing Krebs-bicarbonate solution of the following composition (mM) in distilled water: NaCl, 118: KCl, 4.69: $CaCl_2 2H_2O$, 2.52: $MgSO_4 7H_2O$, 1.18: $KH_2PO_4 2H_2O$ 1.18: $NaHCO_3$, 25: glucose, 11.7, which was maintained at 37 ± 0.5 °C and gassed with 5% CO_2 in oxygen. Both atria and papillary muscles were paced at 2 Hz with square-wave pulses of 5-ms duration at a voltage of 50% above threshold, delivered by a Harvard Research 50-72 stimulator. The resting tensions applied to the tissues were adjusted to give optimum tension development, which occurred in the range 0.5–1.0 g. Isometric tension was recorded on a Devices MT8P polygraph.

2.4. Experimental protocols

2.4.1. Isolated hearts

Before treatment, each heart was allowed to stabilise for 15 min to determine baseline values for haemodynamic parameters. Low-flow ischaemia was induced by a reduction in cardiac afterload from 59 to 7.8 mm Hg by adjustment of the Starling resistance, thus causing a reduction in coronary flow by about 90% (Pijl et al., 1993). Global low-flow ischaemia was maintained for 30 min during which temperature was kept at 37 °C and then

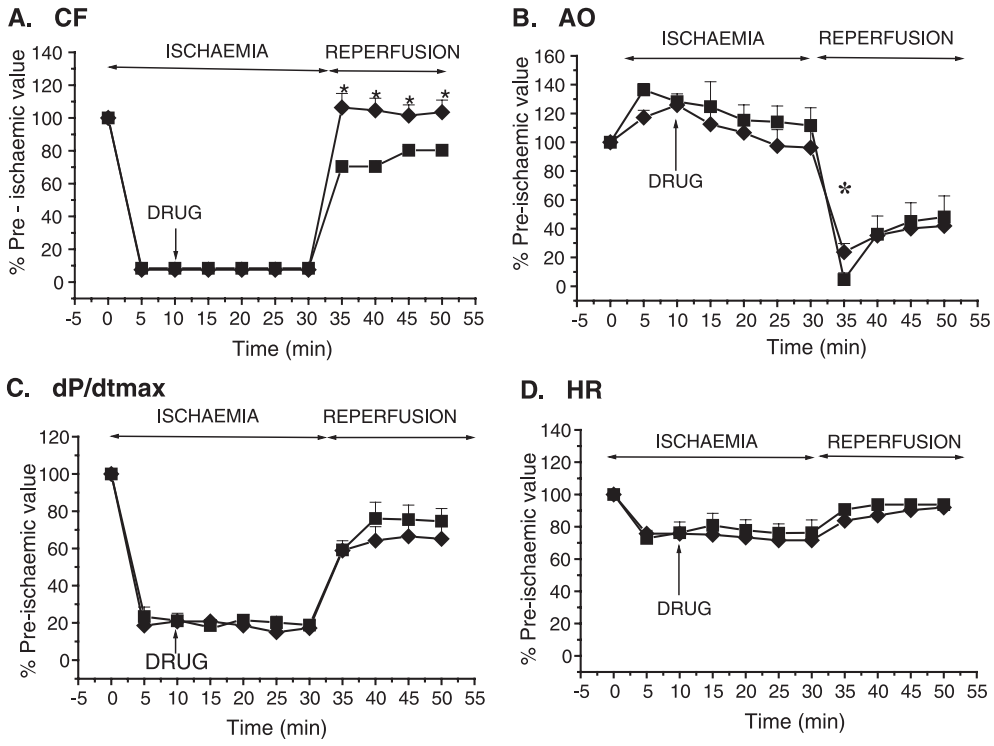

Fig. 1. Effects of IB-MECA (♦, 3×10^{-7} M), or a control in absence of agonist (■) on (A) coronary flow (CF) (B) aortic output (AO) (C) dP/dt_{max} and (D) heart rate (HR) of guinea pig working hearts. Infusions of agonist were commenced at 10 min during a 30-min period of low-flow ischaemia and continued throughout the 20-min reperfusion period. Each point represents the mean ($n = 5$) change from the pre-ischaemic level expressed as a percentage of the pre-ischaemic level (± S.E.M.). Significant differences from control after reperfusion are indicated by $^*P < 0.05$.

Table 1
Effects of an infusion of IB-MECA on various parameters of guinea pig working hearts measured at 5 and 20 min after reperfusion following a 30-min period of ischaemia

	Time (min)	Aortic output	Coronary flow	Cardiac output	Left ventricular pressure	dP/dt_{max}	Heart rate
Control	5	4.5 ± 4.5	70.4 ± 3.2	19.9 ± 5.4	71.4 ± 5.2	59.2 ± 4.9	90.6 ± 2.9
	20	48.2 ± 14.6	80.4 ± 2.3	55.8 ± 12.0	87.9 ± 2.4	74.6 ± 1.8	93.7 ± 1.6
IB-MECA (10 min)	5	23.8 ± 6.0[a]	106.4 ± 8.5[a]	43.1 ± 0.1[a]	81.5 ± 3.9	58.9 ± 2.1	83.7 ± 5.1
	20	41.9 ± 1.9	103.5 ± 7.4[a]	56.4 ± 1.9	88.7 ± 5.3	65.1 ± 7.0	91.9 ± 2.4
IB-MECA (reperfusion)	5	59.0 ± 8.0[a]	77.2 ± 11.0	63.9 ± 7.0[a]	79.2 ± 1.9	62.7 ± 4.4	88.9 ± 3.6
	20	79.6 ± 3.9[a]	109.2 ± 9.6[a]	88.4 ± 2.6[a]	90.3 ± 2.1	74.0 ± 5.0	90.4 ± 4.0

IB-MECA (3×10^{-7} M) was infused from 10 min into the 30-min period of ischaemia or at the onset of reperfusion in endothelium-denuded working hearts. All results are expressed as the mean ($n = 4/5$) change expressed as a percentage of the pre-ischaemic level (± S.E.M.). Statistical significance was performed by analysis of variance (ANOVA) followed by a Student–Newman–Keuls test.

[a] $P < 0.05$ vs. control.

followed by reperfusion for 20 min in the working heart mode. IB-MECA (3×10^{-7} M) was infused at a flow rate of 10% of the basal coronary flow using a low-flow infusion pump (Harvard Apparatus, Type 11 Digital Infusor). Infusions of IB-MECA were started either at 10 min after the initiation of low-flow ischaemia or at the same time as reperfusion was commenced. In both cases, the infusion was maintained throughout the remainder of the experiment.

2.4.2. Isolated atria and papillary muscles

Left atria and papillary muscles were allowed to equilibrate for 15 min with regular changes of the bathing solution. Hypoxia was induced by switching the 5% CO_2 in 95% oxygen gassing the bathing medium to 5% CO_2 in 95% nitrogen followed by several changes in bathing medium. After 30 min of hypoxic gassing, the tissue was reoxygenated by returning to gassing with 5% CO_2 in 95% oxygen. IB-MECA (3×10^{-7} M) was added to the bath either at 10 min into the hypoxic period or at reoxygenation. Control experiments received the appropriate volume of vehicle (polyethylene glycol 400/distilled water). The adenosine A_3 receptor antagonist, 1-propyl-3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)phenylxanthine (I-ABOPX, 1×10^{-5} M) (Linden, 1994) was introduced to the tissue bath immediately prior to induction of hypoxia and remained until the end of the experiment. The effect of I-ABOPX alone or of IB-MECA, added at reoxygenation, in the presence of I-ABOPX was examined.

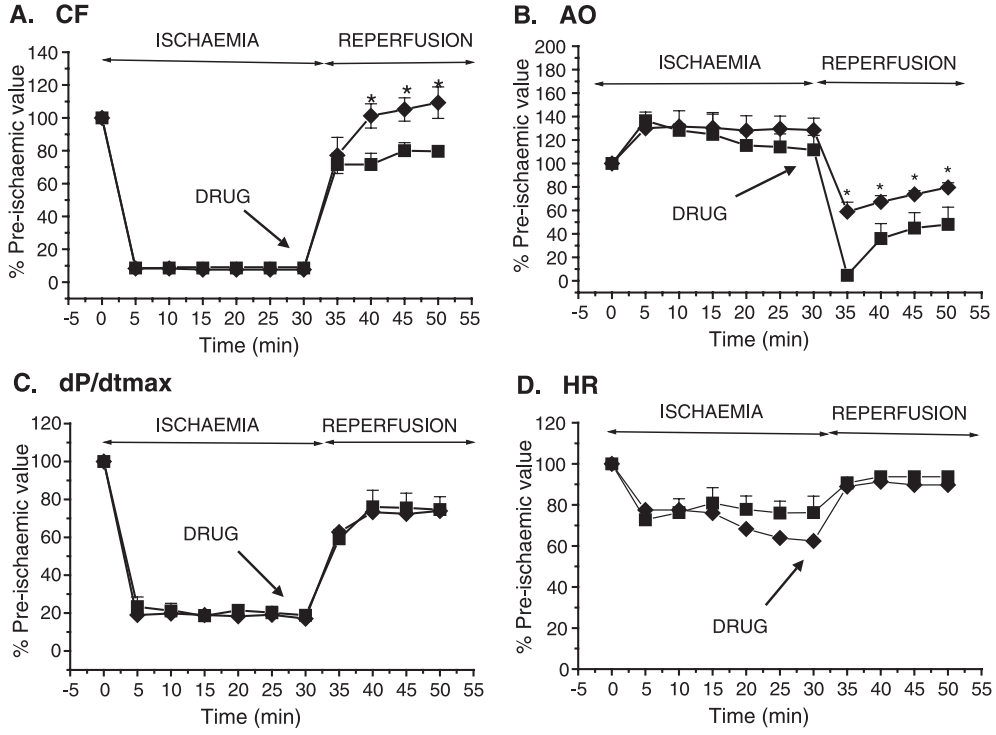

Fig. 2. The effects of IB-MECA (◆, 3×10^{-7} M), or a control in the absence of agonist (■) on (A) coronary flow (CF) (B) aortic output (AO) (C) dP/dt_{max} and (D) heart rate (HR) in guinea pig working hearts. Infusions of agonist were commenced at the onset of reperfusion following a 30-min period of low flow. Each point represents the mean ($n = 5$) change from the pre-ischaemic level expressed as a percentage of the pre-ischaemic level (± S.E.M.). Significant differences from the control during reperfusion indicated by $^*P < 0.05$.

Concentration–response curves for IB-MECA and N^6-cyclopentyladenosine (CPA), as a reference adenosine A_1 receptor selective agonist, were constructed in normally oxygenated isolated atria or papillary muscles following a 15-min equilibration period. Doses were added to the tissue bath cumulatively once the response to the previous dose had reached its plateau effect.

2.5. Drugs

IB-MECA (N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide) was synthesised in house in the Chemistry Department at GlaxoSmithKline, Renne, France and I-ABOPX (1-propyl-3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)phenylxanthine) was kindly provided by Dr. Julian Reeves as a gift from GlaxoSmithKline, Stevenage, Hertfordshire, UK. N^6-cyclopentyladenosine (CPA) was purchased from Sigma, Poole, Dorset, UK. IB-MECA, I-ABOPX and CPA were made up as stock solutions (10^{-3} M) in 50:50 polyethylene glycol 400/distilled water. Serial dilution of IB-MECA was made in Krebs solution and an infusion of Krebs vehicle at 1 ml min^{-1} to working hearts caused no change in parameters other than a small transient injection artefact.

2.6. Statistical analysis

All results are expressed as mean ± S.E.M. for *n* experiments. Statistical analysis was performed using analysis of variance (ANOVA) followed by a Student–Newman–Keuls test for working hearts. In atria and papillary muscles, Student's unpaired *t*-test was used for two-group comparisons, whereas ANOVA followed by a Bonferroni post hoc test was used for multiple comparisons where the same vehicle control was employed. A *P* value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Effects of IB-MECA on low-flow ischaemia and reperfusion of working hearts

A 30-min period of low-flow ischaemia reduced the coronary flow (Fig. 1A) and cardiac contractility (left ventricular pressure, dP/dt_{max}) (Fig. 1C), and to some extent the heart rate (Fig. 1D), but not aortic output (Fig. 1B). On reperfusion, aortic output and cardiac output fell precipitously to 4.5 ± 4.5% and 19.9 ± 5.4% of the pre-ischaemic level at 5 min into the reperfusion, followed by a gradual recovery to 48.2 ± 14.6% and 55.8 ± 12.0% at 20-min of reperfusion (Fig. 1B, Table 1). At 5 min of reperfusion, coronary flow, left ventricular pressure and dP/dt_{max} recovered to 70.4 ± 3.2%, 71.5 ± 5.2% and 59.2 ± 4.9%, respectively, the latter remained at 74.6 ± 6.8% of the pre-ischaemia level at 20 min after reperfusion.

IB-MECA (3×10^{-7} M) was perfused into working hearts commencing at 10 min into the 30-min period of low-flow ischaemia and continuing throughout the subsequent reperfusion. At reperfusion, there was a significant increase in coronary flow at 5 min of reperfusion (106.4 ± 8.5%) in the presence of IB-MECA compared to the control (70.4 ± 3.2%) (Fig. 1A). IB-MECA significantly accelerated the recovery of aortic pressure and cardiac output measured 5 min after the onset of reperfusion (23.8 ± 6.0% and 43.1 + 4.1%, respectively) compared with the control hearts (4.5 ± 4.5% and 19.9 + 5.4%) (Fig. 1B; Table 1). At 20 min after reperfusion commenced, however, aortic pressure (41.9 ± 1.9%) and cardiac output (6.4 + 1.9%) were no different from controls (48.2 ± 14.6% and 55.8 + 12.0%, respectively). The other parameters remained similar to the control in the presence of IB-MECA under these conditions (Fig. 1C,D; Table 1).

Experiments were also conducted in which the infusion of IB-MECA was commenced at the time of onset of reperfusion following low-flow ischaemia. At 5 min after reperfusion, there was no significant increase in coronary flow (77.2 ± 11.0%) in the presence of IB-MECA compared

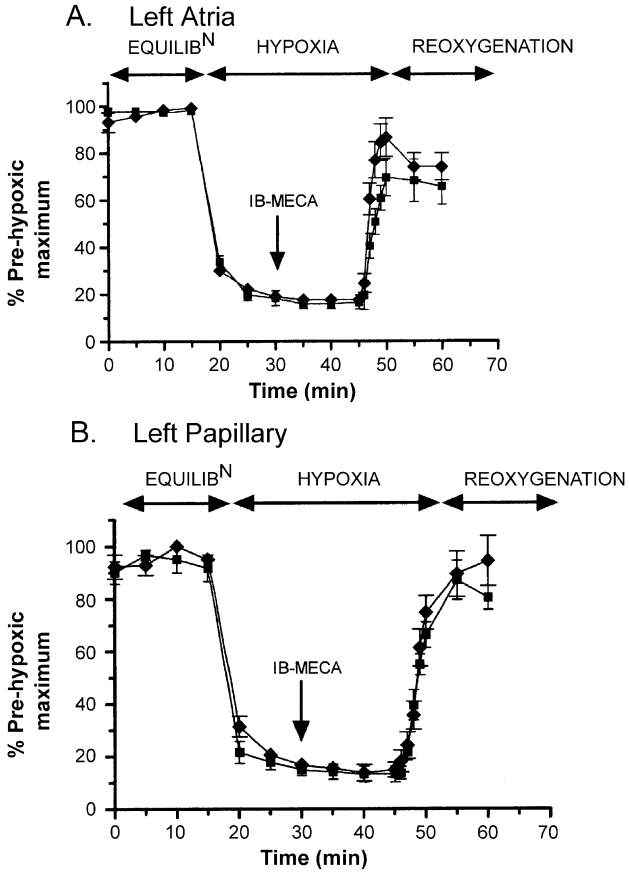

Fig. 3. The effects of IB-MECA (◆, 3×10^{-7} M) added at 10 min into a 30-min period of hypoxia on the recovery of (A) left atria ($n = 4$) and (B) papillary muscles ($n = 4$) caused by reoxygenation compared to controls (■). Each point is the mean (± S.E.M.) developed tension expressed as a percentage of the pre-hypoxia maximum tension.

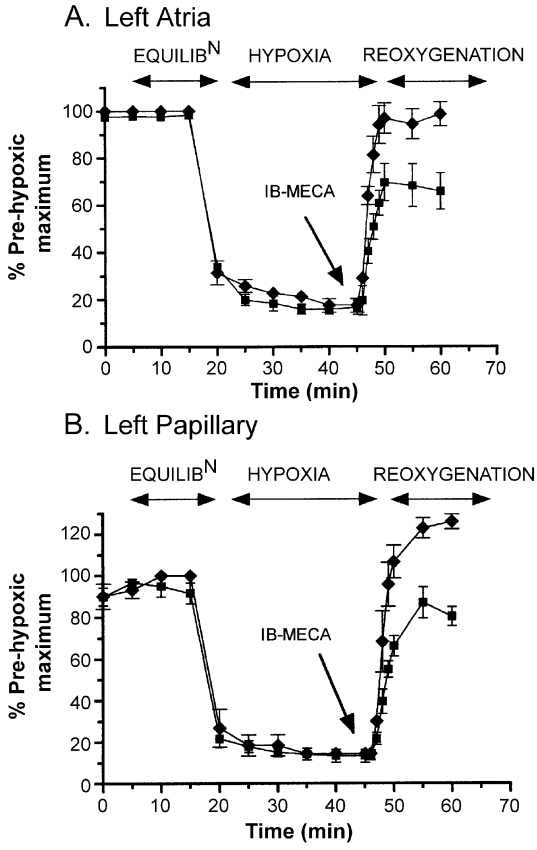

Fig. 4. The effects of IB-MECA (♦, 3×10^{-7} M) added at reoxygenation on the recovery of (A) left atria ($n=4$) and (B) papillary muscles ($n=4$) from a 30-min hypoxic period compared to controls (■). Each point is the mean (± S.E.M.) developed tension expressed as a percentage of the pre-hypoxia maximum tension.

to the control (70.4 ± 3.2%) (Fig. 2A). At 10 min after commencing reperfusion, however, there was a significant increase in coronary flow (101.3 ± 7.4%) compared to the control (70.4 ± 3.2%). IB-MECA given at the onset of reperfusion was also observed to significantly protect against the fall in aortic output (59.0 ± 8.0%) and cardiac output (63.9 ± 7.0%) at 5 min after reperfusion, compared with controls (4.5 ± 4.5% and 19.9 ± 5.4%). The recovery of aortic output and cardiac output was also significantly improved to 79.6 ± 3.9% and 88.4 ± 2.6% at 20 min (compared with controls of 48.2 ± 14.6% and 55.8 ± 12.0%) (Fig. 2B). dP/dt_{max} and heart rate remained similar to the control in the presence of IB-MECA under these conditions (Fig. 2C,D, Table 1). There was a modest but nonsignificant improvement in left ventricular pressure at 5 min into reperfusion (79.1 ± 1.9%) compared with the control (71.4 ± 5.2%) (Table 1).

3.2. Effect of IB-MECA on hypoxia-induced stunning in atria and papillary muscles

Gassing of isolated left atria and papillary muscle with 5% CO_2 in nitrogen instead of 5% CO_2 in oxygen caused falls in developed tension to 16.9 ± 3.4% and 20.3 ± 1.6% of the pre-hypoxic level, respectively. This maximum decrease in tension was reached 20–25 min into the hypoxic period in both left atria and papillary muscle. On reoxygenation, the developed tension returned to 75.4 ± 3.9% and 78.5 ± 5.5% of the pre-hypoxic value in left atria and papillary muscles, respectively, at 5 min. Similar changes occurred in controls with vehicle (polyethylene glycol/water) added 10 min into the hypoxia.

IB-MECA (3×10^{-7} M) added at 10 min into the hypoxic exposure did not affect the fall in developed tension during hypoxia compared to vehicle controls, the values at the end of hypoxia being 17.6 ± 1.3% and 13.8 ± 3.1% of the pre-hypoxic level in left atria and papillary muscles, respectively. On reoxygenation, no significant ($P>0.05$) improvement in developed tension over control values was observed, the values for left atria and papillary muscles being 86.5 ± 8.2% and 74.7 ± 6.3%, respectively, at 5 min compared with vehicle controls (69.4 ± 7.9% and 66.3 ± 4.9%). At 15 min, recovery was to 74.0 ± 5.8% and 94.4 ± 9.5% for left atria and papillary muscles, respectively, compared with vehicle controls (65.7 ± 7.7% and 80.4 ± 4.4%) (Fig. 3).

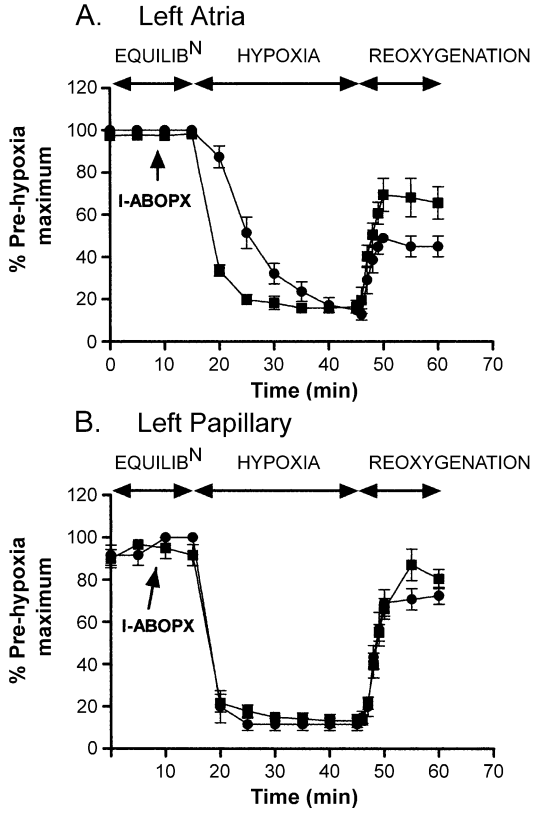

Fig. 5. The effects of I-ABOPX (●, 1×10^{-5} M) added 10 min prior to hypoxia and throughout the remainder of the experiment on the recovery of (A) left atria ($n=4$) and (B) papillary muscles ($n=4$) from a 30-min hypoxic period compared with controls exposed similarly to vehicle (polyethylene glycol 400/water, 50:50%; ■). Each point is the mean (± S.E.M.) developed tension expressed as a percentage of the pre-hypoxia maximum tension.

When IB-MECA (3×10^{-7} M) was added immediately prior to reoxygenation, however, there was improved recovery in developed tension in both left atria and papillary muscles on reoxygenation after the 30-min episode of hypoxia (Fig. 4). The developed tensions were 96.7 ± 6.5% and 106.7 ± 7.8% of the pre-hypoxic values at 5 min of reoxygenation in left atria and papillary muscles, respectively, compared with the vehicle controls (69.4 ± 7.9% and 66.3 ± 4.9%). After 15 min of reoxygenation, the recovery of developed tensions in both left atria (98.5 ± 5.1%) and papillary muscles (125.9 ± 3.5%) were significantly ($P<0.05$) greater than in vehicle controls (65.7 ± 7.7% and 80.4 ± 4.4%, respectively).

The adenosine A_3 receptor antagonist, I-ABOPX (1×10^{-5} M), added immediately prior to hypoxia, did not significantly ($P>0.05$) affect either the speed or extent of recovery from hypoxia in both the left atria and the papillary muscles, when compared with the vehicle controls (polyethylene glycol 400/water, 50:50%) (Fig. 5). The developed tensions at 15 min into reoxygenation were 45.0 ± 4.9% and 72.5 ± 3.9% in atria and papillary muscles in the presence of I-ABOPX and 65.7 ± 7.7% and 80.4 ± 4.4%, respectively, in the presence of the vehicle (polyethylene glycol 400/water, 50:50%).

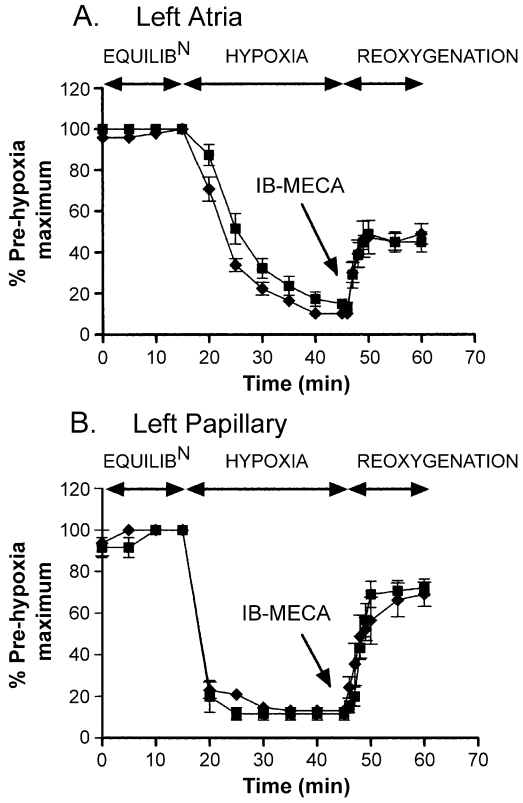

Fig. 6. The effects of IB-MECA (3×10^{-7} M) added at reoxygenation in the presence of I-ABOPX (♦, 1×10^{-5} M) on the recovery of (A) left atria ($n=4$) and (B) papillary muscles ($n=4$) from a 30-min hypoxic period compared to tissues exposed to I-ABOPX alone (■, 1×10^{-5} M). I-ABOPX (1×10^{-5} M) was added at 10 min prior to hypoxia and throughout the remainder of the experiment. Each point is the mean (± S.E.M.) developed tension expressed as a percentage of the pre-hypoxia maximum tension.

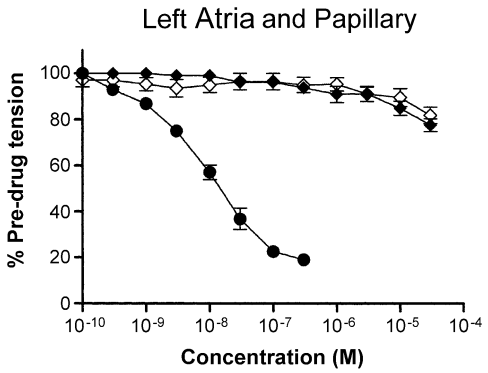

Fig. 7. Concentration–response curves for IB-MECA on guinea pig isolated left atria (◇) and left papillary muscles (♦) and for CPA on left atria (●). Responses are plotted as the reduction in developed tension expressed as a percentage of the resting developed tension (± S.E.M.).

IB-MECA (3×10^{-7} M) added at reoxygenation in the presence of I-ABOPX (1×10^{-5} M), did not significantly affect ($P>0.05$) either the speed or extent of recovery from the 30-min hypoxia in either left atria or papillary muscles (Fig. 6). The developed tension of isolated atria at 15 min into reoxygenation was 48.9 ± 4.9% in the presence of IB-MECA and I-ABOPX, compared with 45.0 ± 4.9% for the I-ABOPX control. In papillary muscles, the developed tension at 15 min into reoxygenation in the presence of IB-MECA and I-ABOPX was 69.2 ± 5.6%, compared with 72.5 ± 3.9% for the I-ABOPX control.

IB-MECA added cumulatively to normally oxygenated left atria and papillary muscles caused small falls in developed tension reaching 74.7 ± 1.9% and 86.0 ± 3.8% of resting developed tension, respectively, at the maximum concentration employed (3×10^{-5} M) (Fig. 7). This compared with the adenosine A_1 receptor-selective agonist, CPA, which caused concentration-related inhibition of atrial developed tension, the maximum inhibition being 24.4 ± 3.6% of developed tension, with an IC_{50} value of 1.5(1.0–2.0) $\times 10^{-8}$ M.

4. Discussion

The impaired contractility and metabolic dysfunction of the heart caused by ischaemia and reperfusion has been termed myocardial stunning (Braunwald and Kloner, 1982). There is incomplete recovery of function even though coronary perfusion may return to normal. In the present study, we used aortic output and cardiac output (the sum of aortic output and coronary flow) as indexes of cardiac performance in working hearts. Aortic output fell almost to zero at the onset of reperfusion and recovered to only 50% of the pre-ischaemic level at 20 min of reperfusion, indicating myocardial stunning. Cardiac output returned to

55% of the pre-ischaemia level at 20 min after reperfusion. Left ventricular pressure and dP/dt_{max} also failed to recover to their pre-ischaemic levels. The hearts were denuded of endothelium in this study by passing a blast of oxygen through the coronary vasculature prior to setting the heart up. We have previously demonstrated the effectiveness of this procedure for removal of the vascular endothelium (Maddock et al., 2002b) and that it does not modify the response to ischaemia or the degree of recovery (Maddock et al., 2001). Although adenosine A_3 receptors have not yet been identified on endothelial cells, the possibility could not be discounted that the adenosine A_3 receptor-selective agonist, IB-MECA (Gallo-Rodriguez et al., 1994), may exert endothelium-dependent effects on the myocardial stunning. Thus, IB-MECA was examined in endothelium-denuded preparations.

4.1. Effect of IB-MECA introduced during low-flow ischaemia or at reperfusion

IB-MECA administered continuously from 10 min into the ischaemic period produced a significant increase in coronary flow throughout reperfusion and produced a small significant protection from the immediate fall in aortic output and cardiac output at 5 min after reperfusion. By 20 min of reperfusion, however, there was no significant effect on aortic output, cardiac output or the other indices of cardiac function. It can therefore be concluded that adenosine A_3 receptor stimulation commenced during ischaemia/hypoxia has no sustained beneficial effect on myocardial function. When IB-MECA was infused into the guinea pig working heart preparation at the onset of reperfusion, there was substantial protection against the immediate falls in aortic and cardiac output. Furthermore, aortic output and cardiac output recovered more completely at 20 min after reperfusion. The other parameters of left ventricular contractility were either unaffected (dP/dt_{max}) or only modestly improved (left ventricular pressure). Aortic output is a more sensitive index of cardiac function in working hearts than left ventricular pressure or $+dP/dt_{max}$. Nifedipine, for example, has been shown to be a significantly more potent negative inotropic agent when measured from aortic output than either left ventricular pressure or $+dP/dt_{max}$ (Pijl et al., 1993), with the dose–response relationship for improvement of aortic output lying to the left of those for other parameters. If this is the case for IB-MECA, it would explain the minimal improvement in the other parameters at the dose of IB-MECA employed. The substantial improvement of aortic output indicates that IB-MECA had protected against the myocardial stunning. Other studies with working hearts also use cardiac output, the sum of coronary flow and aortic output, as an index of cardiac function (Pijl et al., 1993). In the present study, cardiac output was a further indication of the improved recovery of cardiac function by IB-MECA. Coronary flow during reperfusion was also enhanced when IB-MECA was administered during ischaemia and at reperfusion. This could be explained by the improved cardiac output, although it does not explain the persistent improved coronary flow when IB-MECA was administered during ischaemia. The reason for this increased coronary flow during reperfusion remains unclear.

4.2. Effect of IB-MECA on hypoxia-induced stunning

When isolated atria and papillary muscles were exposed to a hypoxic insult, contractility was reduced to 20% of the pre-hypoxic level. Reoxygenation resulted in partial recovery to about 75% of the initial resting level, indicative of a stunned myocardium. The mechanisms underlying myocardial stunning are poorly understood but the process has been shown to be attenuated by Ca^{2+} channel antagonists (Heusch, 1992). These appear to exert cardioprotection by blocking the transient cellular Ca^{2+} overload that immediately follows reperfusion. Consequently, this overload, which may be associated with contracture, could be responsible for the myocardial stunning (Schulz et al., 1995). The onset of contracture has been shown to be delayed in rat isolated perfused hearts by adenosine (Lasley and Mentzer, 1993) and by the adenosine A_1 receptor agonist, *R*-phenylisopropyladenosine (*R*-PIA) (Lasley et al., 1990). Moreover, exogenous adenosine has been shown to augment post-ischaemic recovery in dog hearts in situ (Randhawa et al., 1993. In the present study, the introduction of IB-MECA to isolated atria and papillary muscles during hypoxia failed to improve myocardial contractility at reoxygenation. When the IB-MECA was administered at reoxygenation, however, there was an improved recovery of contractility, which was restored to the pre-hypoxia level.

These results have demonstrated that activation of adenosine A_3 receptors by the selective agonist, IB-MECA, exerts a protective effect against ischaemia-induced myocardial stunning in working hearts when measured from an improved aortic output and cardiac output and hypoxia-induced stunning in isolated cardiac tissues when measured from the tension development. These effects occurred when the agonist was infused at the onset of reperfusion in whole hearts or when added at reoxygenation in myocardial muscle preparations. There was no lasting protection when it was introduced earlier during ischaemia or hypoxia. The protection afforded in isolated cardiac tissues rules out a mechanism involving any coronary vasodilator action of IB-MECA in the working heart. It is also supported by the observation of cardioprotection by adenosine A_3 receptor agonists from cell injury induced by simulated ischaemia in cultured chick embryo myocytes overexpressing human adenosine A_3 receptors (Dougherty et al., 1998) and in neonatal rat myocytes (Safran et al., 2001). IB-MECA may not be selective for adenosine A_3 receptors at the concentration used, since IB-MECA at 300 nM can activate human adenosine A_{2A} receptors (Murphree et al., 2002) and an increase in coronary flow by IB-MECA in rat Langendorff

isolated hearts has been reported and attributed to adenosine A_{2A} receptor stimulation (Lasley et al., 1999). However, the increase in coronary flow at reperfusion in the present study was unlikely to be due to adenosine A_{2A} receptor activation since no improvement of coronary flow has been observed in guinea pig normoxic working hearts (Maddock et al., 2002b). Activation of adenosine A_1 receptors by IB-MECA at 3×10^{-7} M could also be discounted since it was clear that at this concentration there was no negative inotropy in isolated atria (Fig. 7). Furthermore, activation of adenosine A_1 and A_{2A} receptors by IB-MECA at 3×10^{-7} M is unlikely since we have shown previously that in isolated normoxic working hearts it has no effect on coronary flow (adenosine A_{2A} receptors), left ventricular pressure, aortic pressure or heart rate (adenosine A_1 receptors) (Maddock et al., 2002b). Thus, in guinea pig hearts in the present study, adenosine A_1 and A_{2A} receptors do not appear to mediate the protection from stunning.

Protection from myocardial stunning by IB-MECA can be concluded to be mediated via adenosine A_3 receptors and this was confirmed by the fact that the improved recovery of isolated atria and papillary muscles by IB-MECA was abolished by the adenosine A_3 receptor-selective antagonist, I-ABOPX (1×10^{-5} M) (Linden 1994). I-ABOPX alone had no effect on the recovery from hypoxia, indicating that endogenous adenosine was unlikely to exert any protection via adenosine A_3 receptors. It is known that adenosine itself has low affinity for adenosine A_3 receptors (Jacobson et al., 1995) which would explain this lack of action even though adenosine levels would be expected to be raised in hypoxia. I-ABOPX is unlikely to block adenosine A_1 receptors at this concentration since it has a 16-fold adenosine A_3/A_1 selectivity in binding assays (Auchampach et al., 1997a). The pA_2 value of I-ABOPX against the functional tissue response of IB-MECA-induced mast cell degranulation is 6.2 (Reeves et al., 1997), which is consistent with the concentration used in the present study to produce a substantial blockade. An increase in concentration of I-ABOPX by a further 10-fold would therefore be required to produce any blockade of adenosine A_1 receptors. I-ABOPX also has affinity for adenosine A_{2A} and A_{2B} receptors (Linden et al., 1999) and could therefore have blocked these receptors in the present study. It is unlikely, however, that adenosine A_{2A} receptors are involved in the protection from stunning of atria and papillary muscles since the adenosine A_{2A} receptor-selective agonist, CGS21680, at 10^{-7} M, when added at 10 min into hypoxia, failed to improve recovery. The developed tensions at 5 min of reoxygenation were $88.1 \pm 6.9\%$ and $82.1 \pm 6.2\%$, respectively, of the prehypoxic basal levels, compared with the controls ($77.4 \pm 5.0\%$ and $82.3 \pm 7.7\%$). Further support that adenosine A_3 receptors are involved in the protection from stunning in guinea pig isolated atria is our finding that the cardioprotection by IB-MECA is prevented by an alternative adenosine A_3 receptor-selective antagonist, MRS-1220 (Yates and Broadley, 2003).

Adenosine receptor stimulation is believed to be involved in ischaemic preconditioning (Hori et al., 1993; Parratt, 1994; Broadley, 2000; Mubagwa and Flameng, 2001) which protects the myocardium against a subsequent more-prolonged ischaemic insult. Pretreatment of rabbit (Tracey et al., 1997; Hill et al., 1998) or rat (Thourani et al., 1999) isolated perfused hearts with an adenosine A_3 receptor agonist protected the heart against experimental infarction or contractile dysfunction, respectively, and therefore to mimic pre-conditioning. Pretreatment with the adenosine A_3 receptor-selective agonist, IB-MECA, has also been shown to attenuate stunning and infarction after coronary occlusion in conscious rabbits (Auchampach et al., 1997a,b) and to mimic cardioprotection by preconditioning in human isolated atrial trabeculae (Carr et al., 1997). In contrast to these studies, however, the hearts of adenosine A_3 receptor knock-out mice were more resistant to ischaemia and reperfusion injury and the early preconditioning by ischaemic exposures was not prevented as might be predicted if adenosine A_3 receptors were involved (Cerniway et al., 2001; Guo et al., 2001). In all of the above studies showing cardioprotection, the adenosine A_3 receptor-selective agonist was administered *before* the exposure to ischaemia and thus mimics preconditioning. In the present study, however, we administered the adenosine A_3 receptor-selective agonist either during ischaemia/hypoxia or at reperfusion/reoxygenation. In only one previous study has a reduction of infarct size been observed when the adenosine A_3 receptor-selective agonist, 2Cl-IB-MECA (1-[2-chloro-6-[[(3-iodophenyl)methyl]amoni]-9*H*-purin-9-yl]-1-deoxi-*N*-methyl-*b*-D-ribofuranuronamide), was administered at reperfusion but cardiac contractility was not recorded (Maddock et al., 2002a). We considered that it was more clinically relevant to administer an agent to improve recovery of myocardial function after the ischaemic insult than before the event since myocardial infarction is rarely anticipated. The novel finding of this study was therefore that cardioprotection by IB-MECA was only seen when it was administered at reperfusion (hearts) or reoxygenation (isolated atria and papillary muscles). It was not apparent when administered from 10 min into the ischaemia or hypoxia. This is an interesting observation since one would expect the protection to persist during the reperfusion period, the IB-MECA remaining present throughout the reperfusion/reoxygenation period. A possible explanation for this phenomenon is that the adenosine A_3 receptors undergo desensitization with prolonged exposure and this effect occurs when the agonist is added early in the hypoxic/ischaemic period. Agonist-induced desensitization of recombinant human adenosine A_3 receptors occurs within 20 min and is associated with phosphorylation of the C-terminal of the receptor by G-protein receptor-coupled kinases (Jacobson, 1998). The improved coronary flow, however, occurred whether IB-MECA was introduced at reperfusion or early in the ischaemic period. This suggests that desensitization of the coronary flow response does not occur and that it is distinct from

the adenosine A_3 receptor-mediated improvement in cardiac function. Indeed, no tachyphylaxis of the coronary vasodilator effect of IB-MECA in normoxic rat hearts was reported (Lasley et al., 1999).

The mechanism for the improved contractile function of isolated working hearts, isolated atria and isolated papillary muscles following ischaemia or hypoxia remains to be established. Adenosine A_3 receptors do not appear to mediate other myocardial responses in the normoxic heart, which raises the possibility that they might not exist under normoxic conditions but only in hypoxia or ischaemia. The answers to these questions could have implications in terms of understanding cardioprotection by adenosine A_3 receptor activation and how this process can be manipulated for therapeutic benefit.

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