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Preconditioning of rat hearts by adenosine A_1 or A_3 receptor activation

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Abstract

Our study in rat hearts examined whether activation of adenosine A_1 or A_3 receptors improved functional recovery and reduced apoptosis resulting from low-flow ischemia. Prior to 30 min low-flow ischemia (0.6 ml/min; 6% of baseline flow), Langendorff rat hearts were preconditioned with two 5-min cycles of (a) ischemia (PC; $n=7$), (b) infusion of 250 nM adenosine A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA; $n=6$), or (c) infusion of 50 nM adenosine A_3 receptor agonist N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyl-uronamide (IB-MECA; $n=8$). Recovery of function was improved in PC ($71 \pm 3\%$), CCPA ($68 \pm 6\%$) and IB-MECA ($68 \pm 4\%$) groups compared to control hearts ($46 \pm 5\%$; $P<0.05$). Cumulative release of total purines during ischemia–reperfusion was approx. 50% lower in PC, CCPA and IB-MECA groups compared to controls ($P<0.05$) and was significantly correlated to the percentage functional recovery ($R^2=0.55$; $P<0.05$). The number of cytosolic histone-associated-DNA fragments, a hallmark of apoptosis and measured by Enzyme Linked ImmunoSorbent Assay (ELISA), was small and not different between groups after 30 min reperfusion. However, CCPA (0.6 ± 0.1 absorbance units) and MECA (0.7 ± 0.1 units; $P<0.05$ vs. PC) decreased apoptosis after 150 min reperfusion compared to PC (1.4 ± 0.3 units) and control (1.2 ± 0.1 units) hearts. This study shows that adenosine triggers protection of function in preconditioned rat hearts via both the adenosine A_1 and A_3 receptor. In clinical practice, pharmacological stimulation of adenosine A_3 receptors may be advantageous over adenosine A_1 receptor activation due to a lack of contractile side-effects. In contrast to ischemic preconditioning, pharmacological stimulation of adenosine A_1 or A_3 receptors reduced apoptosis. Furthermore, total purine release may serve as a marker of the degree of functional protection.

Keywords: Adenosine A_1 receptor; Adenosine A_3 receptor; Apoptosis; Ischemic preconditioning; (Rat); Ischemia

1. Introduction

Ischemic preconditioning refers to the paradoxical mechanism that short, pre-emptive periods of ischemia protect the heart from a subsequent period of prolonged ischemia (Murry et al., 1986). This phenomenon seems to occur in all vertebrates including humans (Cleveland et al., 1997; Yellon et al., 1993). Of the humoral factors released during its induction that may trigger the event, adenosine is one of the most important. In most animal species and humans (Carr et al., 1997; Lee et al., 1995; Leesar et al., 1997), adenosine's cardioprotective effects are mediated via adenosine A_1 receptors located on the myocardial membrane. The recently discovered adenosine A_3 receptor has also been implicated in preconditioning of chick (Liang and Jacobson, 1998; Stambaugh et al., 1997; Strickler et al., 1996), rabbit (Auchampach et al., 1997b; Hill et al., 1998; Rice et al., 1996; Tracey et al., 1998; Wang et al., 1997), and human (Carr et al., 1997) hearts. However, adenosine-mediated cardioprotection is believed to play no role in rat hearts (Ganote and Armstrong, 2000). In contrast, we (De Jonge et al., 1998, 2001) and others (Ford et al., 1998; Headrick, 1996) observed beneficial effects of adenosine A_1 receptor stimulation in this species. To the best of our knowledge, no study has examined before the role of the adenosine A_3 receptor in preconditioning of rat hearts. However, a one study (Thourani et al., 1999) observed improved functional recovery and reduced creatine kinase release after 30 min no-flow ischemia in isolated rat hearts pretreated with a selective adenosine A_3 receptor agonist for 12 min without a washout period. Apart from reduced ventricular arrhythmias, infarct size, and contractile dysfunctioning, ischemic preconditioning has also been shown to reduce apoptosis in vitro (Gottlieb et al., 1996) and in vivo (Piot et al., 1997). No data exist as to whether ischemic preconditioning effects on apoptosis are also mim-

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icked by stimulation of adenosine A_1/A_3 receptors prior to ischemia. Thus, this study examined the effectiveness of both adenosine A_1 and A_3 receptor stimulation in reducing contractile dysfunction and apoptosis in rat hearts after low-flow ischemia and reperfusion. This study shows that the adenosine A_3 receptor, in addition to the adenosine A_1 receptor, is involved in preconditioning of rat hearts. Although the degree of apoptosis was low, pharmacological preconditioning reduced its occurrence.

2. Methods

All animals were treated in conformation with the guiding principles in the care and use of animals as approved by the American Physiological Society. The Animal Welfare Committee, Erasmus University Rotterdam, approved the protocol.

2.1. Exclusion criteria

During stabilization, hearts were excluded if they met one of the following criteria: (1) unstable contractile function, (2) coronary flows outside the range of 9–19 ml/min, (3) severe arrhythmias, (4) myocardial temperature outside the range 37–39 °C.

2.2. Isolated heart preparation

Fed, male Wistar rats (Wag/Rij inbred, weighing 280–330 g) were obtained from Harlan-CPB (Zeist, The Netherlands). They received a commercial rat chow (Hope Farms AM II, Woerden, The Netherlands) and tap water ad libitum. After anesthesia with an intraperitoneal injection of 0.6 ml sodium pentobarbital (Nembutal®, 60 mg/ml) supplemented with 0.1 ml heparin (Thromboliquine®, 5000 IU/ml), hearts were rapidly excised and arrested in saline (0 °C) until beating ceased. Excess tissue was removed, and the hearts were cannulated within 1 min via the ascending aorta, for retrograde perfusion using a non-recirculating Krebs–Henseleit buffer containing (in mmol/l): NaCl 118, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and D-glucose 11. Insulin (Sigma, St. Louis, MO, USA; 1 U/l) was added to the buffer. Before use, the buffer was filtered through a 45-μm porosity filter to remove any particulate matter, and equilibrated with 5% CO_2/95% O_2, to give a pH of about 7.4 at 37 °C. Myocardial temperature was kept at 37 °C with a water-jacketed heart chamber and buffer reservoir, and regulated with an electric heating coil positioned around the aortic inlet line. The temperature of the outer ventricular wall was monitored with a thermocouple (A-F6, Ellab, Roedovre, Denmark). Global, no-flow ischemia was induced by clamping the aortic line; low-flow ischemia was achieved using a perfusion pump (MV-MS3, Ismatec, Zurich, Switzerland) operating at a flow rate of 0.6 ml/min.

Coronary flow was measured by timed collection of the pulmonary artery effluent. The hearts were allowed to beat spontaneously. Cardiac contractile function was estimated with a force transducer (LVS-50GA, Kyowa Electronic Instruments, Tokyo, Japan) connected to the apex of the heart (De Jonge et al., 1998; Owen et al., 1990). The heart was pre-loaded with an initial resting tension of 2*g*. Systolic tension and diastolic tension were continuously displayed on a recorder (Gould signal conditioner and Gould WindoGraf™ recorder, Valley View, OH, USA). Developed tension was calculated as systolic tension *minus* diastolic tension. Cardiac contractile function was expressed as rate-force product, the product of heart rate and developed tension. We showed before that the recovery of rate-force product after ischemia–reperfusion correlates with the amount of creatine kinase released at reperfusion (De Jonge et al., 1998). Rate-force product was expressed as a percentage of baseline function (20 min stabilization). Perfusion pressure was measured with a disposable pressure transducer (Braun Melsungen, Melsungen, Austria) and kept constant at 65 mm Hg.

2.3. Experimental protocol

After initial isolation and surgical preparation, all hearts were perfused with the modified Krebs–Henseleit buffer and allowed to equilibrate for 20 min followed by a 20-min treatment period. Thereafter, hearts were subjected to 30 min of low-flow ischemia (0.6 ml/min) followed by a reperfusion period of 30 min. The 20-min treatment period prior to low-flow ischemia consisted of (Fig. 1): (1) nor-

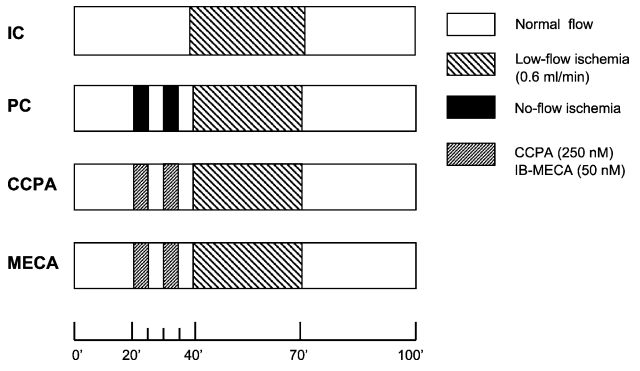

Fig. 1. Diagram showing the different experimental protocols. Each experiment started with a 20-min stabilization period followed by a 20-min treatment period. Thereafter, hearts were subjected to 30 min of low-flow ischemia (0.6 ml/min) followed by a reperfusion period of 30 min. The 20-min treatment period prior to low-flow ischemia consisted of: (1) normoxic perfusion (IC group); (2) preconditioning using two 5-min episodes of no-flow ischemia each interrupted by 5 min of reperfusion (PC group); (3) preconditioning with two 5-min infusions of adenosine A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA group), interspersed by two 5-min periods of drug-free perfusion; (4) preconditioning with two 5-min infusions of adenosine A_3 agonist N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyl-uronamide (IB-MECA group), interspersed by two 5-min periods of drug-free perfusion.

moxic perfusion (IC group; $n=7$); (2) preconditioning using two 5-min episodes of no-flow ischemia each interrupted by 5 min of reperfusion (PC group; $n=7$); (3) preconditioning with two 5-min infusions of 250 nM of the selective adenosine A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA group; $n=6$), interspersed by two 5-min periods of drug-free perfusion; and (4) preconditioning with two 5-min infusions of 50 nM of the selective adenosine A_3 receptor agonist N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (IB-MECA; $n=8$), interspersed by two 5-min periods of drug-free perfusion. The used concentrations of CCPA and IB-MECA were based on their binding activities for (brain) adenosine receptors as indicated by the supplier (CCPA: $K_iA_1=0.4$ μM vs. $K_iA_2=3900$ μM; IB-MECA: $K_iA_3=1.1$ nM vs. $K_iA_1/A_2=50-60$ nM). After 30 min reperfusion, a part of the heart was flash frozen in liquid nitrogen. In a parallel study, hearts were also freeze-clamped after 150 min reperfusion for determination of the degree of apoptosis ($n=4$ per group).

2.4. Analysis of coronary effluent

During ischemia and reperfusion, coronary perfusate samples were continuously collected at 2-, 3-, 5-, or 10-min intervals, depending on the changes expected. Prior to ischemia, several 1-min samples were taken. Lactate in the samples, kept at 0 °C, was determined enzymatically with an Elan auto-analyzer (Eppendorf, Merck, Amsterdam, The Netherlands) according to Sigma procedure 735. The remainder of the samples was stored at −80 °C until further analysis. Purines in coronary perfusate samples were determined by reversed phase high-performance liquid chromatography (HPLC) according to Smolenski et al. (1990). Briefly, a C_{18} column (Hypersil ODS 3 μm, 150 × 4.6 mm, Alltech, Deerfield, IL, USA) was employed combined with a C_{18} guard column (Hypersil ODS 5 μm, 7.5 × 4.6 mm). The system configuration consisted of an AS3000 cooled autosampler, an SCM1000 vacuum membrane degasser, a P2000 gradient pump, a Spectra Focus forward optical scanning detector, and PC1000 software (Spectra-Physics, San Jose, CA, USA). Peaks were detected at 254 nm (hypoxanthine, xanthine, inosine, adenosine) and at 280 nm (uric acid). Perfusate purines were identified based on their co-elution with standards, retention times, and their 254:280 ratios.

2.5. Sandwich enzyme immunoassay

Quantization of DNA fragmentation into cytosolic mononucleosomes and oligonucleosomes was done with an Enzyme Linked ImmunoSorbent Assay (ELISA) kit (Cell Death Detection ELISA^{PLUS}; Boehringer Mannheim, Germany). This test quantifies histone-associated DNA fragments in a sandwich-enzyme-immunoassay using mouse monoclonal antibodies directed against DNA and histones, respectively. At the end of reperfusion, atria were removed and ventricles were frozen in liquid nitrogen and stored at −55 °C. Hearts were ground under liquid nitrogen using pestle and mortar. From the total homogenate, 50 mg was added to 800 μl lysis buffer supplied with the kit and incubated for 30 min at room temperature (ca. 20 °C). After incubation, the homogenate was centrifuged at 13,000 × *g* for 20 min. The supernatant fraction was further diluted 12-fold in phosphate-buffered saline (in mmol/l: NaCl 137, KCl 2.7, Na_2HPO_4 8.1, KH_2PO_4 1.5; pH 7.4) and used as antigen in the ELISA which was performed according to the manufacturer's instructions. Duplicate values of the double absorbance measurements (405–492 nm) were averaged from which the negative control (incubation buffer in stead of sample solution) was substracted.

2.6. Chemicals

CCPA and IB-MECA were obtained from RBI (Natick, MA, USA). Stock solutions (100 μM) of CCPA in deionized water and IB-MECA in dimethylsulfoxide were diluted 400 and 2000 times, respectively, in the perfusion medium. Stock solutions were kept at −55 °C.

2.7. Statistical analysis

The data are expressed as means ± S.E.M., with n = number of hearts. Summary measures were constructed for contractile parameters, lactate release, and total purine release (Matthews et al., 1990). Recovery of rate-force product was expressed as a percentage of baseline value. The sum of lactate and total purines produced during ischemia was calculated as the cumulative release in the venous effluent during 30 min underperfusion and the first 5 min of reperfusion. One-way or two-way analysis of variance with subsequent Student–Newman–Keuls post-hoc tests were used for comparisons between groups. If values were not normally distributed or variances between groups were unequal, Kruskal–Wallis ANalysis Of Variance (ANOVA) on ranks was used. Values of $P<0.05$ (two-tailed test) were regarded as significant.

3. Results

3.1. Contractile function and coronary flow

Since preconditioning may affect recovery of developed pressure and heart rate to a different degree (Bradamante et al., 1993), the rate-force product will be presented in this study as a reliable index of contractile functioning. After 20 min stabilization, there were no differences between groups in rate-force product (mean and S.E.M. of all groups: 4510 ± 224 g/min) or coronary flow rate (10 ± 1 ml/min). Infusion of IB-MECA did not significantly affect preischemic rate-force product (Fig. 2) but increased coronary flow up to 70% (Fig. 3). In contrast, CCPA reduced rate-force product by 83% (Fig. 2) mainly due to a reduction in heart

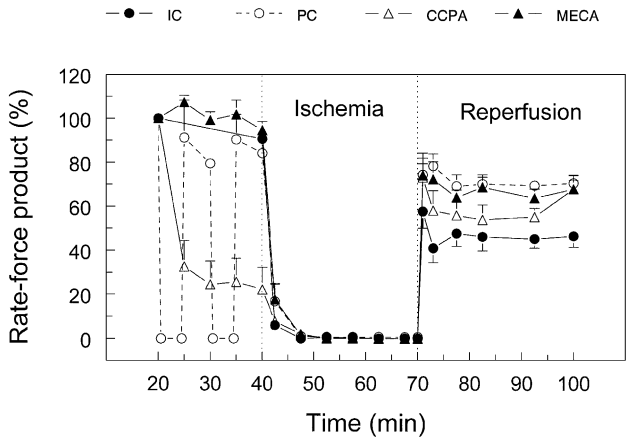

Fig. 2. Changes in rate-force product. In contrast to CCPA, IB-MECA did not affect preischemic contractility. Recovery of rate-force product at the end of reperfusion was significantly improved in PC, CCPA and IB-MECA hearts compared to IC, the controls ($P<0.05$). For abbreviations and protocol, see Fig. 1.

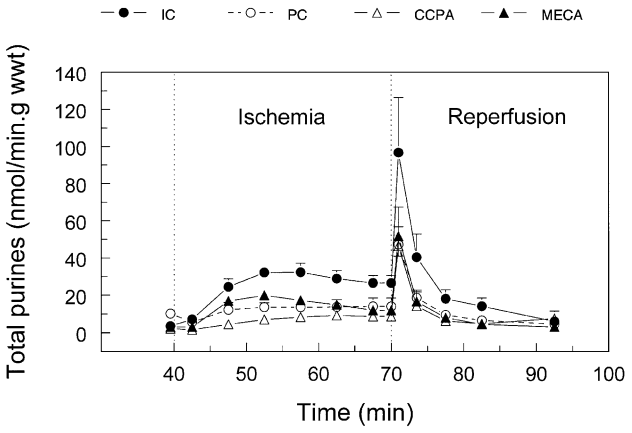

Fig. 4. Release of total purines (adenosine + inosine + hypoxanthine + xanthine + urate) in the coronary effluent. Both pharmacological and ischemic preconditioning reduced the cumulative amount of total purines released during ischemia and reperfusion compared to control hearts ($P<0.05$). For abbreviations and protocol, see Fig. 1.

rate; coronary flow increased during the first infusion (76%) but was reduced 20% vs. pre-drug value during the second infusion (Fig. 3). In the PC group, rate-force product rapidly fell to zero during transient ischemia and recovered to 79% after the second period of reperfusion (Fig. 2). Contractile function fell to 0 within 5 min in all groups at the start of prolonged ischemia. After 30 min reperfusion, rate-force product was improved in PC ($71 \pm 3\%$), CCPA ($68 \pm 6\%$), and MECA ($68 \pm 4\%$) groups compared to controls ($46 \pm 5\%$; $P<0.05$). Early during reperfusion after prolonged ischemia, the extent of reactive hyperemia was similar in all groups (Fig. 3). Coronary flow recovered to normal values after 30 min reperfusion (coronary flow data for 100 min normoxic perfusion not shown) and was not different between groups. Resting tension did not significantly change throughout the protocol in any of the groups.

3.2. Metabolite release

The appearance of purines in the coronary effluent reflects ATP catabolism during ischemia and, hence, the energy status of the cell. Fig. 4 depicts the release of total purines (adenosine + inosine + hypoxanthine + xanthine + urate) throughout the protocol. Cumulative release of total purines (in nmol/g wwt) during long ischemia and reperfusion was approx. 50% lower in PC (672 ± 184), CCPA (486 ± 165) and IB-MECA (690 ± 109) hearts compared to controls (1330 ± 184; $P<0.05$). To determine whether purine release during ische-

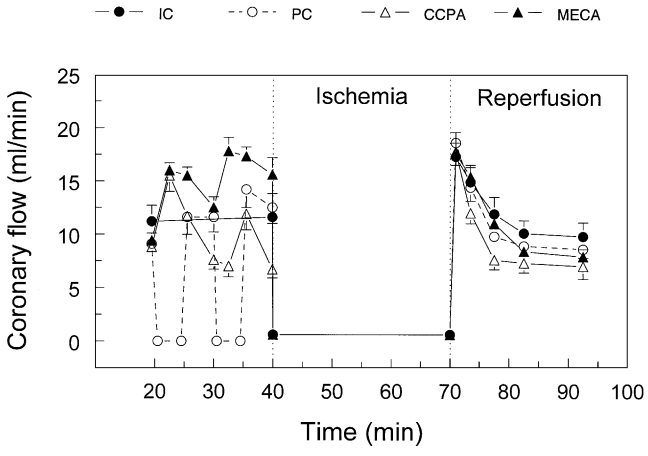

Fig. 3. Changes in coronary flow. IB-MECA infusion resulted in increased coronary flow. Compared to baseline, coronary flow was increased during the first CCPA infusion but was reduced during the second. During the 20-min period of stabilization and during recovery following ischemia, coronary flow was the same between groups. For abbreviations and protocol, see Fig. 1.

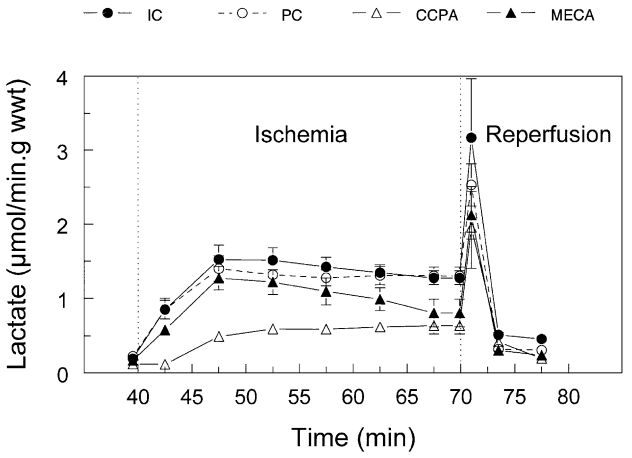

Fig. 5. Release of lactate in the coronary effluent. Lactate release during prolonged ischemia and reperfusion was similar in the IC, PC and IB-MECA groups but was significantly reduced in CCPA hearts ($P<0.05$). For abbreviations and protocol, see Fig. 1.

mia can serve as a marker for the degree of ischemic injury, we correlated recovery of function at the end of reperfusion with the cumulative release of total purines during prolonged ischemia. There was a significant inverse correlation between both variables ($R^2 = 0.55$; $P < 0.05$).

Lactate released in the coronary effluent was taken as measure of anaerobic glycolysis from both endogenous glycogen and exogenous glucose sources. Lactate produced during prolonged underperfusion was not different between IC, PC, and IB-MECA groups (Fig. 5). The sum of lactate released during underperfusion and reperfusion (in μmol/g wwt) was not different between IC (44.5 ± 4.4), PC (40.3 ± 4.5), and IB-MECA (36.6 ± 5.7) groups but was halved in CCPA treated hearts (19.4 ± 2.3; $P < 0.05$ vs. all other groups).

3.3. Apoptosis

The number of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes), a hallmark of apoptosis, was determined by ELISA. Fragmentation of DNA was similar in PC (0.9 ± 0.1 arbitrary absorbance units), CCPA (0.6 ± 0.1 units), IB-MECA (0.8 ± 0.1 units), and IC (0.7 ± 0.1 units) hearts after 30 min reperfusion (Fig. 6). Apoptosis was higher after 150 min reperfusion compared to 30 min reperfusion ($P = 0.001$). Pharmacological preconditioning with CCPA (0.6 ± 0.1) and IB-MECA (0.7 ± 0.1; $P < 0.05$ vs. PC) decreased apoptosis compared to PC (1.4 ± 0.3) and IC (1.2 ± 0.1). The degree of apoptosis appears to be small in hearts subjected to low-flow ischemia and reperfusion since fragmentation in control hearts perfused normoxically for 2 min (NC (2 min); 0.2 units; $n = 1$) or 100 min (NC (100 min); 0.4 ± 0.1 units; $n = 2$) were only 2–4 times lower compared to hearts subjected to ischemia and reperfusion.

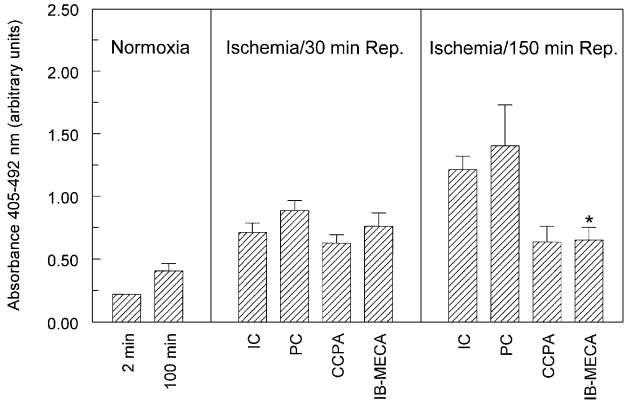

Fig. 6. Number of cytosolic histone-associated DNA fragments, a hallmark of apoptosis, measured by ELISA (absorbance: A405—A492 nm) after 30 and 150 min of reperfusion. The degree of apoptosis is low; pharmacological preconditioning decreased apoptosis. NC (2 min) = 2 min of normoxic perfusion only; NC (100 min) = 100 min of normoxic perfusion; * $P < 0.05$ vs. PC. For other abbreviations and protocol, see Fig. 1.

4. Discussion

4.1. Role of the adenosine receptor in preconditioning of rat hearts

This is the first study in isolated rat hearts indicating that adenosine A_3 receptors are also involved in protection by ischemic preconditioning in rat hearts. Furthermore, in this study we confirm earlier observations of our group (De Jonge and De Jong, 1999; De Jonge et al., 2001) that CCPA, a selective adenosine A_1 receptor agonist, reduces ischemic injury in rat hearts. Infusion of the selective adenosine A_3 receptor agonist IB-MECA prior to ischemia, resulted in a similar degree of myocardial protection to adenosine A_1 receptor stimulation with CCPA (Fig. 2), in line with published reports (Carr et al., 1997; Liang and Jacobson, 1998; Tracey et al., 1997). The absence of any contractile side-effects during infusion of 50 nM IB-MECA indicates that this dose did not activate the adenosine A_1 receptor. Thus, both adenosine A_1 and A_3 receptors trigger preconditioning protection by endogenous adenosine. However, Hill et al. (1998) concluded that the adenosine component of *ischemic* preconditioning is preferentially mediated by the adenosine A_1 receptor since adenosine binds this receptor with a greater affinity than the adenosine A_3 receptor in rabbit hearts ($K_iA_1 = 28$ nM; $K_iA_3 = 532$ nM). Whether both receptors are maximally activated in ischemic preconditioning of rat hearts is unclear since the affinity of adenosine for the adenosine A_3 receptor has not been determined yet in this species. However, higher interstitial adenosine levels during preconditioning ischemia (~ 7 μM) have been reported in the rat than in other species (Headrick, 1996) which makes it likely that both adenosine A_1 and A_3 receptors are maximally activated during ischemic preconditioning, co-operating in its induction (Rice et al., 1996). We showed before using similar models that the non-selective adenosine receptor antagonist 8-(*p*-sulfophenyl)-theophylline abolished protection by ischemic preconditioning completely (De Jonge and De Jong, 1999; De Jonge et al., 2001).

The observation in the present study that both adenosine A_1 and A_3 receptors trigger preconditioning protection in rat hearts is in contrast to other studies that were unable to mimic or abolish ischemic preconditioning with adenosine and adenosine antagonists, respectively (Cave et al., 1993; Li and Kloner, 1993). The inability to abolish ischemic preconditioning with adenosine antagonists could be related to the fact that (1) interstitial adenosine levels during preconditioning are very high in the rat compared to other species which may require increased antagonist concentration (Headrick, 1996), and (2) the use of selective adenosine A_1 receptor antagonists, which do not abolish the adenosine A_3 receptor mediated component of ischemic preconditioning. We showed before that 50 μM 8-sulfophenyltheophylline, a non-selective adenosine antagonist, abolished protection by ischemic preconditioning in rat hearts (De Jonge and De Jong, 1999; De Jonge et al., 2001). Administration of

exogenous adenosine in rats may be limited by the instability of this compound and the high activities of adenosine-degrading enzymes in the coronary endothelium, making the coronary endothelium an active barrier for adenosine transport. This is probably the reason why only studies using selective agonists of the adenosine A_1 receptor, instead of the natural ligand adenosine, have demonstrated protection against ischemic injury (Ford et al., 1998; Headrick, 1996; Liu and Downey, 1992).

Infusion of IB-MECA did not affect contractility in contrast to the large (83%) decrease observed with administration of CCPA (Fig. 2), in line with data obtained in conscious rabbits (Auchampach et al., 1997b). Earlier, we showed that the cardioprotective effects of adenosine A_1 receptor activation are unrelated to the negative chronotropic and dromotropic side-effects of these drugs (De Jonge and De Jong, 1999). IB-MECA increased coronary flow in line with a recent report (Lasley et al., 1999). This effect probably is mediated by the adenosine A_{2A} receptor (Lasley et al., 1999). Activation of the adenosine A_3 receptor induces hypotension by mast cell degranulation in rats in vivo (Auchampach et al., 1997b; Fozard et al., 1996). In humans and dogs, however, mast cell activation is probably mediated by the adenosine A_{2B} receptor, not the adenosine A_3 receptor (Auchampach et al., 1997a; Walker et al., 1997). In contrast, the adenosine A_3 receptor of these species has anti-inflammatory effects by inhibiting eosinophil migration (chemotaxis) (Knight et al., 1997; Walker et al., 1997), neutrophil degranulation (Bouma et al., 1997), and Tumor Necrosis Factor (TNF) release by macrophages (Sajjadi et al., 1996). Thus, although both adenosine A_1 and A_3 receptor activation protect against ischemic injury, agonists of the latter may be more promising as cardioprotective agents in the clinical setting due to a lack of hemodynamic side effects, anti-inflammatory effects, and a more sustained duration of protection than adenosine A_1 receptor agonists (Liang and Jacobson, 1998). Furthermore, selective adenosine A_3 receptor agonists have been reported to protect against both infarction and stunning (Auchampach et al., 1997b) in contrast to ischemic preconditioning, which only reduces infarct-size. This may be a major advantage of selective adenosine A_3 receptor agonists since myocardial stunning is often a greater problem after bypass surgery than infarction, which has a low incidence with current surgical techniques.

4.2. Purine metabolism

Based on the observation that ischemic preconditioning reduces interstitial purine accumulation during regional ischemia in dog hearts, Van Wylen (1994) suggested that ischemic preconditioning improves energy balance and consequently reduces ATP hydrolysis during ischemia. We previously also observed reduced purine release in the coronary effluent of preconditioned rat hearts during low-flow ischemia (De Jonge et al., 1998). In the present study we extend this finding, demonstrating that also hearts protected by adenosine A_1 and A_3 receptor agonists show reduced purine release during ischemia and reperfusion (Fig. 4). Goto et al. (1996) concluded that decreased purine release is an epiphenomenon of ischemic preconditioning and cannot be relied upon to serve as a marker of protection by preconditioning. However, these authors only examined purine release during short preconditioning ischemia and not during long ischemia. The present study strongly suggests that reduced myocardial purine production during ischemia is correlated to the functional recovery at the end of reperfusion, serving as a marker for the degree of ischemic injury.

4.3. Carbohydrate metabolism

This study shows that CCPA decreases lactate production during underperfusion (Fig. 5) compared to the other groups, confirming earlier findings (De Jonge and De Jong, 1999; Finegan et al., 1993). Paced CCPA hearts also showed reduced lactate production during low-flow ischemia (data not shown). Thus, reduced contractility caused by CCPA infusion (Fig. 2) was unrelated to less lactate production during subsequent ischemia. We previously suggested that ischemic preconditioning is mediated by increased glucose uptake during underperfusion without increasing total anaerobic glycolytic flux and that CCPA-induced protection may involve a different mechanism as it decreased glycolysis (De Jonge and De Jong, 1999). Cardioprotection induced by adenosine A_1 receptor agonists has been suggested to involve reduced proton production from glucose metabolism reducing Ca^{2+} overload (Ford et al., 1998). Infusion of IB-MECA prior to underperfusion did not result in reduced anaerobic glycolysis during ischemia as observed with CCPA.

4.4. Apoptosis

Necrosis and apoptosis are two distinct forms of lethal cell injury resulting from ischemia (Fliss and Gattinger, 1996; Kajstura et al., 1996). This is the first study reporting the effects of adenosine analogs on the degree of apoptosis occurring during ischemia and reperfusion. Although ischemic preconditioning improved contractile functioning after ischemia–reperfusion, it did not reduce the degree of apoptosis compared to ischemic control hearts (Fig. 6). This is in contrasts to a study in rat hearts in vivo (Piot et al., 1997) in which both infarct size and apoptosis decreased after ischemic preconditioning. Although we did not assess infarct size in the present study, we showed before that ischemic preconditioning reduces necrosis after 25 min low-flow ischemia as assessed by reduced leakage of creatine kinase (De Jonge et al., 1998). Moreover, less purine release in preconditioned hearts (Fig. 4) also reflects reduced ischemic injury. Thus, we believe that improved functional recovery in preconditioned hearts in the present study for a large part results from a reduction in lethal cell injury. In contrast to ischemic preconditioning, both adenosine A_1 and A_3 receptor agonists decreased apoptosis. Thus, our study provides

evidence for the first time that adenosine analogs may reduce both apoptosis and necrosis (oncosis). In our study, the degree of apoptosis after low-flow ischemia and reperfusion appears low since values were only 2–4 times higher than in rat hearts perfused normoxically (NC 2 and 100 min; Fig. 6). This could be related to the severity of ischemia. In regional ischemic rat hearts in vivo, the degree of apoptosis in ischemic control hearts (Piot et al., 1997), determined with the same commercial ELISA kit as used in the present study, was ca. 15 times higher in the ischemic vs. the nonischemic region. Therefore, the inability of preconditioning protocols to reduce apoptosis in our study may relate to its low incidence after low-flow ischemia and reperfusion. Future studies need to clarify the contribution of apoptosis and necrosis to various forms and degrees of ischemic injury.

4.5. Validity of the model

Whether we may extrapolate our observation concerning cardioprotection by adenosine A_3 receptor activation in the isolated rat heart to the human situation is unclear. Adenosine A_3 receptors have been reported to be present in rat (Zhou et al., 1992), rabbit (Wang et al., 1997), chick (Strickler et al., 1996), and human (Sajjadi and Firestein, 1993) hearts. The fact that both adenosine A_1 (Carr et al., 1997; Ikonomidis et al., 1997; Walker et al., 1994) and adenosine A_3 (Carr et al., 1997) receptors have been implicated in preconditioning of human (Ikonomidis et al., 1997; Leesar et al., 1997; Linden, 1994) and animal hearts indicates that myocardial protection triggered by adenosine receptor activation is a central feature in all species. Our study clearly demonstrates that the rat heart forms no exception to this rule, contrasting other suggestions (Cave et al., 1993; Li and Kloner, 1993). Thus, although species differences with regard to the adenosine-mediated component of ischemic preconditioning exist, they seem to be more of a quantitative than a qualitative nature.

5. Conclusion

The present study demonstrates that adenosine A_1 and A_3 receptors are implicated in preconditioning of rat hearts. The degree of apoptosis was low in hearts subjected to 30 min underperfusion and reperfusion and was reduced by pharmacological preconditioning. Adenosine A_3 receptor agonists may represent a new, potentially useful therapeutic class of agents for providing cardioprotection as they lack cardiovascular side effects associated with adenosine A_1 receptor activation.

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