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European Journal of Pharmacology 414 (2001) 55–62

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Role of adenosine and glycogen in ischemic preconditioning of rat hearts

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Received 20 July 2000; received in revised form 10 November 2000; accepted 14 November 2000

Abstract

We tested whether ischemic preconditioning of the rat heart is mediated by reduced glycogenolysis during ischemia, an event triggered by adenosine A_1 receptor activation. Rat hearts ($n = 40$) were studied with [^{31}P] and [^{13}C] nuclear magnetic resonance (NMR) spectroscopy, using the Langendorff perfusion technique (5.5 mM [1-^{13}C]glucose, 10 U/l insulin). In parallel experiments, hearts ($n = 43$) were freeze-clamped at different time-points throughout the protocol. They were subjected to either ischemic preconditioning (PC), PC in the presence of 50 μM adenosine receptor antagonist, 8-(*p*-sulfophenyl)-theophylline (SPT), or intermittent infusion of 0.25 μM adenosine A_1 receptor agonist, 2-chloro-N^6-cyclopentyladenosine (CCPA). After 30 min ischemia and reperfusion, recovery of heart rate × pressure product was improved in hearts treated with preconditioning (33 ± 13%) or CCPA (58 ± 14%) compared with the SPT and ischemic control (IC) groups, which both failed to recover ($P < 0.05$). CCPA administration induced a 58% increase in pre-ischemic [^{13}C]glycogen ($P < 0.05$ vs. all groups). In the PC and SPT groups, [^{13}C]glycogen decreased by 25 and 47%, respectively ($P < 0.05$) due to the short bouts of ischemia, resulting in lower pre-ischemic glycogen compared to ischemic control and CCPA hearts ($P < 0.05$). The rate of [^{13}C]glycogen utilization during the first 15 min of ischemia (in μmol/min g wwt) was not statistically different between IC (0.42 ± 0.03), PC (0.30 ± 0.04), and CCPA (0.38 ± 0.05) hearts, but was reduced in SPT hearts (0.24 ± 0.05; $P < 0.05$). Total glycogen depletion during 30-min ischemia was reduced in PC hearts (0.61 mg/g wwt) compared to IC (1.84 mg/g wwt) and CCPA (1.75 mg/g wwt) hearts; SPT did not block reduced glycogenolysis during ischemia in PC hearts (0.77 mg/g wwt vs. IC). This study adds further strong evidence that in rat hearts, adenosine is involved in ischemic preconditioning. However, protection is unrelated to pre-ischemic glycogen levels and glycogenolysis during ischemia.

Keywords: Adenosine A_1 receptor; Glycogen; Ischemic preconditioning; [^{31}P] NMR (nuclear magnetic resonance); [^{13}C] NMR; (Rat); Ischemia

1. Introduction

Ischemic preconditioning (Murry et al., 1986) occurs in many animal species, and most likely also in humans (Yellon et al., 1993). Many substances like adenosine, bradykinin, and catecholamines released during preconditioning ischemia may trigger protection (Goto et al., 1995). Adenosine, probably the most important, triggers and mediates preconditioning by stimulating the adenosine A_1 receptor (Thornton et al., 1993). Also, the adenosine A_3 receptor has been implicated in preconditioning protection (Carr et al., 1997). Adenosine mediates preconditioning in most animal species tested including humans (Lee et al., 1995; Leesar et al., 1997). However, for rat heart such a role is controversial (Asimakis et al., 1993; Cave et al., 1993; Cleveland et al., 1996; Headrick, 1996; Li and Kloner, 1993; Yellon et al., 1998). Our first objective was to test whether the adenosine A_1 receptor is involved in protection induced by ischemic preconditioning in the rat heart.

Glycogen depletion by preconditioning ischemia results in less accumulation of glycolytic end products during ischemia; it may mediate protection (Wolfe et al., 1993). Moreover, diminished protection parallels the time course of glycogen recovery before sustained ischemia (Wolfe et al., 1993); prolonged preconditioning results in glycogen depletion and infarct-size reduction (Barbosa et al., 1996). This 'glycogen hypothesis' is controversial (Asimakis, 1996; King and Opie, 1996; McNulty et al., 1996; Soares et al., 1997). A [^{13}C] nuclear magnetic resonance (NMR)

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0014-2999/01/$ - see front matter
PII: S0014-2999(00)00875-X

study (Weiss et al., 1996) indicates that preconditioning depresses glycogenolysis during ischemia. Interestingly, adenosine influences carbohydrate metabolism during normoxia (Finegan et al., 1996) and ischemia (Finegan et al., 1993). We argued that if reduced glycogen utilisation during ischemia mediates ischemic preconditioning, reduced glycogenolysis should also occur in hearts pharmacologically preconditioned with a selective adenosine A_1 receptor agonist. Moreover, if the glycogen hypothesis of preconditioning is true, an adenosine receptor antagonist should block both improved functional recovery and reduced glycogenolysis during ischemia. Thus, our second objective was to check whether reduced pre-ischemic glycogen and reduced ischemic glycogenolysis mediate ischemic preconditioning, manipulating the adenosine A_1 receptor. This study shows that adenosine is indeed involved in preconditioning of rat hearts and that neither pre-ischemic glycogen nor glycogen depletion during ischemia is related to preconditioning-induced cardioprotection.

2. Methods

2.1. Materials

The following 99% ^{13}C-enriched materials were from Cambridge Isotope Laboratories, [1-^{13}C]glucose and [2-^{13}C]ribose. The synthetic drugs 2-chloro-N^6-cyclopentyladenosine (CCPA) and 8-(*p*-sulfophenyl)-theophylline (SPT) were obtained from RBI (Natick, MA, USA).

2.2. Heart perfusion

Fed, male Sprague–Dawley rats, weighing 250–300 g, were stunned and bled. The heart was rapidly isolated and arrested in ice-cold perfusion fluid. The aorta was cannulated and the heart was Langendorff-perfused at constant pressure (70 mm Hg) for 20 min in order to allow coronary flow to be measured. This value (9–11 ml/min) was applied to the subsequent 10 min constant flow perfusion period. The perfusion medium was a modified Krebs–Henseleit solution (composition in mmol/l): NaCl 137; KCl 5.4; $MgCl_2$ 1.2; $CaCl_2$ 1.8; NaH_2PO_4 0.46; $NaHCO_3$ 12; α-D-glucose 5.5 (or [1-^{13}C]glucose), with 10 U/l insulin and saturated at 37°C with O_2–CO_2 (95–5%); pH 7.4. High insulin was used to stimulate incorporation of [^{13}C]glucose in the glycogen pool during normoxic perfusion, according to established techniques (Weiss et al., 1996). To minimize temperature changes during ischemia, the heart was immersed in 37°C perfusate, maintained by a Bruker temperature controller accessory. Myocardial function (heart rate, developed pressure, and rate-pressure product) was assessed using a pressure transducer connected to an intraventricular balloon filled to give an end-diastolic pressure of 4–8 mm Hg.

2.3. [^{31}P] and [^{13}C] NMR spectroscopy

The heart was inserted in a broad-band 20-mm probe of a Bruker AMX 500 wide-bore NMR spectrometer operating at 11.74 T. Field homogeneity was optimized by shimming the water proton signal using the decoupling coil. [^{31}P] NMR spectra were obtained at 202.4 MHz with a 60° pulse of 20 μs, and a 2-s delay using blocks of 60–90–150 transients corresponding to 2–3–5 min of accumulation time. Relative metabolite quantification was obtained as already reported (Bradamante et al., 1992, 1993) and the results expressed as a percentage of the baseline value (100%). Intracellular pH was calculated from the chemical shift (δ) of P_i relative to phosphocreatine using the equation $pH = 6.77 - \log(\delta - 5.78)/(3.27 - \delta)$. Zero ppm was assigned to phosphocreatine. The validity of this equation has been checked for our experimental condition (Flaherty et al., 1982).

^{1}H-decoupled ^{13}C spectra were obtained at 125.72 MHz using the MLEV-16 sequence. Free induction decays were collected in 2K data points and zero-filled to 8K, using blocks of 100–150–250 transients corresponding to 2–3–5 min of accumulation time. The pulse width was 23 μs (60°) and the recycling time 1.1 s. A line broadening of 25 Hz was introduced before Fourier transformation. The chemical shifts are reported in relation to tetramethylsilane, using β-C1 glucose at 97 ppm as the internal reference standard. A capillary tube filled with a solution containing 100 mmol/l [2-^{13}C]ribose was used as the external standard. Metabolite concentrations were measured by integrating the areas under the individual peaks and were expressed as a percentage of the baseline value (100%) at 30 min of stabilization. Corrections from the nuclear Overhauser effect and partial saturation effects were performed. Absolute concentrations were calculated using the 100 mmol/l [2-^{13}C]ribose solution.

2.4. Experimental protocol

The protocol is shown in Fig. 1. The hearts were randomly assigned to four groups: IC, PC, CCPA and SPT, which all underwent 30 min stop-flow ischemia and 30 min reperfusion. Pretreatment consisted of (for [^{31}P] NMR and [^{13}C] NMR: $n = 4$–6 per group; $n = 40$): (1) Group IC, ischemic control: 42 min stabilization; (2) group PC, ischemic preconditioned: 30 min stabilization, four cycles of 2 min ischemia and 3 min reperfusion; (3) group CCPA, adenosine A_1 receptor agonist treated: 30 min stabilization, four cycles of 2 min CCPA (0.25 μM) and 3 min normal perfusion; (4) group SPT, adenosine receptor antagonist treated: 30 min stabilization, four cycles of 2 min ischemia and 3 min reperfusion. SPT (50 μM) was added to the perfusion medium 2 min before and during intermittent ischemia–reperfusion. The CCPA dose was based on the adenosine A_1 receptor affinity ($K_iA_1 = 0.4$ nM); SPT

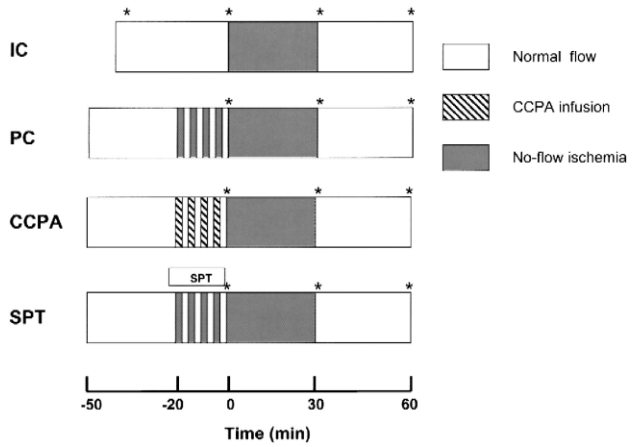

Fig. 1. Experimental protocols. All hearts were subjected to 30 min no-flow ischemia and 30 min reperfusion. Hearts ($n = 4–6$ per group in [^{31}P] and [^{13}C] NMR experiments: $n = 40$) were preconditioned with either four cycles of 2 min ischemia and 3 min reperfusion (PC group) or four 2-min infusions of 0.25 μM adenosine A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA group), followed by 3-min drug-free perfusion. In one group of ischemic preconditioned hearts, 50 μM of the adenosine receptor antagonist 8-(*p*-sulfophenyl)theophylline (SPT group) was present in the perfusion medium 2 min before and during intermittent ischemia. In [^{13}C] NMR experiments, 5.5 mM [1-^{13}C]glucose was present in the perfusion buffer throughout the protocol. Because PC hearts were perfused with labeled glucose during preconditioning, the ischemic control hearts (IC group) received a 42-min stabilization period to ensure that hearts received equal amounts of labelled glucose before ischemia. In parallel experiments, hearts were freeze-clamped at different time-points throughout the protocol ($n = 3–5$ per group: $n = 43$; indicated with *).

dose was chosen based on the study of Headrick (Headrick, 1996).

The hearts were weighed at the end of the experiment. Reperfusion was performed at constant pressure (70 mm Hg) for 10 min and continued at constant flow. 5.5 mM [1-^{13}C]glucose was present throughout the protocol in [^{13}C] NMR experiments. In parallel experiments, hearts ($n = 43$) were freeze-clamped at the indicated time-points (see Fig. 1) for determination of total glycogen.

2.5. Biochemical analysis

Total glycogen in freeze-clamped hearts was determined as previously described (De Jonge et al., 1998). Freeze-clamped hearts were ground under liquid nitrogen using pestle and mortar. One milliliter homegenate was sonicated for 4×20 s on ice and neutralized with 5 M NaOH, and used for glycogen determination, with rabbit liver glycogen (Boehringer Mannheim) as a standard. After treatment with amyloglucosidase, glucose was assayed spectrophotometrically.

2.6. Statistical analysis

The values are expressed as means ± S.E.M. ANOVA and Student–Newman–Keuls' *t*-test were used for evaluating their statistical significance. P values of < 0.05 were considered statistically significant. NS indicates not statistically significant.

3. Results

3.1. Contractile function

Fig. 2 presents the time-dependent changes in rate-pressure product, a reflection of ventricular systolic functioning. Since preconditioning may affect recovery of heart rate and developed pressure to a different degree (Bradamante et al., 1993), the rate-pressure product will be presented in this study as a reliable index of contractile functioning. After 30 min stabilization, there were no differences between groups in rate-pressure product (mean and S.E.M. of all groups: 32,433 ± 3006 mm Hg/min). With the short periods of intermittent ischemia in the PC and SPT groups, rate-pressure product rapidly fell to near zero values. Rate-pressure product recovered to 90% in the PC hearts and to 100% in the SPT hearts (NS vs. IC) after the fourth 2-min period of ischemia. The first 2-min infusion period of CCPA induced a 47% decrease in contractility, which did not recover during the first 3-min period of CCPA-free perfusion. The subsequent cycles of CCPA infusion further decreased rate-pressure product to a pre-ischemic value of 23% ($P < 0.05$ vs. PC, SPT, IC). With the onset of prolonged no-flow ischemia, rate-pressure product fell to zero within 5 min in all groups. Upon reperfusion after long ischemia, functional recovery was significantly improved in PC (33 ± 13%) and CCPA (58 ± 14%) hearts compared to IC (0 ± 0%; $P < 0.05$). SPT abolished preconditioning protection (0.4 ± 0.4%; $P <$ 0.05 vs. PC).

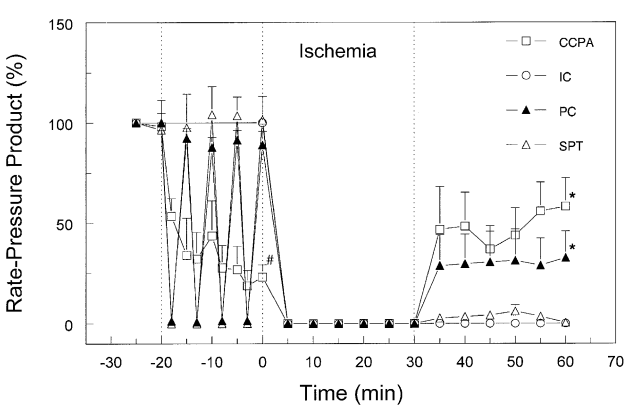

Fig. 2. Changes in rate-pressure product expressed as a percentage of the baseline value (100%). Recovery of rate-force product at the end of reperfusion following ischemia was improved in CCPA and PC hearts compared to SPT and IC groups. $^{\#}P < 0.05$ vs. IC, PC, SPT; $^{*}P < 0.05$ vs. IC, SPT. For abbreviations and protocol, see legend to Fig. 1.

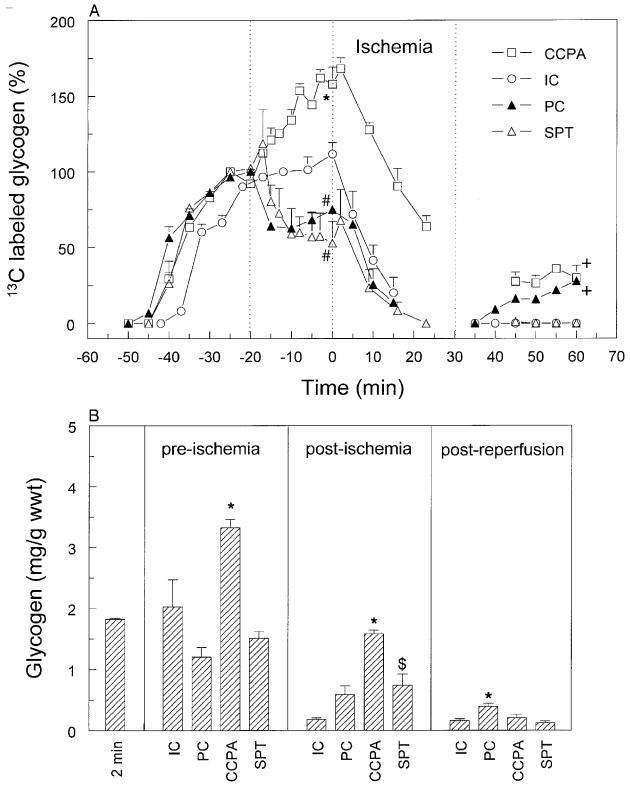

Fig. 3. [^{13}C] labeling and mobilization of glycogen (A) expressed as a percentage of the baseline value (100%) and total glycogen in hearts freeze-clamped at different time-points throughout the protocol (B). Functional recovery was unrelated to preischemic glycogen. 2 min = 2 min normoxic perfusion (B); $^{*}P < 0.05$ vs. IC, PC, SPT; $^{\#}P < 0.05$ vs. CCPA, IC; $^{+}P < 0.05$ vs. IC, SPT; $^{\$}P < 0.05$ vs. IC. For abbreviations and protocol, see legend to Fig. 1.

3.2. Glycogen

The time-dependent changes in [^{13}C]glycogen are presented in Fig. 3A. The presence of 10 U/l insulin in the perfusion medium induced a marked increase in total glycogen with the concurrent incorporation of labelled glucose residues, as monitored in the ^{13}C spectra by the growth of the signal at 100.6 ppm. The [^{13}C]glycogen signal levelled off after 30 min of normoxic perfusion. Short intermittent ischemia resulted in a 25% decrease in [^{13}C]glycogen in PC hearts ($P < 0.05$ vs. IC). This decrease was not abolished by SPT; pre-ischemic [^{13}C]glycogen was 53% of the value after stabilization in SPT hearts ($P < 0.05$ vs. IC). CCPA induced a 58% increase in [^{13}C]glycogen resulting in significantly higher pre-ischemic glycogen levels compared to IC hearts ($P < 0.05$ vs. IC). Changes in [^{13}C]glycogen reflected those of total glycogen determined in freeze-clamped hearts during parallel experiments (Fig. 3B): short intermittent ischemia decreased total glycogen by 40% in PC hearts and by 25% in SPT hearts whereas CCPA infusion increased total glycogen by 65%. [^{13}C]glycogen utilization was delayed during the first 2–5 min of prolonged ischemia in PC and CCPA groups (Fig.

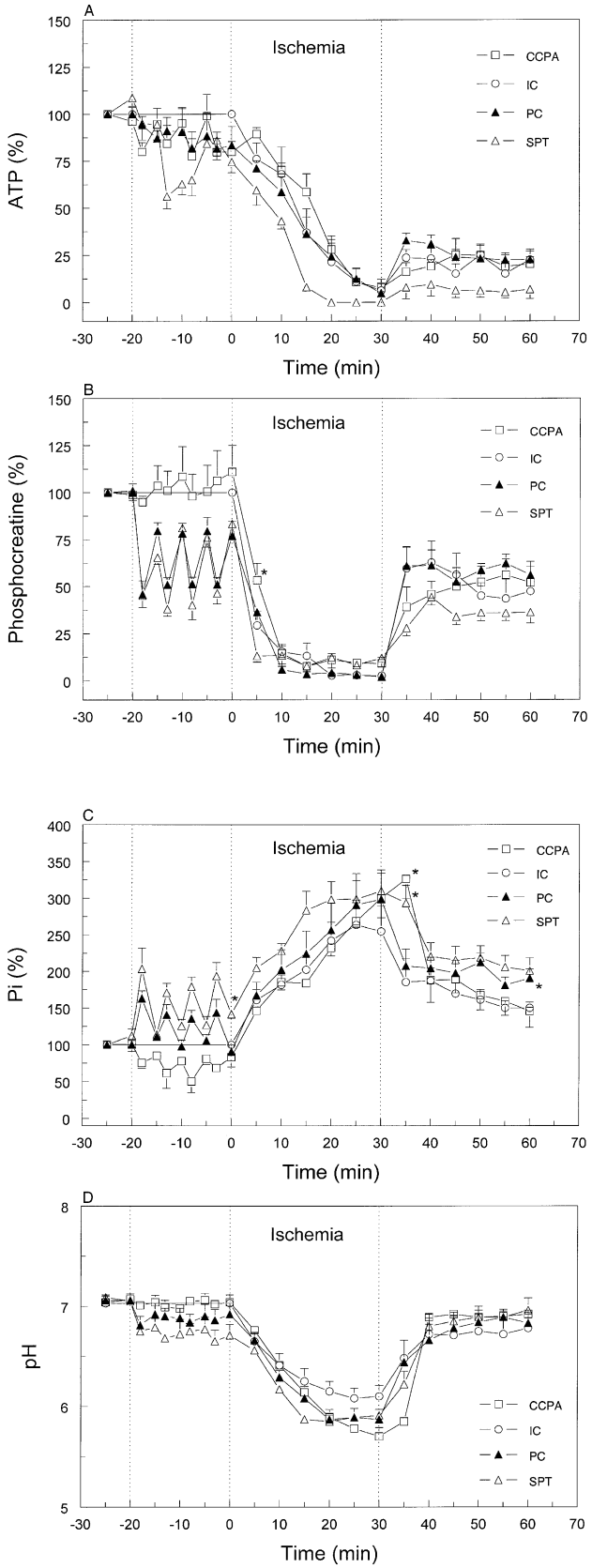

Fig. 4. Changes in (A) ATP, (B) phosphocreatine, (C) inorganic phosphate [P_i], and (D) pH. ATP, phosphocreatine, and P_i are expressed as a percentage of the baseline value (100%). $^{*}P < 0.05$ vs. IC. For abbreviations and protocol, see legend to Fig. 1.

3A), this delay was not blocked by SPT. [^{13}C]glycogen utilization during the first 15 min of prolonged ischemia (in μmol/min g wwt) was similar in PC (0.30 ± 0.04), IC (0.42 ± 0.03), and CCPA (0.38 ± 0.05) hearts, but was lower in SPT hearts (0.24 ± 0.05; $P < 0.05$ vs. IC). At the end of ischemia, [^{13}C]glycogen was almost completely exhausted in the PC, IC and SPT groups, whereas it was still 64% of the stabilization value in CCPA-treated hearts (Fig. 3A). In CCPA hearts, freeze-clamped at the end of sustained ischemia, total glycogen was 87% of the 2-min stabilization value (Fig. 3B). Upon reperfusion, only PC and CCPA hearts resumed [^{13}C]glycogen synthesis compared to IC and SPT groups ($P < 0.05$; Fig. 3A). This was partly reflected in total glycogen values determined after reperfusion (Fig. 3B): glycogen was higher in PC hearts compared to all other groups $P < 0.05$).

3.3. High energy phosphates

After 30 min stabilization, absolute values (mean ± S.E.M. for all groups) for phosphocreatine (5.55 ± 0.37 μmol/g wwt), ATP (2.82 ± 0.21 μmol/g wwt) and Pi (2.94 ± 0.24 μmol/g wwt) were similar between groups. Intermittent ischemia and reperfusion induced cyclic variations in phosphocreatine and P_i in PC and SPT groups (Fig. 4B and C, respectively); during short ischemia, phosphocreatine decreases with a concomitant increase in P_i in order to buffer the ATP needs of the tissue (Bradamante et al., 1993, 1995); hence, ATP levels are not severely affected in the PC and SPT groups. However, pre-ischemic phosphocreatine was not different between groups; pre-ischemic P_i was higher in SPT hearts ($P < 0.05$ vs. IC, PC, CCPA). ATP levels (Fig. 4A) and pH (Fig. 4D) were not affected by any of the treatments prior to ischemia.

The fall in ATP during prolonged ischemia was similar in all groups (Fig. 4A); at the end of ischemia, ATP levels were depleted in all hearts. During early ischemia, phosphocreatine levels decreased more slowly in CCPA hearts ($P < 0.05$ vs. IC) although phosphocreatine depletion was similar in all groups at the end of ischemia. During ischemia, P_i rose similarly in all groups. Ischemic acidification was similar in all groups but tended to be less in IC hearts.

Upon reperfusion, recovery of ATP, phosphocreatine, and pH was similar in all groups. At the end of reperfusion, recovery of P_i was worse in PC hearts ($P < 0.05$ vs. IC).

4. Discussion

4.1. Adenosine preconditions rat heart

Fig. 2 shows that the ischemic preconditioning of rat hearts can be mimicked by pre-treatment with a selective adenosine A_1 receptor agonist. CCPA induced marked negative chronotropic and dromotropic effects, with reduced pre-ischemic contractility (Fig. 2). However, these contractile side-effect were unrelated to its cardioprotective effects since protection was also present in paced CCPA-treated hearts (De Jonge and De Jong, 1999). Furthermore, the non-selective adenosine receptor antagonist SPT abolished ischemic preconditioning. Our conclusion —adenosine is involved in preconditioning of rat hearts—contrasts literature data (Ganote and Armstrong, 2000; see also Introduction). Possible reasons include: (1) Interstitial adenosine is much higher in rat than, e.g., rabbit heart, which may require more adenosine receptor antagonist (Headrick, 1996). (2) In the rat, sub-optimal adenosine A_1 receptor activation could be due to active endothelial adenosine degrading enzymes (Olsson and Pearson, 1990), the endothelial barrier for adenosine transport (Cambray et al., 1998), and the short half-life of blood adenosine (Möser et al., 1989). (3) Selective adenosine A_1 receptor antagonists or non-selective xanthine derivatives like SPT, which weakly bind the adenosine A_3 receptor in rat hearts (van Galen et al., 1994; Zhou et al., 1992), do not abolish the adenosine A_3 receptor-mediated component of ischemic preconditioning. Taken together, we conclude that adenosine is involved in preconditioning of rat hearts.

4.2. Glycogen and ischemic preconditioning

We tested whether ischemic preconditioning is mediated by reduced pre-ischemic glycogen and ischemic glycogenolysis, resulting in attenuated glycolytic catabolite accumulation and development of intracellular acidosis ('glycogen hypothesis of ischemic preconditioning' (Murry et al., 1990; Wolfe et al., 1993)). Furthermore, we assessed whether reduced glycogen depletion during ischemia is mediated by adenosine A_1 receptor activation. Our study shows that pre-ischemic glycogen is unrelated to preconditioning protection since both high (CCPA group) and low (PC group) pre-ischemic glycogen (Fig. 3) are associated with improved functional recovery (Fig. 2). A comparison of the PC and IC hearts shows that glycogenolysis during early ischemia (first 5 min) was attenuated in PC hearts, in line with other reports (King and Opie, 1996; McNulty et al., 1996; Murry et al., 1990; Weiss et al., 1996). CCPA also reduced glycogenolysis during early ischemia (Fig. 3). However, although SPT prevented preconditioning protection, it did not abolish reduced glycogen utilization after 5 min of ischemia (Fig. 3). Since we previously have shown that preconditioning primarily affects carbohydrate metabolism during the first 15 min of ischemia (De Jonge and De Jong, 1999), we calculated [^{13}C]glycogen utilization during this period. [^{13}C]glycogen utilization during the first 15 min of sustained ischemia was not different between PC, IC, and CCPA groups, and was even reduced in SPT hearts compared to IC hearts (Fig. 3A). Analysis of glycogen in freeze-clamped hearts (Fig. 3B) showed that total glycogen depletion during 30 min ischemia was

drastically reduced in PC and SPT hearts, compared to IC and CCPA hearts. Thus, pre-ischemic glycogen and ischemic glycogenolysis can be dissociated from protection. Moreover, also against the glycogen hypothesis is our observation that pre-ischemic glycogen is not necessarily related to the rate of its depletion during ischemia. Other studies also have failed to observe a relation between functional recovery after prolonged ischemia and pre-ischemic glycogen levels (Asimakis, 1996; King and Opie, 1996; Soares et al., 1997) or glycogen depletion during ischemia (King and Opie, 1996). Furthermore, we did not find any differences between groups in the degree of acidosis during ischemia (Fig. 4D). We previously also showed no difference in pH between PC and IC hearts (Bradamante et al., 1993). Moreover, we now add to these observations that pharmacologically preconditioned hearts also do not show less acidosis during ischemia. Thus, the degree of acidosis during ischemia does not play a role in preconditioning protection. Our observation contrasts many studies showing reduced accumulation of glycolytic end-products (lactate, protons, sugar phosphates) in ischemically preconditioned hearts during ischemia (Asimakis, 1996; De Albuquerque et al., 1994; Murry et al., 1990; Soares et al., 1997; Steenbergen et al., 1993; Weiss et al., 1996). However, Soares et al. (1997) and Schaefer et al. (1995) showed that although pre-ischemic glycogen content relates to pH during ischemia, it is unrelated to functional recovery upon reperfusion. Moreover, several groups (Cave and Garlick, 1997; Harrison et al., 1998) were also unable to find a relation between preconditioning protection and reduced acidosis during ischemia in line with the present study. Cross et al. (1996) showed improved functional recovery in hearts displaying greater degrees of acidosis during ischemia. Lundmark et al. (1999) dissociated a higher pH during ischemia from reduced ischemic injury. We also found the rate of high-energy phosphate depletion (Fig. 4A and B) during ischemia to be similar in all groups. In conclusion, this study provides evidence that preconditioning protection against sustained ischemia is not mediated by (1) reduced glycogen utilization and consequent attenuation of glycolytic catabolite accumulation, and (2) reduced rate of high-energy phosphates depletion, as proposed by Murry et al. (1990). Hence, both correlations merely represent epiphenomena of ischemic preconditioning.

Surprisingly, CCPA increased glycogen synthesis resulting in relatively high pre-ischemic tissue glycogen (Fig. 3A and B). This may be due to stimulated glycogen synthesis and inhibited glycogen breakdown. Both adenosine and a CCPA analogue reduce glycolysis during normoxia (Finegan et al., 1996). Due to high pre-ischemic glycogen in CCPA hearts, post-ischemic glycogen content was much higher than that in the other groups (Fig. 3B). Cross et al. (1996) showed that although pre-ischemic glycogen is unrelated to functional recovery after ischemia, glycogen depletion and cessation of glycolytic ATP production during ischemia is detrimental to the heart. Thus, CCPA may protect by preventing glycogen depletion and cessation of glycolytic flux during ischemia.

4.3. Adenosine and glycogen

Few studies have examined the effect of adenosine preconditioning directly on ischemic glycogenolysis. Adenosine prior to no-flow ischemia reduces ischemic lactate accumulation (Fralix et al., 1993). CCPA pretreatment inhibits anaerobic glycolysis and glycolysis-from-glucose during low-flow ischemia (De Jonge and De Jong, 1999; Finegan et al., 1993; Lasley and Mentzer, 1992). In ischemic dog hearts, adenosine did not affect the rate of glycogen depletion (Vander Heide et al., 1993). CCPA mimicked PC in the initial delay in ischemic glycogenolysis; however, SPT did not block delayed glycogenolysis in preconditioned hearts (Fig. 3A). Moreover, overall glycogen depletion during 30 min no-flow ischemia was higher in the CCPA and IC hearts compared to SPT and PC groups (Fig. 3A and B).

4.4. Critique of methods

We used the non-selective adenosine receptor antagonist SPT to block adenosine receptors. Adenosine A_1, A_2 and A_3 receptors are involved in protection by ischemic preconditioning. Thus, selective adenosine A_1 receptor blockade (e.g., with 8-Cyclopentyl-1,3-dipropylxanthine; DPCPX) probably would not abolish ischemic preconditioning due to residual activation of other cardioprotective adenosine (A_3) receptors (Rice et al., 1996). The dose used in this study (50 μM) was based on experimental data (Headrick, 1996) and theoretical calculations (Ganote and Armstrong, 2000). High interstitial adenosine (7 μM; Headrick, 1996) during preconditioning in the rat heart requires increased receptor antagonist concentration. Moreover, our laboratory (van den Doel et al., 1999) recently showed that high dose but not low dose SPT abolished preconditioning protection induced by N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyl-uronamide (IB-MECA), a selective adenosine A_3 receptor agonist. Thus, 50 μM SPT blocks all adenosine receptors sufficiently.

To verify whether adenosine A_1 receptors are involved in preconditioning of rat hearts, we used the selective adenosine A_1 receptor agonist CCPA. The dose selected (0.25 μM) was based on maximal activation of adenosine A_1 receptors and is commonly used in preconditioning experiments (see e.g., (Headrick, 1996)). The short infusion–reperfusion periods and the barrier function of the vasculature makes it difficult to predict the actual CCPA concentration at the site of the adenosine A_1 receptor, the interstitial space. We used relatively high dose CCPA (250 nM); It should be kept in mind that in ischemic preconditioning even higher interstitial adenosine levels (7000 nM; $K_iA_1 = 0.1–10$ nM) are reached. Thus, in ischemic pre-

conditioning, most likely adenosine receptor number/affinity rather than receptor agonist concentration limits adenosine-induced preconditioning. The marked negative chronotropic/inotropic effects observed upon infusion of CCPA (see Fig. 2) indicate that the dose used was effective.

4.5. Conclusion

In conclusion, adenosine mediates protection by ischemic preconditioning in rat hearts. However, glycogen depletion prior to ischemia and reduced glycogenolysis during ischemia are not causally related to preconditioning protection.

Acknowledgements

We gratefully acknowledge the support of the Netherlands Heart Foundation (NHS 94.043), the European Community (Biomed II, BMH4-CT950838) and NATO (LST.CLG.976634).

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