ELSEVIER

European Journal of Pharmacology 320 (1997) 241–248

ejp

Dual activation of adenosine A_1 and A_3 receptors mediates preconditioning of isolated cardiac myocytes

Jianxun Wang [a], Lisa Drake [a], Fereydoun Sajjadi [b], Gary S. Firestein [b], Kevin M. Mullane [a], David A. Bullough [a,*]

[a] *Department of Cardiovascular Pharmacology, Gensia Inc., 9360 Towne Center Drive, San Diego, CA, USA*
[b] *Department of Immunology, Gensia Inc., San Diego, CA, USA*

Received 23 September 1996; accepted 12 November 1996

Abstract

Ischemic preconditioning reduces post-ischemic myocardial injury by activating myocellular adenosine A_1 receptors. Adenosine A_3 receptors have also been implicated but there is no evidence for A_3 receptors in cardiac myocytes. The aim of this study was to develop a model of preconditioning in isolated cardiac myocytes to evaluate the role of the adenosine A_1 and A_3 receptors in preconditioning-induced protection from ischemic injury. Reverse transcription polymerase chain reaction (PCR) was also employed to establish the presence of adenosine A_3 receptors in these cells. In the preconditioning studies, ischemic injury was simulated by exposing isolated rabbit myocytes (placed in the cell chamber and paced at 1 Hz) to buffer containing (in mM) 2′-deoxyglucose (20), NaCN (1), Na^+-lactate (20), KCl (10) at pH 6.6 (37°C). Changes of diastolic and systolic cell length were monitored with an optical-video edge imaging system, and hypercontracture was assessed as an index of irreversible cell injury. Preconditioning (2 min brief ischemia and 15 min reperfusion) significantly reduced cell injury resulting from a subsequent prolonged ischemia (10 min) and reperfusion (15 min), as indicated by a reduction in the incidence of cell hypercontracture from $67 \pm 6\%$ to $29 \pm 5\%$ ($P < 0.001$). Preconditioning-induced cardioprotection was only partially blocked by a maximally effective concentration (100 nM) of the adenosine A_1 receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (cell hypercontracture = $43 \pm 3\%$, $P < 0.05$ vs. control) but completely blocked by either the combination of DPCPX (100 nM) with the adenosine A_1/A_3 receptor antagonist DPCPX + 8-(4-carboxyethylphenyl)-1,3-dipropylxanthine (BWA1433; 1 μM) or the non-selective adenosine receptor antagonist, 8-(*p*-sulfophenyl)theophylline (8-SPT; 100 μM) (cell hypercontracture = $64 \pm 4\%$, $59 \pm 5\%$, respectively; P = NS vs. control). In non-hypercontractured myocytes, preconditioning also substantially enhanced the recovery of the contractile amplitude and, similarly, this effect was only partially blocked by DPCPX but completely blocked by either the combination of DPCPX with BWA1433, or 8-SPT. These studies suggest that preconditioning protects isolated cardiac myocytes from ischemic injury independent of other cell types, and that maximal preconditioning-induced cardioprotection requires activation of both adenosine A_1 and A_3 receptors. Reverse transcription-PCR using primers for the rabbit receptor provide evidence for the presence of adenosine A_3 receptors in these cells.

Keywords: Adenosine receptor; Ischemic preconditioning; Cardiac myocyte, isolated

1. Introduction

Ischemic preconditioning of the heart is a phenomenon by which a brief period of ischemia followed by reperfusion protects the myocardium from irreversible injury or infarction that results from a subsequent more prolonged or severe ischemic insult. It was first described by Murry and colleagues in the dog (Murry et al., 1986) and has since been observed in a variety of species including the rat (Liu and Downey, 1992), rabbit (Liu et al., 1991), pig (Schott et al., 1990) and more recently in humans (Deutsch et al., 1990; Yellon et al., 1993). An exact mechanism to explain the protective actions of myocardial preconditioning has not been elucidated, although recent evidence strongly implicates a role for adenosine (Downey et al., 1993). For instance, adenosine or adenosine receptor agonists can substitute for the conditioning period, whereas adenosine receptor antagonists block the salutary effects of preconditioning (Liu et al., 1991; Thornton et al., 1992; Auchampach and Gross, 1993; Yao and Gross, 1993). However, despite evidence that adenosine receptor activa-

* Corresponding author. Tel.: (1-619) 622-3931; Fax: (1-619) 622-5546; e-mail: david.bullough@gensia.com

PII S0014-2999(96)00901-6

tion is involved, the specific contributions of individual receptor subtypes are less well defined.

Four adenosine receptors have been cloned and designated A_1, A_{2a}, A_{2b} and A_3 (Tucker and Linden, 1993). Of these, the adenosine A_1 receptor on the cardiac myocyte has been most extensively implicated in the protective effects of adenosine and ischemic preconditioning (Liu et al., 1991; Downey et al., 1993; Thornton et al., 1992; Auchampach and Gross, 1993). This conclusion has been challenged by studies in which the adenosine A_1/A_2-selective receptor antagonist, 8-(*p*-sulfophenyl)theophylline (8-SPT), and the adenosine A_1-selective antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), failed to abrogate the beneficial effects of ischemic preconditioning (Downey et al., 1993; Li and Kloner, 1993). Two recent studies have suggested that activation of adenosine A_3 receptors might be involved (Armstrong and Ganote, 1994; Liu et al., 1994).

The aim of the present study was to establish a model of preconditioning in isolated cardiac myocytes to assess the relative contributions of the adenosine A_1 and A_3 receptors in preconditioning-induced protection from ischemic injury. A second goal of this study was to employ reverse transcription-polymerase chain reaction (RT-PCR) using primers for the rabbit receptor to assess the presence of adenosine A_3 receptors in these cells. Together, the results suggest that preconditioning-induced protection may require activation of both adenosine A_1 and A_3 receptors and provide the first direct evidence that adenosine A_3 receptors may be present in isolated cardiac myocytes.

2. Materials and methods

2.1. Isolation of myocytes (Powell, 1988; Wahler, 1992)

Male New Zealand white rabbits (2.0–2.5 kg) were anesthetized with intravenous sodium pentobarbital (30 mg/kg). The heart was quickly removed and mounted on a Langendorff perfusion apparatus and perfused with modified Krebs Ringer buffer containing (in mM) 118.5 NaCl, 2.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 11 glucose and 0.5 g bovine serum albumin (fatty acid free) at pH 7.4 for 7 min followed by modified Krebs Ringer buffer containing 113 U/ml collagenase II and 5 μM $CaCl_2$ for 30 min. The heart was then transferred to a petri dish with 10 ml of storage solution containing (in mM) 40 KCl, 20 KH_2PO_4, 50 L-glutamic acid, 20 taurine, 0.5 EGTA, 10 glucose, 10 Na^+-Hepes, 77 KOH and 3 $MgCl_2$ at pH 7.4. Pieces were cut from the left ventricle and transferred to another petri dish containing 10 ml of storage solution. The pieces were then gently minced and transferred to a 20 ml beaker which was placed in a water jacket for 12 min at 37°C. The tissue suspension from the beaker was filtered through a Nitex mesh. Cells were sedimented by centrifugation ($90 \times g$) for 90 s, resuspended in storage solution and rested for 1 h. Preparations were > 70% rod-shaped cells and cell viabilities were > 70% as measured by trypan blue exclusion.

2.2. Cell injury and contractility measurements

A drop of isolated myocyte suspension was placed on a cover slip located in the myocyte chamber. The chamber was temperature-controlled (at 37°C) and a pair of platinum electrodes was installed. Cells in the chamber were electrically stimulated at 1 Hz and superfused at 1 ml/min with a buffer (control) containing (in mM) 130 NaCl, 3.58 KCl, 1 $MgSO_4$, 11 glucose, 10 Na^+-Hepes, 2.5 $CaCl_2$, at 37°C, pH 7.4. When the contractile amplitude of myocytes was stable (usually after 10 min), the cells were exposed to a simulated ischemic buffer containing (mM) 110 NaCl, 10 KCl, 1 $MgSO_4$, 10 Na^+-Hepes, 2.5 $CaCl_2$, 20 2′-deoxyglucose, 20 Na^+-lactate and 1 NaCN, at pH 6.6 (37°C) for 10 min followed by 15 min reperfusion using the non-ischemic (control) buffer. Usually 8–12 beating cells from each cover slip were monitored. Hypercontracture (cell diastolic length shortened to less than 60% of its original length) was measured as an index of irreversible cell injury using an edge detector coupled to a microscope-video-monitor system (Nishida et al., 1993; Esumi et al., 1991). Contractile function of single myocytes was assessed using a phase-contrast microscope-video motion detection system (Nishida et al., 1993; Esumi et al., 1991; Wang et al., 1994).

2.3. Experimental protocols for cell injury studies

Five groups of cells were evaluated for their effects on cell injury as shown in Fig. 1. Each treatment group was subjected to 10 min of ischemia followed by 15 min of reperfusion. In preconditioned cells this was preceded by 2 min of ischemia followed by 15 min of reperfusion. DPCPX, DPCPX + 8-(4-carboxyethylphenyl)-1,3-

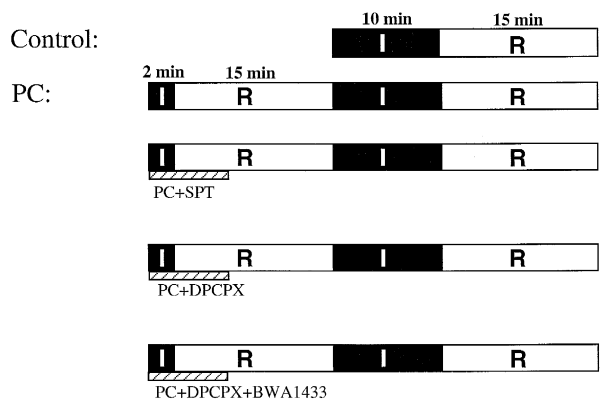

Fig. 1. Experimental protocols used to evaluate the effects of preconditioning in isolated myocytes and the influence of adenosine receptor antagonists on preconditioning in these cells. I, ischemia; R, reperfusion; PC, preconditioned cells.

dipropylxanthine (BWA1433), or 8-SPT were dissolved in the superfusion buffer and cells were treated during the ischemic period of preconditioning and 5 min of reperfusion, and were then allowed to wash out for 10 min prior to subjecting the cells to prolonged ischemia and reperfusion. DPCPX and BWA1433 were added from concentrated solutions prepared in 10% (v/v) dimethylsulfoxide (DMSO) such that the final concentration of DMSO was 0.01% (v/v). DPCPX (250 nM), BWA1433 (1 μM), 8-SPT (100 μM) or DMSO (0.1% v/v) had no effect on basal myocyte contraction.

DPCPX concentrations sufficient to antagonize adenosine A_1 receptors were selected based on their ability to attenuate inhibition of isoprenaline-enhanced contraction by the adenosine agonist, 2-chloro-N^6-cyclopentyladenosine (CCPA) in isolated rabbit myocytes. Initially different concentrations of CCPA were evaluated to establish a minimum concentration that maximally inhibited isoprenaline-enhanced contraction. As shown in Fig. 2a, 25 nM CCPA maximally inhibited contraction, whereas a higher concentration of CCPA (125 nM) showed no additional inhibition. Incubation of CCPA-treated cells with increasing concentrations of DPCPX prior to addition of isoprenaline, showed 50 nM DPCPX was sufficient to prevent inhibition of isoprenaline-enhanced contraction by CCPA (Fig. 2b). In similar experiments performed with BWA1433, CCPA-inhibited contraction was only partially inhibited by 0.1–1 μM BWA1433 (Fig. 2c). These results are therefore consistent with 50 nM DPCPX being sufficient to antagonize the adenosine A_1 receptor in rabbit myocytes, while 0.1–1 μM BWA1433 had minimal effect.

2.4. Reverse transcription PCR

Isolated myocytes were purified using three successive gradient centrifugation steps ($50 \times g$, 30 s) followed by three differential attachment steps on 10 cm petri dishes (each 30 min at 37°C). One thousand cells were counted and no fibroblasts or non-myocyte cells were observed. Isolation of mRNA from these myocytes was achieved using total RNA and mRNA purification kits (Qiagen, Chattsworth, CA, USA) and then reverse transcribed using oligo-dT and reverse transcriptase according to the manufacturers instructions (Perkin-Elmer, Foster City, CA, USA). Approximately 0.3 μg of mRNA was used as a template for RT-PCR in a final volume of 20 μl. Half of this reaction was used for PCR analysis as recommended by the manufacturer of the RT-PCR kit in a volume of 50 μl. Reverse transcribed cDNA was subjected to PCR amplification with the following primers and conditions: A-(5′-CTGAAGACCACCACGTTCTACTTC); B-(5′-GCAAGAACCAGAAACAGCGACTTG); E-(5′-AATCACGATCGGCTTCTACAGCTG); denaturation at 95°C for 25 s, hybridization at 60°C for 25 s and extension at 72°C for 25 s (30 cycles each per primer pair), using a Perkin Elmer 480 instrument and reagents (Perkin Elmer). PCR products were concentrated on micron 50 filters (Amicon, Beverly, CT, USA), electrophoretically separated on a 0.9% agarose gel, and visualized with ethidium bromide. G3PDH primers served as positive controls for the PCR reaction (Clontech, Palo Alto, CA, USA) and the presence of genomic DNA. The sequence of the rabbit adenosine A_3 cDNA was kindly provided by Dr Steven Reppert (Harvard, Cambridge, MA, USA) and will be reported elsewhere.

2.5. Statistical analysis

Data are expressed as means ± S.E.M. n = number of experiments using batches of cells isolated from individual hearts. Results were analyzed by analysis of variance

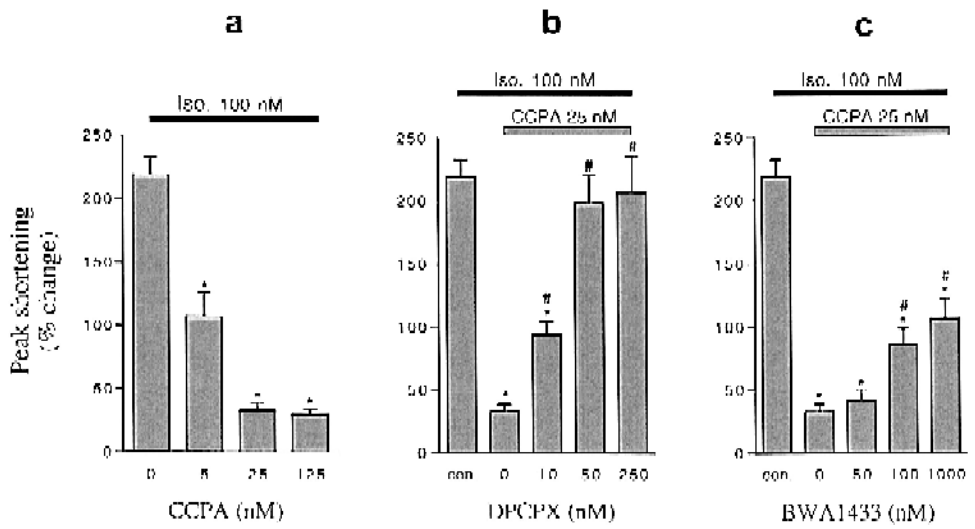

Fig. 2. Determination DPCPX and BWA1433 concentrations that attenuated inhibition of isoprenaline (ISO)-stimulated contraction by CCPA. Isolated myocytes were pretreated for 5 min with: (a) CCPA (0, 5, 25, 125 nM), (b) CCPA (25 nM) plus DPCPX (0, 10, 50, 250 nM) and (c) CCPA (25 nM) plus BWA1433 (0, 50, 100, 1000 nM) prior to addition of isoprenaline (100 nM). In each group 7–8 cells were evaluated. * $P < 0.05$ vs. Iso. 100 nM only; # $P < 0.05$ vs. Iso. 100 nM plus CCPA 25 nM.

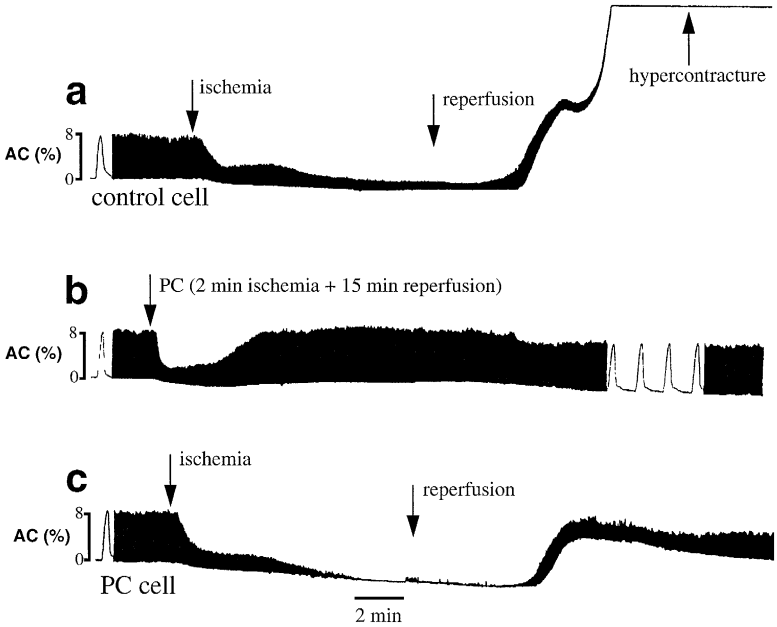

Fig. 3. Typical continuous recordings of amplitude of contraction (AC) from single isolated myocytes subjected to (a) 10 min ischemia and 15 min reperfusion, (b) preconditioning PC; 2 min ischemia, 15 min reperfusion) and (c) preconditioning followed by 10 min ischemia and 15 min reperfusion. AC was expressed as the percent shortening of the diastolic cell length.

(ANOVA) and subsequent comparison of means using Scheffe's *F*-test; $P < 0.05$ was considered significant.

2.6. Other reagents

Isoprenaline was obtained from Sigma (St. Louis, MO, USA). CCPA, DPCPX, BWA1433 and 8-SPT were from Research Biochemicals International (Natick, MA, USA). Collagenase II was purchased from Worthington Biochemical (Malvern, PA, USA).

3. Results

3.1. Effect of preconditioning on ischemia / reperfusion-induced myocyte injury

Fig. 3 shows typical recordings of myocyte contraction for preconditioned and non-preconditioned control cells. When isolated beating myocytes were subjected to 10 min of simulated ischemia and reperfused, hypercontracture (Fig. 3a) occurred in 67 ± 6% of cells ($n = 12$, total cells

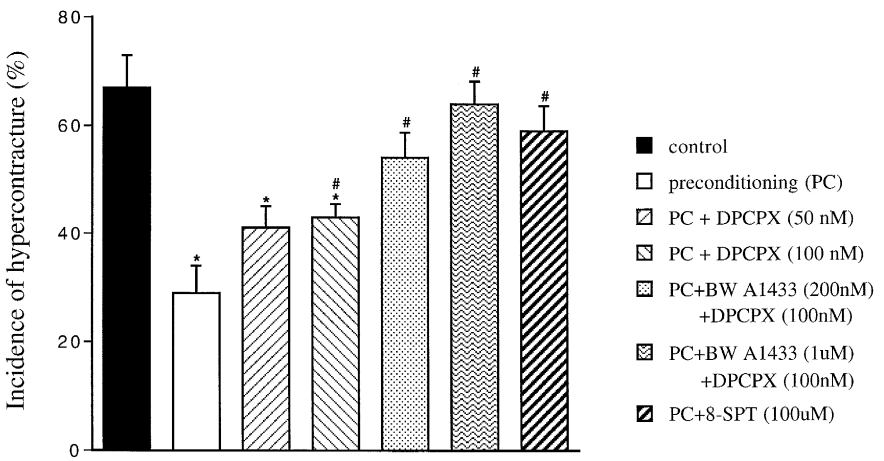

Fig. 4. Effect of adenosine receptor antagonists on preconditioning-attenuated hypercontracture. Isolated myocytes were subjected to 2 min ischemia and 15 min reperfusion in the presence of DPCPX (50 or 100 nM), DPCPX (100 nM) plus BWA1433 (200 nM or 1 μM), or 8-SPT (100 μM) as described in Section 2. Incidence of hypercontracture in cells subjected to 10 min ischemia and 15 min reperfusion is expressed as a percentage of the cells monitored in each experiment. * $P < 0.05$ vs. control, # $P < 0.05$ vs. preconditioning group.

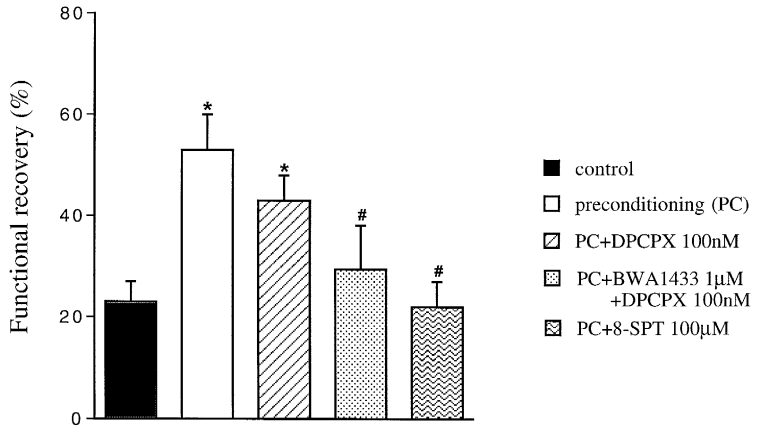

Fig. 5. Effect of adenosine receptor antagonists on preconditioning-augmented contractile function. Isolated myocytes were subjected to preconditioning in the presence of DPCPX (100 nM), DPCPX plus BWA1433 (1 μM), or 8-SPT as described in the legend to Fig. 3. Contractile amplitude of a single isolated myocyte from each cover slip was monitored continuously as described in Section 2. Post-ischemic functional recovery (at the end of reperfusion) is expressed as a percentage of pre-ischemic levels. * $P < 0.05$ vs. control. # $P < 0.05$ vs. preconditioning group.

analyzed = 110) (Fig. 4). In contrast, when myocytes were preconditioned with 2 min of ischemia and 15 min of reperfusion (Fig. 3b), hypercontracture resulting from a subsequent prolonged ischemia and reperfusion was reduced to $29 \pm 5\%$ ($n = 11$, total cells = 101, $P < 0.001$ vs. non-preconditioned control cells) (Fig. 4). Preconditioning had no effect on basal myocyte function (Fig. 3b), but significantly enhanced the recovery of function (Fig. 3c) in surviving myocytes subjected to prolonged ischemia and reperfusion ($53 \pm 7\%$, of pre-ischemic function in preconditioned cells, cells = 12 vs. $23 \pm 4\%$ in controls, cells = 11, $P < 0.05$) (Fig. 5). These results demonstrate that preconditioning protects isolated myocytes from ischemia/reperfusion-induced cell injury.

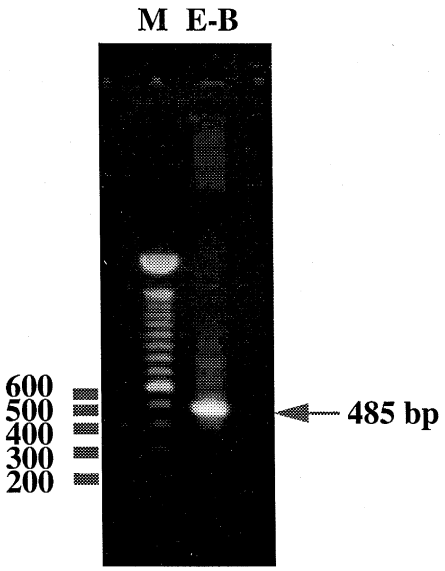

Fig. 6. Expression of A_3 receptors in rabbit cardiac myocytes. M, 1 μg of 100-bp ladder size markers. E-B, PCR reaction of primers E and B obtained using the RT-PCR products or products of primers A and B as template. The position of the expected 485-bp fragment is indicated by the arrow. Marker sizes (in bp) are indicated to the left of the gel.

3.2. Role of adenosine receptors in preconditioning-induced myocyte protection

Fig. 4 and Fig. 5 summarize the effects of adenosine receptor antagonists on preconditioning-attenuated hypercontracture and preconditioning-augmented functional recovery. Incubation of cells with the adenosine A_1-selective antagonist DPCPX (50 or 100 nM) only partly prevented inhibition of hypercontracture by preconditioning ($41 \pm 4\%$, $n = 10$, total cells = 80, or $43 \pm 3\%$, $n = 11$, total cells = 88, respectively), whereas DPCPX (100 nM) combined with the adenosine A_1/A_3-antagonist BWA1433 (1 μM) or the non-selective antagonist 8-SPT (100 μM) completely prevented the protective effects of preconditioning on ischemia/reperfusion-induced hypercontracture ($64 \pm 4\%$, $n = 9$, total cells = 82, or $59 \pm 5\%$, $n = 10$, total cells = 86, respectively; each $P < 0.05$ vs. preconditioned cells and P = NS vs. controls). Similarly, DPCPX (100 nM) only partly attenuated preconditioning-increased functional recovery in the surviving myocytes ($43 \pm 5\%$, cells = 9; $P < 0.05$ vs. control cells but P = NS vs. preconditioned cells), whereas DPCPX combined with BWA1433, or 8-SPT, completely attenuated preconditioning-increased functional recovery ($22 \pm 5\%$, cells = 7, or $29 \pm 9\%$, cells = 8; each $P < 0.05$ vs. preconditioned cells and P = NS vs. control cells). Thus, these results demonstrate that preconditioning protects myocytes by activating adenosine receptors and suggests that both A_1 and A_3 receptors may be involved.

3.3. Evidence for adenosine A_3 receptors in rabbit myocytes by PCR

Reverse transcription-PCR was carried out on rabbit myocyte mRNA using primers specific to the A_3 adenosine receptor sequence to amplify a 583-bp region of the coding sequence of the receptor (primers A and B). No

visible DNA fragments were visualized on the ethidium gel following 30 cycles of PCR. However, PCR amplification of the same cDNA mixture with primers specific to the G3PDH gene, yielded a product of accurate size, with low yield (data not shown), suggesting a low abundance of mRNA in rabbit myocytes. Subsequent amplification of the A-B PCR reaction using primers E (immediately downstream of primer A) and B produced a 485-bp fragment, that was of correct sign, following nested PCR, indicating the presence of adenosine A_3 receptor mRNA (see Fig. 6).

4. Discussion

Ischemic preconditioning has been extensively studied and there is convincing evidence that adenosine receptor activation contributes to its protective effects on the ischemic myocardium (Downey et al., 1993). Two adenosine receptor subtypes, the A_1 and A_3 receptors, have been implicated but their relative contributions are not clearly defined. Consequently, an isolated cell model of preconditioning was developed to enable the specific roles of the A_1 and A_3 receptors to be critically evaluated. Major accomplishments of this study include, (i) establishing an isolated myocyte model of preconditioning in which simulated ischemia conferred protection from a subsequent more severe ischemic insult, (ii) providing evidence that activation of both adenosine A_1 and A_3 receptors, but not the adenosine A_1 receptor alone, is responsible for these protective effects and, (iii) demonstrating that the adenosine A_3 receptor gene is expressed by cardiac myocytes.

In earlier studies that reported preconditioning in isolated cardiac myocytes, Armstrong and Ganote (1994) found that glucose-free preincubation protected rabbit myocytes from ischemic pelleting-induced injury, whereas Ikonomidis et al. (1994) showed that anoxic perfusion was protective in cultured human cardiac myocytes. In these studies non-beating cells were used and cell injury was assessed by trypan blue exclusion. Consistent with these earlier studies, the present studies demonstrate that preconditioning is observed in isolated cardiac myocytes independent of other cell types, i.e., in the absence of neutrophils as well as vascular or neural influences that have been implicated in cardiac ischemic injury. However, they contrast with previous studies because the protective effects of preconditioning were demonstrated in beating myocytes, employed metabolic inhibition to precondition the cells and assessed cellular integrity both morphologically and functionally using video-imaging techniques. In the present studies, preconditioning decreased both the number of myocytes that developed hypercontracture in response to ischemia and improved functional recovery of surviving cells. The magnitude of this protection (50–60%) is similar to that reported in the intact heart, and the mechanism by which these beneficial effects was elicited, i.e., by transient metabolic inhibition, is consistent with recent isolated perfused heart studies (De Albuquerque et al., 1994) providing evidence that preconditioning can occur in the absence of interrupted flow.

The present studies demonstrate that preconditioning is prevented by the combination of A_1- and A_1/A_3-selective antagonists, DPCPX and BWA1433, or the non-selective antagonist, 8-SPT, to demonstrate that adenosine receptor activation protects the myocytes from ischemic injury. However, they extend earlier studies (Armstrong and Ganote, 1994; Liu et al., 1994) by showing that maximal protection elicited by preconditioning requires activation of both adenosine A_1 and A_3 receptors. Interpretation of these studies is complicated because the receptor antagonists employed have poorly defined receptor subtype-specific activity. A large species variability, especially with the adenosine A_3 receptor (Ji et al., 1994), exacerbates this problem. For example, DPCPX binds to the human and rat adenosine A_3 receptors with affinities of 0.8 μM and >100 μM, respectively (Linden, 1994). In the present study, the receptor antagonists were independently evaluated for subtype selectivity using a functional assay for adenosine A_1 receptor antagonism in the same rabbit myocytes used in the preconditioning studies. In this assay, 50 nM DPCPX completely prevented attenuation of isoprenaline-stimulated myocyte contraction by the adenosine A_1-selective receptor agonist, CCPA. Consequently, independent evidence suggests that the partial prevention of preconditioning-induced myocyte protection by 50 or 100 nM DPCPX can be attributed to maximal antagonism of the adenosine A_1 receptor. In contrast, 0.1–1 μM BWA1433 only partially prevented CCPA-induced attenuation of isoprenaline-stimulated contraction. Thus, blockade of a component of preconditioning-induced myocyte protection by 1 μM BWA1433 implies an adenosine A_1-independent mechanism.

BWA1433 binds to cloned sheep and human adenosine A_3 receptors expressed in Chinese hamster ovary cells with an affinity consistent with BWA1433 binding to adenosine A_3 receptors (K_i values 21 nM and 53 nM, respectively; Ji et al., 1994). In contrast, BWA1433 exhibited a lower affinity for the cloned rat adenosine A_3 receptor or the adenosine A_3 receptor in membranes isolated from rabbit brains (K_i values 15 μM and 25 mM, respectively; Ji et al., 1994). It is difficult to reconcile these receptor binding data with the potency of functional antagonism by 1 μM BWA1433 in the present studies. However, it has been suggested by Linden (1994) that the rat adenosine A_3 receptor may be a different subtype of the adenosine A_3 receptor because a structurally-related 8-arylxanthine containing a carboxylic group, BWA522, also exhibited lower affinity for the rat adenosine A_3 receptor in radioligand binding studies, but was a functional antagonist in the presumed A_3-mediated hypotensive response in the rat (Fozard and Hannon, 1994). Moreover, in the rabbit A_3 receptor binding studies the ligand employed, 3-iodo-4-aminobenzyladenosine-5′-*N*-methyl-

uroamide (AB-MECA), has a low selectivity for adenosine A_3 receptors (Ji et al., 1994), and may also bind preferentially to receptor subtypes other than A_3. Furthermore, BWA1433 and the non-selective antagonist 8-SPT, which also prevented the protective effects of preconditioning, were functional antagonists of adenosine A_3 agonist-induced myocyte protection in the rabbit at the same concentrations used in the present study (Armstrong and Ganote, 1994; Liu et al., 1994). Consequently, the present studies provide evidence for both an A_1-dependent component and A_1-independent component of preconditioning-induced protection, and suggest that the A_1-independent component may involve the adenosine A_3 receptor.

These results do not directly address a potential involvement of the adenosine A_{2a} or A_{2b} receptors. However, DPCPX and BWA1433, which together completely block the effects of preconditioning, do not bind with high affinity to A_2 receptors (Linden, 1994; Yakel et al., 1994). In addition, Armstrong and Ganote (1994) showed that the A_{2a} receptor agonist, CGS21680, did not precondition isolated myocytes. Moreover, A_2 receptors couple to G_s proteins which are not pertussis toxin-sensitive and do not couple to protein kinase C (Fleming et al., 1992). Thornton et al. (1993) have shown that preconditioning is pertussis toxin-sensitive and prevented by inhibitors of protein kinase C (Ytrehus et al., 1994; Speechly-Dick et al., 1994), indicative of involvement of G_i-G_o proteins and more consistent with involvement of either adenosine A_1 or A_3 receptors.

The present studies are the first to demonstrate the existence of adenosine A_3 receptor gene expression in cardiac myocytes with the caveat that the high sensitivity of PCR might allow detection of the A_3 mRNA transcript in small numbers of contaminating cells that remain despite efforts to minimize contamination. The finding of adenosine A_3 mRNA supports a role for the adenosine A_3 receptor in preconditioning of these cells. It also provides the first evidence that the rabbit heart contains adenosine A_3 receptors. Messenger RNA for the adenosine A_3 receptor is also present in the rat (Zhou et al., 1992) and human (Sajjadi and Firestein, 1993; Salvatore et al., 1993) heart suggesting that the involvement of the adenosine A_3 receptor in preconditioning-induced protection may be conserved in all species. A physiological role for the adenosine A_3 receptor has not been firmly established. In anesthetized rats, pretreated to antagonize adenosine A_1, A_{2a} and A_{2b} receptors, the non-selective adenosine A_3 agonist, APNEA, induced hypotension that was attributed to histamine release from mast cells (Hannon et al., 1995). Evidence that activation of adenosine A_3 receptors triggers mast cell degranulation was also reported in RBL-2H3 cells, a tumor cell line derived from rat mast cells (Ramkumar et al., 1993), although Marquardt et al. (1994) have suggested other adenosine receptor subtypes may be involved.

In summary, the present studies provide evidence that both adenosine A_1 and A_3 receptors may contribute to preconditioning-induced protection of isolated cardiac myocytes from ischemic injury. These studies also provide the first evidence that adenosine A_3 receptors are present in cardiac myocytes. Further proof of the involvement of adenosine A_3 receptors in preconditioning of myocytes will require highly selective adenosine A_3 receptor antagonists that are currently not available, as well as established species- and receptor-subtype specific binding or functional assays.

References

Armstrong, S. and C.E. Ganote, 1994, Adenosine receptor specificity in preconditioning of isolated rabbit cardiomyocytes: evidence of A_3 receptor involvement, Cardiovasc. Res. 28, 1049.

Auchampach, J.A. and G.J. Gross, 1993, Adenosine A_1 receptors, K_{ATP} channels, and ischemic preconditioning in dogs, Am. J. Physiol. 264, H1327.

De Albuquerque, C.P., G. Gerstenblith and R.G. Weiss, 1994, Importance of metabolic inhibition and cellular pH in mediating preconditioning contractile and metabolic effects in rat hearts, Circ. Res. 74, 139.

Deutsch, E., M. Berger, W.G. Kussmaul, J.W. Hirshfeld Jr., H.C. Herrmann and W.R. Laskey, 1990, Adaptation to ischemia during percutaneous transluminal coronary angioplasty: clinical, hemodynamic, and metabolic features, Circulation 82, 2044.

Downey, J.M., G.S. Liu and J.D. Thornton, 1993, Adenosine and the anti-infarct effects of preconditioning, Cardiovasc. Res. 27, 3.

Esumi, K., M. Nishida, D. Shaw, T.W. Smith and J.D. Marsh, 1991, NADH measurements in adult rat myocytes during simulated ischemia, Am. J. Physiol. 29, H1743.

Fleming, J.W., P.L. Wisler and A.M. Watanabe, 1992, Signal transduction by G proteins in cardiac tissues, Circulation, 85, 420.

Fozard, J.R. and J.P. Hannon, 1994, BW-A522 blocks adenosine A_3 receptor-mediated hypotensive responses in the rat, Eur. J. Pharmacol. 252, R5.

Hannon, J.P., H.J. Pfannkuche and J.R. Fozard, 1995, A role for mast cells in adenosine A_3 receptor-mediated hypotension in the rat, Br. J. Pharmacol. 115, 945.

Ikonomidis, J.S., L.C. Tumiati, R.D. Weisel, A.G. Mickle and R.K. Li, 1994, Preconditioning human ventricular cardiomyocytes with brief periods of simulated ischaemia, Cardiovasc. Res. 28, 1285.

Ji, X., D. Von Lubitz, M.E. Olah, G.L. Stiles and K.A. Jacobson, 1994, Species differences in ligand affinity at central A_3-adenosine receptors, Drug Dev. Res. 33, 51.

Li, Y. and R.A. Kloner, 1993, The cardioprotective effects of ischemic "preconditioning" are not mediated by adenosine receptors in rat hearts, Circulation 87, 1642.

Linden, J., 1994, Cloned adenosine A_3 receptors: pharmacological properties, species differences and receptor functions, Trends Pharmacol. Sci. 15, 298.

Liu, Y. and J.M. Downey, 1992, Ischemic preconditioning protects against infarction in rat heart, Am. J. Physiol. 263, H1107.

Liu, G.S., J. Thornton, D.M. Van Winkle, A.W.H. Stanley, R.A. Olsson and J.M. Downey, 1991, Protection against infarction afforded by preconditioning is mediated by A_1 adenosine receptors in rabbit heart, Circulation 84, 350.

Liu, G.S., S.C. Richards, R.A. Olsson, K. Mullane, R.S. Walsh and J.M. Downey, 1994, Evidence that the adenosine A_3 receptor may mediate the protection afforded by preconditioning in the isolated rabbit heart, Cardiovasc. Res. 28, 1057.

Marquardt, D.L., L.L. Walker and S. Heinemann, 1994, Cloning of two adenosine receptor subtypes from mouse bone marrow-derived mast cells, J. Immunol. 152, 4508.

Murry, C.E., R.B. Jennings and K.A. Reimer, 1986, Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium, Circulation 76, 1124.

Nishida, M., S. Borzak, B. Kraemer, J. Navas, R.A. Kelly, T.A. Smith and J.D. Marsh, 1993, Role of action gradients in hypercontracture of myocytes during simulated ischaemia and reperfusion, Am. J. Physiol. 264, H1896.

Powell, T., 1988, Methods for the isolation and preparation of single adult myocytes. In: Biology of Isolated Adult Cardiac Myocytes (Elsevier, New York, NY) p. 9.

Ramkumar, V., G.L. Stiles, M.A. Beaven and H. Ali, 1993, The A_3 receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells, J. Biol. Chem. 268, 16887.

Sajjadi, F. and G.S. Firestein, 1993, cDNA cloning and sequence analysis of the human A_3 adenosine receptor, Biochim. Biophys. Acta 1179, 105.

Salvatore, C.A., M.A. Jacobsen, H.E. Taylor, J. Linden and R.G. Johnson, 1993, Molecular cloning and characterization of the human adenosine A_3 receptor, Proc. Natl. Acad. Sci. USA 90, 10365.

Schott, R.J., S. Rohmann, E.R. Braun and W. Schaper, 1990, Ischemic preconditioning reduces infarct size in swine myocardium, Circ. Res. 66, 1133.

Speechly-Dick, M.E., M.M. Mocanu and D.M. Yellon, 1994, Protein kinase C: its role in ischemic preconditioning in the rat, Circ. Res. 75, 586.

Thornton, J.D., G.S. Liu, R.A. Olsson and J.M. Downey, 1992, Intravenous pre-treatment with A_1 selective adenosine analogues protects the heart against infarction, Circulation 85, 659.

Thornton, J.D., G.S. Liu and J.M. Downey, 1993, Pre-treatment with pertussis toxin blocks the protective effects of preconditioning: evidence for a G-protein mechanism, J. Mol. Cell. Cardiol. 25, 311.

Tucker, A.L. and J. Linden, 1993, Cloned receptors and cardiovascular responses to adenosine, Cardiovasc. Res. 27, 62.

Wahler, G.M., 1992, Developmental increases in the inwardly rectifying potassium current of rat ventricular myocytes, Am. J. Physiol. 262, C1266.

Wang, J.X., K. Flemal, Z.H. Qiu, L. Ablin, W. Grossman and J.P. Morgan, 1994, Ca^{2+} handling and myofibrillar Ca^{2+} sensitivity in ferret cardiac myocytes with pressure-overload hypertrophy, Am. J. Physiol. 267, H918.

Yakel, J.L., R.A. Warren, S.M. Reppert and R.A. North, 1994, Functional expression of adenosine A_{2b} receptor in Xenopus oocytes, Mol. Pharmacol. 43, 277.

Yao, A. and G.J. Gross, 1993, Glibenclamide antagonizes adenosine A_1 receptor-mediated cardioprotection in stunned canine myocardium, Circulation 88, 235.

Yellon, D.M., A.M. Alkhulaifi and W.B. Pugsley, 1993, Preconditioning the human myocardium, Lancet 342, 276.

Ytrehus, K., Y. Liu and J.M. Downey, 1994, Preconditioning protects ischemic rabbit heart by protein kinase C activation, Am. J. Physiol. 266, H1145.

Zhou, Q.Y., C. Li, M.E. Olah, R.A. Johnson, G.L. Stiles and O. Civelli, 1992, Molecular cloning and characterization of an adenosine receptor: the A_3 adenosine receptor, Proc. Natl. Acad. Sci. USA 89, 7432.