Role of large-conductance Ca^{2+}-activated K^+ channels in adenosine A_1 receptor-mediated pharmacological postconditioning in H9c2 cells

Laurice Fretwell and John M. Dickenson

Abstract: Ischaemic postconditioning is a phenomenon whereby short periods of ischaemia applied during the start of reperfusion protect the myocardium from the damaging consequences of reperfusion. As such, pharmacological-induced postconditioning represents an attractive therapeutic strategy for reducing reperfusion injury during cardiac surgery and following myocardial infarction. The primary aim of this study was to determine the role of large-conductance Ca^{2+}-activated potassium channels (BK_{Ca} channels) in adenosine A_1 receptor-induced pharmacological postconditioning in the rat embryonic cardiomyoblast-derived cell line H9c2. H9c2 cells were exposed to 6 h hypoxia (0.5% O_2) followed by 18 h reoxygenation (H/R) after which cell viability was assessed by monitoring lactate dehydrogenase (LDH) release and caspase-3 activation. The adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA; 100 nmol/L) or the BK_{Ca} channel opener NS1619 (10 μmol/L) were added for 30 min at the start of reoxygenation following 6 h hypoxic exposure. Where appropriate, cells were treated (15 min) before pharmacological postconditioning with the BK_{Ca} channel blockers paxilline (1 μmol/L) or iberiotoxin (100 nmol/L). Pharmacological postconditioning with CPA or NS1619 significantly reduced H/R-induced LDH release. Treatment with paxilline or iberiotoxin attenuated adenosine A_1 receptor and NS1619-induced pharmacological postconditioning. These results have shown for the first time that BK_{Ca} channels are involved in adenosine A_1 receptor-induced pharmacological postconditioning in a cell model system.

Key words: adenosine, H9c2 cells, large-conductance Ca^{2+}-activated potassium channel, hypoxia, adenosine A_1 receptor, pharmacological postconditioning.

Résumé : Le postconditionnement ischémique est un phénomène par lequel de brèves périodes d'ischémie appliquées au début de la reperfusion protègent le myocarde contre les effets préjudiciables de la reperfusion. En tant que tel, le postconditionnement induit de manière pharmacologique représente une stratégie thérapeutique attrayante pour diminuer la lésion de reperfusion durant une intervention cardiaque et après un infarctus du myocarde. La présente étude a eu comme principal objectif de déterminer le rôle des canaux potassiques de grande conductance activés par le Ca^{2+} (canaux BK_{Ca}) dans le postconditionnement pharmacologique induit par les récepteurs A_1 de l'adénosine dans la lignée de cellules H9c2 dérivée de cardiomyoblastes embryonnaires de rats. On a exposé les cellules H9c2 à 6 h d'hypoxie (0,5 % O_2), puis à 18 h de réoxygénation (H/R) ; on a ensuite évalué la viabilité cellulaire en examinant la libération de la lactate déshydrogénase (LDH) et l'activation de la caspase-3. On a ajouté l'agoniste des récepteurs A_1 de l'adénosine, N^6-cyclopentyladénosine (CPA ; 100 nmol/L), ou l'ouvreur de canaux BK_{Ca}, NS1619 (10 μmol/L), durant 30 min, au début de la réoxygénation qui a suivi l'exposition hypoxique de 6 h. Les cellules appropriées ont été traitées (15 min) avant le postconditionnement ischémique avec les bloqueurs des canaux BK_{Ca} paxilline (1 μmol/L) et ibériotoxine (100 nmol/L). Le postconditionnement pharmacologique avec la CPA ou le NS1619 a réduit significativement la libération de LDH induite par l'H/R. Le traitement avec la paxilline ou l'ibériotoxine a atténué le postconditionnement pharmacologique induit par le récepteur A_1 de l'adénosine ou le NS1619. Ces résultats sont les premiers à démontrer le rôle des canaux BK_{Ca} dans le postconditionnement pharmacologique induit pat les récepteurs A_1 de l'adénosine dans un modèle cellulaire.

Mots-clés : adénosine, cellules H9c2, canal potassique de grande conductance activé par le Ca^{2+}, hypoxie, récepteur A_1 de l'adénosine, postconditionnement pharmacologique.

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Introduction

Ischaemic preconditioning is a well-characterized phenomenon in which brief periods of ischaemia can protect the myocardium from infarction induced by a subsequent and prolonged ischaemic attack (Murry et al. 1986). More recently, Zhao et al. (2003) reported another form of cardioprotection, termed ischaemic postconditioning, which in-

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volves short periods of ischaemia applied during the start of reperfusion after a prolonged ischaemic insult. From a clinical viewpoint, ischaemic postconditioning represents an attractive therapeutic approach for reducing reperfusion-induced myocardial injury.

The molecular mechanisms underlying ischaemic preconditioning have been extensively studied, resulting in a number of membrane-bound receptors, predominantly belonging to the G protein-coupled receptor (GPCR) superfamily, being implicated in cardioprotection including adenosine A_1 and A_3 receptors (Sommerschild and Kirkebøen 2000; Headrick et al. 2003; Peart and Headrick 2007). The cardioprotective effects of ischaemic preconditioning can be mimicked either by direct stimulation of the above-mentioned receptors or via activation of the signal transduction pathways involved, using selective pharmacological agents (pharmacological preconditioning). Many of the receptors and key signalling pathways associated with ischaemic preconditioning are also involved in cardioprotection induced by ischaemic postconditioning (Burley and Baxter 2009).

The cardioprotective signal transduction mechanisms triggered by the adenosine A_1 receptor have been widely studied and include the activation of several protein kinases, including protein kinase C, extracellular signal-regulated kinase 1/2 (ERK1/2), and p38 MAPK (Henry et al. 1996; Dana et al. 2000; Germack and Dickenson 2005). These pathways enhance cell survival and modulate mitochondrial function in part through the opening of mitochondrial ATP-sensitive potassium channels (mK_{ATP} channels) located on the inner mitochondrial membrane (Liang 1996) and inhibition of the mitochondrial permeability transition pore (Juhaszova et al. 2004).

Large-conductance Ca^{2+}-activated potassium channels (BK_{Ca} channels), which are abundantly expressed on the plasma membrane of vascular smooth muscle cells (Ko et al. 2008), have recently been implicated in cardioprotection against ischaemia and reperfusion injury in isolated perfused guinea-pig heart (Xu et al. 2002). However, evidence to date suggests that in cardiac myocytes BK_{Ca} channels are expressed only on the inner mitochondrial membrane and not at the cell surface (Xu et al. 2002; Wang et al. 2008). Since the observation by Xu et al. (2002), BK_{Ca} channels have been implicated in ischaemic preconditioning against ischaemia and reperfusion injury in isolated rat and mouse hearts, anaesthetized dogs, and isolated cardiac myocytes (Shintani et al. 2004; Wang et al. 2004; Cao et al. 2005*a*; Sato et al. 2005).

In studies using the rat embryonic cardiomyoblast-derived cell line H9c2, we recently showed that BK_{Ca} channels are involved in adenosine A_1 receptor-induced pharmacological preconditioning (Fretwell and Dickenson 2009). These cells, derived from embryonic rat heart tissue (Kimes and Brandt 1976), are increasingly used as an in vitro model for studies exploring cardioprotection, since they display similar morphological, electrophysiological, and biochemical properties to primary cardiac myocytes (Hescheler et al. 1991). Therefore, the primary aim of this study was to determine the role of BK_{Ca} channels in adenosine A_1 receptor-induced pharmacological postconditioning in H9c2 cells. The results obtained indicate that adenosine A_1 receptor activation triggers postconditioning in H9c2 cells via the opening of BK_{Ca} channels.

Materials and methods

Materials

CPA (N^6-cyclopentyladenosine), DPCPX (1,3-dipropylcyclopentylxanthine), and NS1619 (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-trifluoromethyl-2Hbenzinorbinaltorphimine) were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Paxilline and iberiotoxin were from Tocris (Bristol, UK). Dulbecco's modified Eagle's medium (DMEM), foetal calf serum, trypsin (10x), L-glutamine (200 mmol/L), penicillin (10 000 U/mL) / streptomycin (10 000 μg/mL) were purchased from BioWhittaker UK Ltd (Berkshire, UK). All other chemicals were of analytical grade. Stock concentrations of CPA (10 mmol/L), DPCPX (10 mmol/L), and NS1619 (10 mmol/L) were dissolved in DMSO. The final concentration of DMSO was ≥0.1%.

Cell culture

Rat embryonic cardiomyoblast-derived H9c2 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). Cells were cultured in DMEM supplemented with 2 mmol/L L-glutamine, 10% (*v*/*v*) foetal calf serum, and penicillin (100 U/mL) / streptomycin (100 μg/mL). Cells were maintained in a humidified incubator (95% air / 5% CO_2) at 37 °C until 70%–80% confluent and subcultured (1:5 split ratio) using 0.05% trypsin (*w*/*v*) / 0.02% EDTA (*w*/*v*).

Experimental protocol

H9c2 cells in glucose-free and serum-free DMEM were exposed to 6 h hypoxia using a hypoxic incubator (5% CO_2 / 0.5% O_2) at 37 °C, where O_2 was replaced by N_2. Following hypoxic exposure, cells were reoxygenated in DMEM containing 1% foetal calf serum for 18 h in a humidified atmosphere (5% CO_2 / 95% air) at 37 °C. For pharmacological postconditioning, cells were exposed to the adenosine A_1 receptor agonist CPA (100 nmol/L) or the BK_{Ca} channel opener NS1619 (10 μmol/L) for 30 min at the start of reoxygenation following hypoxia exposure. Where appropriate, cells were treated for 15 min with the BK_{Ca} channel blockers paxilline (1 μmol/L) or iberiotoxin (100 nmol/L) before the addition of CPA or NS1619. After the above treatments, the medium containing agonists, openers, and inhibitors was removed and replaced with fresh DMEM containing 1% (*v*/*v*) foetal calf serum for the remainder of the reoxygenation period.

Cell viability

H9c2 cells were plated in 96-well flat-bottomed plates at a final density of 5000 cells/well and cultured for 24 h in fully supplemented DMEM, after which the medium was replaced with glucose- and serum-free DMEM containing 2 mmol/L L-glutamine and penicillin/streptomycin (100 U/mL). Cell viability assays were performed by determining the activity of lactate dehydrogenase (LDH) released into the media and detected colourimetrically (CytoTox 96 Non-Radioactive Cytotoxicity LDH assay, Promega, Southamp-

ton, UK). Assays were performed according to the manufacturer's instructions.

Caspase 3 activity

H9c2 cells were cultured in 60 mm tissue culture dishes, and caspase-3 activity was measured following 6 h hypoxia using a fluorescence-based assay. Cells were harvested using 0.05% trypsin (*w*/*v*) / 0.02% EDTA (*w*/*v*), centrifuged (1000*g* for 5 min), and the resulting cell pellets resuspended in 120 μL lysis buffer (25 mmol/L HEPES, 2.5 mmol/L CHAPS, 2.5 mmol/L DDT, pH 7.4) and incubated on ice for 20 min. Cell lysates were centrifuged (14 000*g* at 4 °C) and the supernatant collected and stored on ice. Aliquots of supernatant (30 μL) were transferred to black 96-well microplates (Greiner Bio-One, Stonehouse, Gloucestershire, UK) and 50 μL assay buffer (20 mmol/L HEPES, 5 mmol/L DDT, 0.1% CHAPS, 2 mmol/L EDTA, pH 7.4) and 20 μL caspase-3 substrate (0.5 mmol/L; acetyl-DEVD-7-amidomethylcoumarin) were added. Fluorescence readings (λ_{ex} = 355 nm and λ_{em} = 460 nm) were taken over a 2 h period. Protein level was determined using the Bio-Rad DC Protein assay kit (Bio-Rad Laboratories, Hertfordshire, UK) and caspase-3 activity per min per μg protein determined and expressed as a percentage of basal activity.

Data analysis

Statistical significance was determined by ANOVA with a post hoc Tukey's test ($p < 0.05$ was considered statistically significant). All data are presented as means ± SEM. The *n* in the text refers to the number of separate experiments.

Results

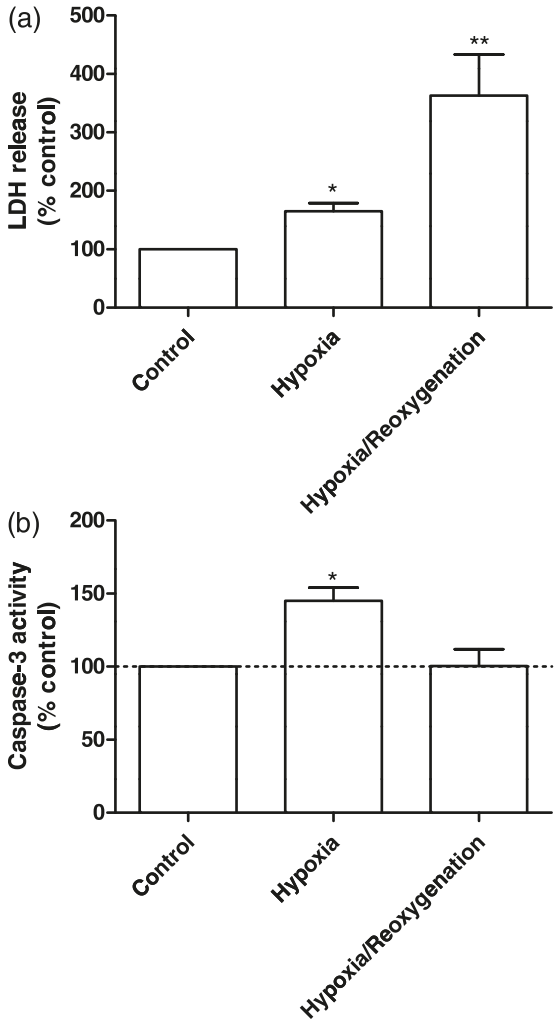
Hypoxia/reoxygenation-induced cell death in H9c2 cells

Cell-based systems using hypoxia/reoxygenation protocols are widely used as an in vitro model of ischaemia/reperfusion injury. In this study, H9c2 cells were exposed to 6 h hypoxia (0.5% O_2) in serum-free and glucose-free medium, followed by 18 h reoxygenation in medium containing glucose and 1% serum, as described previously (Germack and Dickenson 2005). The effects of hypoxia and reoxygenation on cell viability were assessed by monitoring LDH activity released into the medium and caspase-3 activity. As shown in Fig. 1, 6 h hypoxia followed by 18 h reoxygenation (H/R) induced a marked increase in LDH release compared with cells exposed to 6 h hypoxia. In marked contrast, there was no measurable increase in caspase-3 activity following H/R, suggesting that apoptosis had not occurred.

Role of BK_{Ca} channels in NS1619-induced postconditioning in H9c2 cells

The ability of BK_{Ca} channel activation to trigger postconditioning was assessed by treating H9c2 cells during the first 30 min of reoxygenation with the BK_{Ca} channel opener NS1619 (10 μmol/L). We have previously shown that this concentration of NS1619 triggers pharmacological preconditioning against 6 h hypoxia-induced cell death in H9c2 cells (Fretwell and Dickenson 2009). As shown in Fig. 2, postconditioning with NS1619 significantly reversed H/R-induced LDH release ($n = 7$, $p < 0.001$). Furthermore, the protection afforded by NS1619 was reversed by the BK_{Ca} channel blockers paxilline (1 μmol/L; $n = 5$, $p < 0.05$) and iberiotoxin (10 nmol/L; $n = 5$, $p < 0.05$), confirming the involvement of the BK_{Ca} channel (Fig. 2).

Fig. 1. Cell death induced by hypoxia/reoxygenation in H9c2 cells. Cells were exposed to 6 h normoxia (control), 6 h hypoxia, or 6 h hypoxia followed by 18 h reoxygenation, then cell death was assessed by (*a*) lactate dehydrogenase (LDH) release or (*b*) caspase-3 activity. Data are expressed as percentage of the control. Each bar represents the mean ± SEM from 6 separate experiments; for each experiment, the mean was taken from 2 (caspase-3) or 6 (LDH) replicates. *, $p < 0.05$ and **, $p < 0.01$ vs. control.



Role of BK_{Ca} channels in adenosine A_1 receptor-induced postconditioning in H9c2 cells

We recently reported that the selective adenosine A_1 receptor agonist CPA triggers pharmacological preconditioning against 6 h hypoxia-induced cell death in H9c2 cells (Fretwell and Dickenson 2009). In this study, pharmacological postconditioning with CPA triggered protection against H/R-induced LDH release (Fig. 3). CPA-mediated protection was reversed by the adenosine A_1 receptor antagonist DPCPX (10 μmol/L; $n = 6$, $p < 0.01$), confirming the involvement of the A_1 receptor. Furthermore, the BK_{Ca} channel blockers paxilline (1 μmol/L; $n = 6$, $p < 0.05$) and

Fig. 2. The role of large-conductance Ca^{2+}-activated potassium channels (BK_{Ca} channels) in 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-trifluoromethyl-2Hbenzinor-binaltorphimine (NS1619)-mediated postconditioning in H9c2 cells. Cells were exposed to 6 h hypoxia, followed by 18 h reoxygenation. For the first 45 min of reoxygenation, cells were treated with paxilline (Pax; 1 μmol/L) or iberiotoxin (IbTx; 10 nmol/L) with the addition of NS1619 (10 μmol/L) for the final 30 min of treatment. Data are expressed as percentage of the untreated control cells exposed to hypoxia/reoxygenation alone (=100%). Each bar represents the mean ± SEM of 5 separate experiments, and for each experiment the mean was taken from 6 replicates. *, $p < 0.05$ vs. control; #, $p < 0.05$ vs. NS1619.

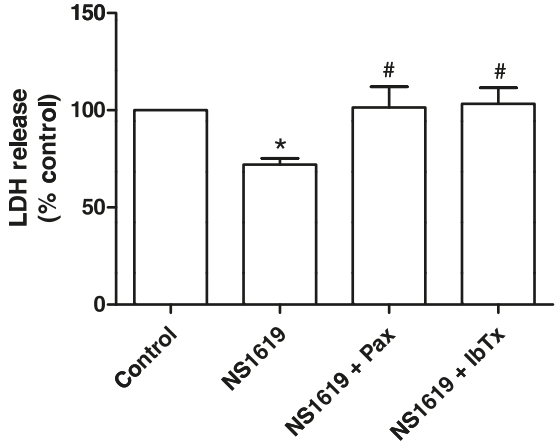
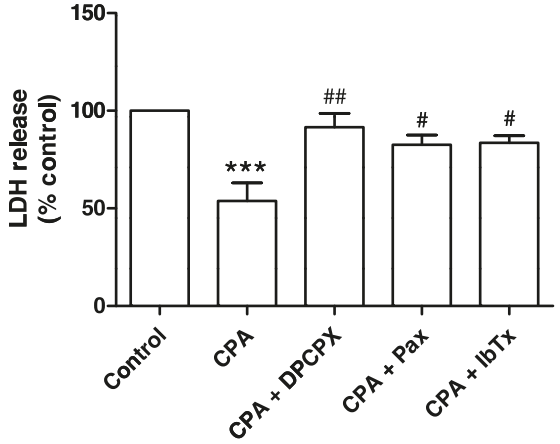


Fig. 3. The role of BK_{Ca} channels in adenosine A_1 receptor-mediated postconditioning in H9c2 cells. Cells were exposed to 6 h hypoxia, followed by 18 h reoxygenation. For the first 45 min of reoxygenation, cells were treated with 1,3-dipropylcyclopentylxanthine (DPCPX; 10 μmol/L), paxilline (Pax; 1 μmol/L), or iberiotoxin (IbTx; 10 nmol/L), with the addition of N^6-cyclopentyladenosine (CPA; 100 nmol/L) for the final 30 min of treatment. Data are expressed as percentage of the untreated control cells exposed to hypoxia/reoxygenation alone (=100%). Each bar represents the mean ± SEM of 5 separate experiments, and for each experiment, the mean was taken from 6 replicates. ***, $p < 0.001$ vs. control; ##, $p < 0.01$ vs. NS1619; and #, $p < 0.05$ vs. 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-trifluoromethyl-2Hbenzinor-binaltorphimine (NS1619).



iberiotoxin (10 nmol/L; $n = 6$, $p < 0.05$) significantly attenuated the CPA-mediated response, suggesting a role for the BK_{Ca} channel in adenosine A_1 receptor-mediated postconditioning in H9c2 cells.

Discussion

We recently described a role for the BK_{Ca} channel in adenosine A_1 receptor-induced pharmacological preconditioning in H9c2 cells (Fretwell and Dickenson 2009). The primary aim of this study was to investigate the potential role of the BK_{Ca} channel in adenosine A_1 receptor-mediated postconditioning.

Ischaemic postconditioning is a newly described phenomenon that involves short intermittent periods of ischaemia applied at the start of reperfusion, protecting the myocardium from the damaging consequences of reperfusion (Zhao et al. 2003). Since its initial discovery, ischaemic postconditioning has been observed in many animal models (Burley and Baxter 2009) and, importantly, in the human heart (Staat et al. 2005). Identifying the molecular mechanisms that trigger postconditioning may facilitate the discovery of suitable therapeutic targets for clinical use against reperfusion injury, either during surgical procedures or following acute myocardial infarction. Indeed, many of the receptors and key signalling pathways traditionally associated with ischaemic preconditioning are also involved in cardioprotection induced by ischaemic postconditioning (Burley and Baxter 2009).

The roles of the adenosine A_1 receptor in ischaemic preconditioning and associated signal transduction pathways have been extensively studied in many model systems (Sommerschild and Kirkebøen 2000; Headrick et al. 2003; Peart and Headrick 2007). However, the involvement of the adenosine A_1 receptor in ischaemic postconditioning is not clear-cut (Burley and Baxter 2009). Xi et al. (2008) reported that the infarct limiting protection of ischaemic postconditioning was absent in adenosine A_1 receptor knockout mice. Similarly, the ischaemic postconditioning in normal and hypercholesterolemic rabbits was abolished by the adenosine A_1 receptor antagonist DPCPX (Donato et al. 2007). Studies using isolated rat ventricular myocytes have also demonstrated that pharmacological postconditioning with adenosine is blocked by DPCPX (Lu et al. 2006). In marked contrast, several studies suggest that the adenosine A_1 receptor is not involved in ischaemic postconditioning (Kin et al. 2005; Philipp et al. 2006). The reasons for these differences may reflect differences in experimental protocol and model systems used. However, the data presented in this study, using the rat embryonic cardiomyoblast-derived cell line H9c2, support the role of the adenosine A_1 receptor in triggering postconditioning.

Although a number of studies have implicated a role of the mitochondrial BK_{Ca} channel in both ischaemic and pharmacological preconditioning (Xu et al. 2002; Shintani et al. 2004; Wang et al. 2004; Cao et al. 2005*a*; Sato et al. 2005), there are no reports to date detailing its role in postconditioning. In this study, we have shown that opening of the BK_{Ca} channel during the onset of reoxygenation (achieved using NS1619) attenuates H/R-induced cell injury. Importantly, paxilline and iberiotoxin inhibited the ability of

NS1619 to block H/R-induced cell death, confirming for the first time a role for the BK_{Ca} channel in postconditioning. Studies using BK_{Ca} channel blockers in conjunction with NS1619 are important since NS1619 also mediates cytoprotection via BK_{Ca} channel-independent pathways (Cancherini et al. 2007; Gáspár et al. 2008). For example, NS1619 promotes nonselective permeabilization of the inner mitochondrial membrane to ions in isolated heart mitochondria and BK_{Ca} channel-independent delayed preconditioning in rat cortical neuronal cultures (Cancherini et al. 2007; Gáspár et al. 2008). Furthermore, since evidence to date suggests that BK_{Ca} channel expression in cardiac myocytes is restricted to the inner mitochondrial membrane, the effects of NS1619, iberiotoxin, and paxilline are likely to be entirely mitochondrial (Xu et al. 2002; Wang et al. 2008). This is in contrast to the K_{ATP} channel that mediates cardioprotection via plasma membrane and mitochondrial-based pathways (Hanley and Daut 2005).

We recently reported a role for the BK_{Ca} channel in adenosine A_1 receptor-induced pharmacological preconditioning in H9c2 cells (Fretwell and Dickenson 2009). In this study, we have shown for the first time that the BK_{Ca} channel is also involved in adenosine A_1 receptor-induced postconditioning. However, at present, the signal transduction pathway(s) linking adenosine A_1 receptor activation with BK_{Ca} channel opening are not known. Previous studies have shown that BK_{Ca} channel activation via the κ-opioid receptor and adrenomedullin involve PKC and PKA, respectively (Cao et al. 2005*a*; Nishida et al. 2008). It will be interesting to establish whether any of the protein kinases (PKC, ERK1/2, and p38 MAPK) associated with the cardioprotective effects of the adenosine A_1 receptor trigger BK_{Ca} channel opening.

We previously showed that BK_{Ca} channel opening attenuates hypoxia-induced caspase-3 activation in H9c2 cells, suggesting anti-apoptotic effects (Fretwell and Dickenson 2009). However, in this study, we did not observe caspase-3 activation following hypoxia/reoxygenation challenge. This is surprising, since other studies, including work from our laboratory using neonatal rat cardiomyocytes, have shown caspase-3 activation following hypoxia/reoxygenation (Germack and Dickenson 2005; Kang et al. 2000). Indeed, previous studies using H9c2 cells have reported that 2 h hypoxia followed by 16 h reoxygenation triggers caspase-3 activation and apoptotic cell death (Chiu et al. 2008). The reason(s) for the apparent lack of caspase-3 activity in this study is not known, but may reflect the differing period of hypoxic exposure (6 h vs. 2 h). Alternatively, apoptotic cell death may not contribute to cell death in H9c2 cells exposed to 6 h hypoxia prior to 16 h reoxygenation. Detailed time-course analysis experiments (varying hypoxia exposure times vs. varying reoxygenation times) measuring necrotic and apoptotic cell death may shed light on this issue.

At present, the downstream cardioprotective mechanisms associated with mitochondrial BK_{Ca} channel opening are not fully understood. However, several studies have indicated that inhibition of mitochondrial permeability transition pore opening is downstream of this channel (Cao et al. 2005*a*, 2005*b*; Gao et al. 2005). The recent demonstration that a single dose of cyclosporine A given at the time of reperfusion to patients undergoing percutaneous coronary intervention for acute myocardial infarction results in infarct size reduction (Piot et al. 2008) is an indication of the therapeutic potential of inhibiting the mitochondrial permeability transition pore. Additional protective mechanisms associated with BK_{Ca} channel opening include reduction in mitochondrial Ca^{2+} overload (Kang et al. 2007), which would aid mitochondrial permeability transition pore closure, and the generation of reactive oxygen species (Heinen et al. 2007). Clearly, further studies are required to ascertain whether postconditioning induced by the adenosine A_1 receptor involves mitochondrial permeability transition pore inhibition downstream from the BK_{Ca} channel.

In summary, we have shown for the first time that BK_{Ca} channel opening is involved in adenosine A_1 receptor-induced postconditioning. Future experiments will endeavour to characterize the signalling pathways that link adenosine A_1 receptor activation to BK_{Ca} channel opening and to elucidate the cardioprotective mechanisms downstream of channel opening in this model cell system.

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