Infarct size limitation by adrenomedullin: protein kinase A but not PI3-kinase is linked to mitochondrial K$_{Ca}$ channels†

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Received 27 March 2007; revised 25 July 2007; accepted 26 July 2007

Time for primary review: 29 days

Aim Adrenomedullin (ADM) has been shown to protect the heart against ischaemic injury, but little is known of the underlying mechanism. Mitochondrial Ca$^{2+}$-activated K$^{+}$ (mitoK$_{Ca}$) channels play a key role in cardioprotection. This study examined whether mitoK$_{Ca}$ channel is involved in the protection afforded by ADM.

Methods Flavoprotein fluorescence in rabbit ventricular myocytes was measured to assay mitoK$_{Ca}$ channel activity. Infarct size in the isolated perfused rabbit hearts subjected to 30-min global ischaemia and 120-min reperfusion was determined by triphenyltetrazolium chloride staining.

Results The mitoK$_{Ca}$ channel opener NS1619 (30 μM) partially oxidized flavoprotein. ADM (10 nM) augmented the NS1619-induced flavoprotein oxidation when applied after the effect of NS1619 had reached steady state. This potentiating effect of ADM was prevented by the protein kinase A (PKA) inhibitor KT5720 (200 nM), but not by the phosphatidylinositol 3-kinase (PI3-K) inhibitor LY294002 (5 μM). The mitoK$_{Ca}$ channel blocker paxilline (PX, 2 μM) completely blocked the oxidative effects of NS1619 in the presence of ADM. Treatment with ADM for 10 min before ischaemia significantly reduced infarct size after ischaemia/reperfusion from 63 ± 3% in controls to 32 ± 4% (P < 0.01). This infarct size-limiting effect of ADM was abolished by PX (61 ± 2%), as well as by KT5720 (62 ± 3%). ADM treatment for the first 10 min of reperfusion significantly reduced infarct size compared with controls (42 ± 3%, P < 0.01). This cardioprotective effect of ADM was unaffected by PX (38 ± 4%), but was abolished by LY294002 (60 ± 4%).

Conclusions ADM augments the opening of mitoK$_{Ca}$ channels by PKA activation, but not by PI3-K activation. ADM treatment prior to ischaemia reduces infarct size via PKA-mediated activation of mitoK$_{Ca}$ channels. On the other hand, ADM treatment upon reperfusion reduces infarct size via a PI3-K-mediated pathway without activating mitoK$_{Ca}$ channels.

1. Introduction

Adrenomedullin (ADM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma tissue by monitoring cyclic 3',5'-adenosine monophosphate (cAMP) levels in platelet. Later, the peptide was shown to increase the level of cAMP in a wide variety of cells, including smooth muscle cells, endothelial cells, and cardiac cells. Thus, it was initially postulated that cAMP might be the primary second messenger involved in ADM-induced vasorelaxation. However, it has been shown that ADM induces endothelium-dependent vasodilatation through a phosphatidylinositol 3-kinase (PI3-K)-dependent pathway. Recently, evidence has been accumulating that ADM has more pleiotropic effects than initially expected as a vasodilator. The plasma ADM levels increase in patients with various cardiovascular diseases, including hypertension, heart failure, and myocardial infarction. Therefore, endogenous ADM, which is ubiquitously distributed, is thought to be involved in the regulation of cardiovascular functions. Furthermore, exogenous ADM administration exerts beneficial cardiovascular effects. It has been reported that ADM inhibits apoptosis of rat cardiac myocytes via a cAMP-dependent pathway. More recently, Okumura et al. reported that intravenous administration of ADM attenuated myocardial ischaemia/reperfusion injury via a PI3-K-dependent pathway. However, the mechanisms that underlie the cardioprotective effects of ADM remain obscure.

†This article was published online by Elsevier on 2 August, 2007.

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Large conductance Ca\(^{2+}\)-activated K\(^+\) channels of cardiac myocytes are present in the mitochondrial inner membrane (mitoK\(_{Ca}\) channels) and have been implicated in cardioprotection.\(^{16,17,18}\) Our previous study demonstrated that, with the use of the flavoprotein fluorescence method, mitoK\(_{Ca}\) channel opening could be potentiated by cAMP-dependent protein kinase (PKA).\(^{19}\) It is therefore reasonable to assume that ADM (through the activation of PKA) acts on mitoK\(_{Ca}\) channels and may confer cardioprotection. Besides PKA, ADM has been shown to activate PI3-K in the heart.\(^{15,20}\) PI3-K/Akt is one of the pro-survival kinases which have been termed the reperfusion injury salvage kinases (RISK), and the activation of the PI3-K/Akt pathway promotes cell survival during early reperfusion.\(^{21}\) Accordingly, it would be of great interest to know the link between the activation of PI3-K/Akt and the opening of mitoK\(_{Ca}\) channels. However, it remains unclear whether activation of PI3-K by ADM opens mitoK\(_{Ca}\) channels and thereby leads to cardioprotection. Therefore, the present study was undertaken to characterize the effect of ADM on mitoK\(_{Ca}\) channels and to determine the contribution of PKA- and/or PI3-K-mediated pathways.

2. Methods

All procedures complied with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996 revision), and were approved by the Institutional Animal Care and Use Committee of Chiba University.

2.1 Isolation of rabbit ventricular myocytes

Rabbit ventricular myocytes were isolated by collagenase digestion. Female rabbits (Japanese White) weighing 2.5–3.5 kg were anesthetized by intravenous injection of 30 mg/kg pentobarbital sodium. Excised hearts were mounted on a Langendorff apparatus and perfused with modified HEPES-buffered Tyrode’s solution composed of (in mM) NaCl 143, KCl 5.4, CaCl\(_2\) 1.8, NaH\(_2\)PO\(_4\) 0.33, MgCl\(_2\) 0.5, glucose 5.5, and HEPES 5 (pH 7.4). The perfusate was bubbled with 100% O\(_2\) and maintained at 37°C. After 5 min perfusion, hearts were perfused without Ca\(^{2+}\) for another 10 min. The perfusion solution was then switched to one containing collagenase (0.25 mg/ml, Wako type I). After 25–30 min of digestion, the heart was perfused with the high-K\(^+\) low-Cl\(^-\) (modified KB) solution containing (in mM) KOH 70, L-glutamic acid 50, KCl 40, taurine 20, K\(_2\)HPO\(_4\) 20, MgCl\(_2\) 3, glucose 10, EGTA 1, and HEPES-KOH buffer 10 (pH 7.4). Ventricular tissue was cut into small pieces in the modified KB solution, and the pieces were gently agitated to dissociate cells. Cells were then filtered through nylon mesh. Once isolated, the cells were suspended in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum at room temperature (≈22°C) until use.

2.2 Flavoprotein fluorescence measurement

To index mitoK\(_{Ca}\) or mitochondrial ATP-sensitive K\(^+\) (mitoK\(_{ATP}\)) channel activity, the autofluorescence of mitochondrial flavoprotein was measured by a modification of method described by Sato et al.\(^{19,22}\) Briefly, the cells were superfused with glucose-free Tyrode’s solution containing (in mM) NaCl 143, KCl 5.4, CaCl\(_2\) 1.8, NaH\(_2\)PO\(_4\) 0.33, MgCl\(_2\) 0.5 and HEPES 5 (pH 7.4) at room temperature (≈22°C). Flavoprotein fluorescence was excited at 480 nm (for 200 ms every 10 s) and emitted at 520 nm. Relative fluorescence was calibrated with signals recorded after application of the mitochondrial uncoupler 2,4-dinitrophenol (DNP, 100 μM). Emitted fluorescence was monitored with cooled charge-coupled device digital camera (C4742-95, Hamamatsu Photonics, Hamamatsu, Japan). The imaging of flavoprotein was analyzed for average pixel intensities of regions of interest drawn to include whole cell and expressed as a percentage of the DNP-induced maximal oxidation, using an Aquacosmos image-processing system (Hamamatsu Photonics).

2.3 Langendorff perfused rabbit hearts

Female rabbits (Japanese White, 2.5–3.5 kg) were anesthetized by intravenous injection of 30 mg/kg pentobarbital sodium. The hearts were then dissected out, and mounted on a Langendorff apparatus for perfusion with a modified Krebs-Henseleit solution composed of (in mM) NaCl 119, KCl 4.8, K\(_2\)HPO\(_4\) 1.2, MgSO\(_4\) 1.2, CaCl\(_2\) 2.5, NaHCO\(_3\) 24.9, glucose 10, and gassed with 95% O\(_2\)-5% CO\(_2\) (pH 7.4, 36°C). Krebs-Henseleit solution was delivered at a constant rate of 25–30 ml/min that established an initial coronary perfusion pressure (COP) of ≈40 mm Hg. To measure left ventricular pressure, a fluid-filled balloon was inserted via the left atrium and positioned in the left ventricle. The balloon was expanded with distilled water to achieve an initial baseline left ventricular end-diastolic pressure (LVDP) between 4 and 8 mm Hg. Hemodynamic parameters, i.e., COP, heart rate (HR), left ventricular developed pressure (LVP; difference between left ventricular end-systolic pressure and LVEDP), velocity of contraction (dP/dt) were monitored continuously using a PowerLab data acquisition system (AD Instruments, Castle Hill, Australia).

2.4 Experimental protocols

Langendorff perfused hearts were stabilized and subjected to 30 min of normothermic global ischaemia followed by 120 min of reperfusion. Global ischaemia was achieved by complete interruption of coronary perfusion. As illustrated in Figure 1, hearts were divided into 7 experimental groups. ADM was administered before ischaemia in protocol 1 (Groups 2–4) and upon reperfusion in protocol 2 (Groups 5–7), respectively. Group 1, CONT (n = 8): no treatment. Group 2, ADM (n = 7): ADM (10 nM) was added directly to the perfusate 10 min before ischaemia, and it was perfused until the onset of ischaemia. Group 3, ADM + PX (n = 7): treatment with the mitoK\(_{Ca}\) channel blocker paxilline (PX, 2 μM) during ADM. Group 4, ADM + KT (n = 7): treatment with the PKA inhibitor KT5720 (KT, 200 nM) 5 min prior to and during ADM. Group 5, ADM (n = 7): ADM (10 nM) was administered for the first 10 min of reperfusion. Group 6, ADM + PX (n = 7): treatment with PX during reperfusion.

![Figure 1](https://example.com/figure1.png)
ADM. Group 7, ADM + LY (n = 7): treatment with the PI3-K inhibitor LY294002 (LY, 5 μM) during ADM.

2.5 Infarct size measurement

After 120 min of reperfusion, the heart was removed from the Langendorff apparatus, and then cut into six to eight transverse slices from apex to base. The slices were incubated for 5 min at 37°C in a 1% solution of triphenyltetrazolium chloride to visualize infarcts. All slices were weighed and photographed after staining. The areas of infarct and the ventricles in each slice were measured by computed planimetry (Aquiscom; Hamamatsu Photonics). Infarct weight was determined with the following equation: % infarct area = weight of each slice, as described previously, and expressed as a percentage of the total tissue weight.

2.6 Chemicals

ADM, diazoxide, NS1619, paxilline, KT5720, 8-bromoadenosine 3′-cyclic monophosphate (8Br-cAMP), and LY294002 were purchased from Sigma-Aldrich (St. Louis, MO, USA). DNP was purchased from Wako Pure Chemicals (Osaka, Japan). Erythropoietin was a kind gift from Chugai Pharmaceutical (Tokyo, Japan). Diazoxide, NS1619, paxilline, KT5720, 8Br-cAMP, and LY294002 were dissolved in dimethyl sulfoxide (DMSO) before they were added to the experimental solution, and the final concentration of DMSO was <0.1%. ADM, DNP, and erythropoietin were dissolved in the perfusate.

2.7 Statistical analysis

Data are expressed as mean ± SEM, and the number of cells or experiments is shown as n. Statistical comparisons were made using Student’s t-test or ANOVA combined with Fisher post hoc test, as appropriate. A value of P < 0.05 was regarded as significant.

3. Results

3.1 Effects of ADM on flavoprotein oxidation

It has been shown that in guinea pig ventricular myocytes the flavoprotein fluorescence is a useful index to assay not only mitoKATP but also mitoKCa channel activity. We therefore examined the effects of ADM on flavoprotein oxidation in rabbit ventricular cells. Figure 2A shows the time course of changes in flavoprotein fluorescence in a cell exposed to diazoxide and ADM. Opening of mitoKATP channels by diazoxide (100 μM) oxidized flavoprotein and increased the fluorescence intensity. Subsequent exposure to ADM (10 nM) did not affect the oxidative effect of diazoxide. As summarized in Figure 2B, diazoxide alone increased flavoprotein oxidation to 29.0 ± 5.6% of the DNP value (n = 4). Although ADM alone did not affect the flavoprotein fluorescence (3.6 ± 2.8% of the DNP value, n = 5, data not shown), the diazoxide-induced flavoprotein oxidation was not affected by ADM (27.5 ± 5.7% of the DNP value, n = 4). The lack of potentiating effect of ADM was not due to the maximum oxidation by 100 μM diazoxide because ADM did not increase the flavoprotein oxidation during application of 30 μM diazoxide (18.3 ± 3.3% vs. 16.4 ± 2.8% of the DNP value, n = 5, data not shown). Furthermore, the mitoKATP channel blocker paxilline (2 μM) did not inhibit the flavoprotein oxidation during the application of diazoxide and ADM (26.2 ± 3.1%, n = 4). Figure 2C shows the time course of changes in flavoprotein fluorescence in a cell exposed to NS1619 and ADM. The mitoKCa channel opener NS1619 (30 μM) partially oxidized flavoproteins, as we have reported previously. ADM augmented the NS1619-induced flavoprotein oxidation when applied after the effect of NS1619 had reached steady state. As summarized in Figure 2D, NS1619 alone increased flavoprotein oxidation to 20.7 ± 2.3% of the DNP value (n = 6). ADM significantly increased the NS1619-induced flavoprotein oxidation to 41.5 ± 4.7% of the DNP value (n = 6, P < 0.05). Paxilline completely blocked the oxidative effects of NS1619 in the presence of ADM (2.7 ± 2.4% of the DNP value, n = 5). These results indicate that ADM augments the opening of mitoKCa, but not mitoKATP channels.

3.2 Effects of PKA and PI3-K on mitoKCa channel activation

In the following experiments we tested whether ADM augmented the oxidative effect of NS1619 via activation of PKA and/or PI3-K. As shown in Figure 3A and B, when the PKA inhibitor KT5720 (200 nM) was administered 5 min prior to and during ADM, ADM failed to augment the oxidative effect of NS1619 (26.3 ± 4.9% vs. 31.1 ± 7.1% of the DNP value, n = 4, P = NS). Furthermore, consistent with our earlier findings, the cell-permeable CAMP analogue 8Br-cAMP (0.5 mM) mimicked the potentiating effect of ADM and significantly increased the NS1619-induced flavoprotein oxidation from 23.3 ± 3.5% to 43.0 ± 2.2% of the DNP value (n = 5, P < 0.05). These results suggest that ADM-induced potentiation of mitoKCa channels is mediated by CAMP/PKA pathway. Contrarily, as shown in Figure 3C, D, ADM significantly increased the oxidative effect of NS1619 even in the presence of LY294002 (5 μM), a potent inhibitor of PI3-K (23.8 ± 2.0% vs. 42.9 ± 3.4% of the DNP value, n = 7, P < 0.05). Moreover, erythropoietin (10 units/ml), a potent activator of PI3-K, did not augment the NS1619-induced flavoprotein oxidation (24.0 ± 4.4% vs. 30.7 ± 6.6% of the DNP value, n = 4, P = NS). These results suggest that mitoKCa channel is not regulated by PI3-K.

3.3 ADM reduces infarct size when given before ischaemia (protocol 1)

We next examined the effect of ADM on infarct size in the isolated perfused rabbit hearts subjected to ischaemia/reperfusion. ADM alone produced no significant effects on CCP, heart rate, LVDP, and +dP/dt max. We also found no significant differences in hemodynamic parameters among the groups before the start of ischaemia (Table 1). As shown in Figure 4, administration of ADM to the heart for 10 min prior to ischaemia significantly reduced infarct size compared with the control group (31.8 ± 4.4% for Group 2 vs. 63.4 ± 3.2% for Group 1, P < 0.01). Moreover, ADM treated hearts showed improved recovery of postischaemic LVDP and +dP/dt max compared with control hearts (Table 1). To test whether the infarct size-limiting effect of ADM is dependent on PKA-mediated activation of mitoKCa channels, we treated the hearts with paxilline, a blocker of mitoKCa channels, or KT5720, a cell-permeable inhibitor of PKA. Infarct size in the group treated with paxilline alone (63.7 ± 2.1%, n = 4) or KT5720 alone (62.1 ± 4.3%, n = 4) was not significantly different from that of the control group (data not shown). Treatment with paxilline blocked the ADM-induced reduction in infarct size (60.6 ± 1.8% for Group 3) and improvement in postischaemic LVDP and +dP/dt max (Table 1). Similarly, treatment with KT5720 abolished the
Infarct size-limiting effect of ADM (62.0 ± 3.1% for Group 4) and blocked the ADM-induced improvement in postischaemic contractile function (LVDP and +dP/dt\text{max}). However, treatment with the PI3-K inhibitor L Y294002 (5 μM) 5 min prior to and during ADM did not abolish the infarct size-limiting effect of ADM (36.6 ± 4.0%, n = 4, data not shown). These results indicate that ADM treatment prior to ischaemia provides cardioprotection, and that the protection is mediated by PKA-dependent activation of mitoKCa channels.

3.4 ADM reduces infarct size when given at reperfusion (protocol 2)

The cardioprotective effect of ADM at reperfusion was examined. There was no significant difference in hemodynamic parameters among the groups before ischaemia (Table 1). As shown in Figure 5, administration of ADM for the first 10 min of reperfusion significantly reduced infarct size compared with the control group (41.5 ± 3.2% for Group 5 vs. 63.4 ± 3.2% for Group 1, P < 0.01). However, LVDP and +dP/dt\text{max} after ischaemia/reperfusion were not significantly different between groups (Table 1). In contrast to the results seen in protocol 1, infarct size limitation by ADM was not attenuated by paxilline (38.0 ± 3.8% for Group 6). These results indicate that opening of mitoKCa channels is not involved in the ADM-mediated cardioprotection when given at the onset of the reperfusion. Next, we examined the involvement of PI3-K in the cardioprotective effect of ADM upon reperfusion using the PI3-K inhibitor LY294002. Infarct size in the group treated with LY294002 alone for
the first 10 min of reperfusion was not significantly different from that of the control group (61.2 ± 4.3%, n = 4, data not shown). Treatment with LY294002 blocked the ADM-induced reduction in infarct size (59.7 ± 4.0% for Group 7), suggesting the cardioprotective effect of ADM at reperfusion is dependent on PI3-K pathway.

### 4. Discussion

Our previous study has shown that mitochondrial oxidation induced by opening of mitoK\textsubscript{ATP} and mitoK\textsubscript{Ca} channels occurs independently of each other.\textsuperscript{19} In the present study, the effects of ADM on mitoK\textsubscript{ATP} and mitoK\textsubscript{Ca} channels in rabbit ventricular myocytes were investigated by measuring flavoprotein fluorescence. The mitochondrial redox potential is an indirect way of detecting mitoK\textsubscript{ATP} or mitoK\textsubscript{Ca} channel activity and may be affected even by the K\textsuperscript{+} channel-independent uncoupling of mitochondria. Despite this limitation, the mitochondrial flavoprotein oxidation state has provided the only convenient measure of mitoK\textsubscript{ATP} or mitoK\textsubscript{Ca} channel opening in intact cells.\textsuperscript{18}

The concentration of ADM (10 nM) used in this study was based on previous studies which showed that the concentration of ≥10 nM ADM is needed to activate both cAMP/PKA and PI3-K/Akt pathways in cardiac myocytes.\textsuperscript{15,26,27} We found that flavoprotein oxidation induced by the mitoK\textsubscript{Ca} channel opener diazoxide was not affected by ADM (Figure 2A and B). Therefore, it seems unlikely that activation of cAMP and/or PI3-K by ADM is linked to mitoK\textsubscript{ATP} channels. The salient experimental observations were that ADM potentiated the flavoprotein oxidation induced by the mitoK\textsubscript{Ca} channel opener NS1619 (Figure 2C). The effects of ADM and NS1619 reflected mitoK\textsubscript{Ca} channel activation, because they could be blocked by the mitoK\textsubscript{Ca} channel blocker paxilline (Figure 2D). The PKA inhibitor KT5720 prevented the ability of ADM to augment the oxidative effect of NS1619 (Figure 3A). Moreover, the NS1619-induced flavoprotein oxidation was similarly potentiated by the cell-permeable cAMP analogue 8Br-cAMP (Figure 3B), as we have seen in previous study.\textsuperscript{19} These results suggest that ADM modulates mitoK\textsubscript{Ca} channels through cAMP/PKA.

#### Table 1 Hemodynamic Parameters

<table>
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<tr>
<td>+ LY</td>
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Values are mean ± SEM. ADM indicates adrenomedulline; PX, paxilline; KT, KT5720; LY, LY294002; CCP, coronary perfusion pressure; LVDP, left ventricular developed pressure; and +dP/dt, positive change in pressure over time. *P < 0.05 vs. control.
although ADM itself can not open the mitoKCa channels directly. Previous studies have shown that PKC activators, phorbol 12-myristate 13-acetate and adenosine, do not suffice to open mitoKATP channels, they shift channels into the primed state, from which channels can be opened much more readily by endogenous stimuli during ischaemia. It is reasonable to suppose that, as in the case of mitoKATP channel, ADM activates PKA and primes the mitoKCa channel to open earlier and more intensely in response to elevation of mitochondrial Ca2+ during ischaemia. Therefore, ADM can be cardioprotective without direct mitoKCa channel opening.

It has been demonstrated that PKA potentiates mitoKCa channel activation, whereas PKC and PKG potentiate mitoKATP channel activation. However, the possible role of PI3-K in the regulation of mitoKCa as well as mitoKATP channels has not yet been investigated. In the present study, we found that ADM augmented the NS1619-induced flavoprotein oxidation even in the presence of the PI3-K inhibitor LY294002 (Figure 3C). These results suggest that activation of PI3-K does not contribute to the potentiating effect of ADM. Further support for this notion comes from the observation that the NS1619-induced flavoprotein oxidation was not affected by erythropoietin, a potent activator of PI3-K. PI3-K/Akt is known to stimulate nitric oxide synthase to produce nitric oxide (NO). It has also been reported that NO activates sarcolemmal BKCa channels in smooth muscle cells. Although NO has been shown to activate mitoKATP channels, the possible role of NO in the regulation of mitoKCa channels remains to be clarified.

In this study, we found that in isolated rabbit hearts administration of ADM for 10 min before ischaemia significantly reduced infarct size after ischaemia/reperfusion (Figure 4). Since the coronary flow rate was maintained constant throughout the experiments using a roller pump, cardioprotective effects of ADM are not due to vasodilation in our experimental conditions. ADM-induced cardioprotection was attenuated by administration of the mitoKCa channel blocker paxilline and the PKA inhibitor KT5720. These results suggest that ADM treatment prior to ischaemia reduced infarct size via PKA-mediated activation of mitoKCa channels. There is support for the concept that activation of PKA prior to ischaemia can protect hearts against ischaemia/reperfusion injury. It has been reported that brief exposure to β-adrenergic receptor agonist isoproterenol prior to ischaemia is protective against ischaemic injury in rat and murine hearts. Furthermore, activation of cAMP/PKA is required for ischaemic preconditioning, in which lethal injury to the heart can be dramatically blunted by brief conditioning periods of ischaemia. Recent study by Cao et al. demonstrated that in rat hearts cardioprotection conferred by ischaemic preconditioning was abolished by paxilline administered before ischaemia. Considering above findings together with the effects of ADM on mitoKCa channels, we conclude that cAMP/PKA-mediated priming of mitoKCa channels is involved in the cardioprotection that observed when ADM was administered before ischaemia. It has been proposed that PI3-K is involved in mediating ischaemic preconditioning. Contrary to previous reports, however, the infarct size-limiting effect of ADM was not abolished by LY294002 administered before ischaemia. Although the reason for this discrepancy in experimental results is not clear, our results suggest that activation of PI3-K/Akt pathway before ischaemia may not be involved in the preconditioning-like effect of ADM. In agreement with this suggestion, Hanlon et al. reported that LY294002 did not block the cardioprotection afforded by treatment with erythropoietin prior to ischaemia.

The mechanisms by which opening of mitoKCa channels could protect the hearts remain to be elucidated. It seems that mitoKCa channel activation confers cardioprotection in a manner similar to but independent of mitoKATP channel activation. We previously reported that opening of mitoKCa channels attenuates the mitochondrial Ca2+ overload with accompanying depolarization of mitochondrial membrane. Recent study by Stowe et al. reported that opening of mitoKCa channels by NS1619 generates reactive oxygen species (ROS) to initiate pharmacological preconditioning, as in the case of mitoKATP channel activation. It has been shown that ROS per se increases the open probability of mitoKATP channel, and activates the downstream kinases that feed back in a positive manner and keep the channel open. However, there is a need to determine whether such a mechanism could offer explanations for our findings, because conflicting data can be found in the literature. It has been reported that ROS production is attenuated by ADM, and ROS rather inhibits the opening of sarcolemmal BKCa channels.

Another important finding of this study was that ADM given at the onset of reperfusion results in a significant reduction in infarct size (Figure 5). This finding is consistent with the previous study in rat hearts. We further found that the cardioprotective effect of ADM at reperfusion was not affected by the mitoKCa channel blocker paxilline, but was completely abolished by the PI3-K inhibitor LY294002. These observations reinforce our notion that PI3-K is not linked to mitoKCa channels. Furthermore, it is concluded that activation of PI3-K, but not mitoKCa channel, is involved in the cardioprotective effect of ADM at reperfusion. PI3-K/Akt pathway is a component of the RISK pathway, and much attention has been paid in recent years to the cardioprotective role of PI3-K at the time of reperfusion. Like ADM, it has been shown that cardioprotective effect of erythropoietin given at the onset of reperfusion is mediated by activation of PI3-K. Although the present study did not investigate the downstream effectors of PI3-K, recent study suggest that the activation of RISK pathway confers its cardioprotection through the inhibition of mitochondrial permeability transition pore (PTP). PTP promotes the release of pro-apoptotic signaling molecules from the mitochondria and triggers apoptosis. Indeed, ADM has been shown to reduce myocardial apoptotic death through the PI3-K/Akt-dependent pathway. However, previous reports have not provided evidence that ADM exerts its ant apoptotic effect by the inhibition of PTP. Recent evidence suggests that ADM exerts its cardioprotective action during early reperfusion through a NO-dependent mechanism. Although NO has been shown to inhibit PTP opening, it remains unclear whether NO generation by ADM inhibits PTP in the reperfused myocardium.
channel acts as a common downstream effectors of cAMP/PI3-K/Akt-mediated cardioprotection. It has been demonstrated that PI3-K/Akt pathway is involved in cardioprotection through the inhibition of mitochondrial permeability transition pore (PTP).

Figure 6 Schematic representation of the mechanism of cardioprotection afforded by ADM. ADM administered prior to ischaemia potentiates the opening of mitoKCa channels. ADM administered upon reperfusion confers cardioprotection through PI3-K/Akt-mediated pathway. However, PI3-K/Akt is not linked to mitoKCa channels. It has been demonstrated that PI3-K/Akt pathway is involved in cardioprotection through the inhibition of mitochondrial permeability transition pore (PTP).

Acknowledgments
We thank M. Tamagawa and I. Sakashita for excellent technical and secretarial assistance.

Conflict of interest: none declared.

Funding
This study was supported in part by Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science, K. Watanabe Research Foundation, and the Vehicle Racing Commemorative Foundation.

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