Role of mitochondrial and sarcolemmal \( K_{\text{ATP}} \) channels in ischemic preconditioning of the canine heart

SHOJI SANADA,1 MASAFUMI KITAKAZE,2 HIROSHI ASANUMA,1 KENGO HARADA,1 HISAKAZU OGITA,1 KOICHI NODE,1 SEIJI TAKASHIMA,1 YASUHIKO SAKATA,1 MASANORI ASAKURA,1 YOSHIRO SHINOZAKI,2 HIDEZO MORI,2 TSUNEHIKO KUZUYA,1 AND MASATSUGU HORI1

1Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871; and 2Department of Physiological Science, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan

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Sanada, Shoji, Masafumi Kitakaze, Hiroshi Asanuma, Kengo Harada, Hisakazu Ogita, Koichi Node, Seiji Takashima, Yasuhiko Sakata, Masanori Asakura, Yoshiro Shinozaki, Hidezo Mori, Tsunehiko Kuzuya, and Masatsugu Hori. Role of mitochondrial and sarcolemmal \( K_{\text{ATP}} \) channels in ischemic preconditioning in the canine heart. Am J Physiol Heart Circ Physiol 280: H256–H263, 2001. We tested whether mitochondrial or sarcolemmal \( K_{\text{ATP}} \)-sensitive \( K^+ \) (\( K_{\text{ATP}} \)) channels play a key role in ischemic preconditioning (IP) in canine hearts. In open-chest beagle dogs, the left anterior descending artery was occluded four times for 5 min each with 5-min intervals of reperfusion (IP), occluded for 90 min, and reperfused for 6 h. IP as well as cromakalim and nicorandil (nonspecific \( K_{\text{ATP}} \) channel openers) markedly limited infarct size (6.3 ± 1.2, 8.9 ± 1.9, and 7.2 ± 1.6%, respectively) compared with the control group (40.9 ± 4.1%). A selective mitochondrial \( K_{\text{ATP}} \) channel blocker, 5-hydroxydecanoate, partially blunted the limitation of infarct size in the animals subjected to IP and those treated with cromakalim and nicorandil (21.6 ± 3.8, 25.1 ± 4.6, and 19.8 ± 5.2%, respectively). A nonspecific \( K_{\text{ATP}} \) channel blocker, glibenclamide, completely abolished the effect of IP (38.5 ± 6.2%). In conclusion, mitochondrial and sarcolemmal \( K_{\text{ATP}} \) channels independently play an important role in the limitation of infarct size by IP in the canine heart.

In brief, periods of ischemia that precede sustained ischemia can markedly decrease infarct size. This phenomenon is known as ischemic preconditioning (IP) (23, 29, 37), and the underlying mechanisms have been studied extensively (30, 34, 38). Gross and Auchampach (8) and Grover et al. (12) first proposed a role for the \( K_{\text{ATP}} \) channels in IP, which has been confirmed by us (20) and many other investigators (3, 9, 24, 27, 40). \( K_{\text{ATP}} \) channels are located on the sarcolemmal membrane (31) and inner mitochondrial membrane (16, 32). Several investigators have suggested that mitochondrial \( K_{\text{ATP}} \) channels are important for triggering or mediating the infarct size-limiting effect of IP, but it remains controversial whether mitochondrial or sarcolemmal channels are more important, especially in larger mammals.

To answer this question, we tested the influence of opening mitochondrial or sarcolemmal \( K_{\text{ATP}} \) channels on IP and assessed the involvement of each channel in the infarct size-limiting effect of IP. We used diazoxide, a selective mitochondrial \( K_{\text{ATP}} \) channel opener (7), and 5-hydroxydecanoate (5-HD), a selective mitochondrial \( K_{\text{ATP}} \) channel blocker (17, 36), to study IP in the canine heart.

MATERIALS AND METHODS

All procedures were performed in conformance with the Guide for the Care and Use of Laboratory Animals [DHHS Publ. No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, Bethesda, MD].

Instrumentation

Beagle dogs weighing 9–14 kg were anesthetized, intubated, and ventilated as described previously (21). The chest was opened, the heart was suspended and catherized, and the fluid balance was maintained as described previously (21). In protocols I and II, an occluder was placed on the left anterior descending artery (LAD) next to the first diagonal branch, and the left carotid artery was catherized for monitoring aortic blood pressure (ABP). In protocols III–V, after intravenous administration of heparin (500 U/kg), the LAD was cannulated and perfused using a bypass tube, and ABP was monitored as described previously (21). In all experiments, the mean ABP, heart rate, and \( P_{\text{O}_2} \) of systemic arterial blood under control conditions averaged 108 ± 1.2 mmHg, 136 ± 1.3 beats/min, and 108 ± 3.8 mmHg, respectively, and did not change significantly throughout the protocol.
Experimental Protocols

Protocol I: effect of intravenous 5-HD on IP. After hemodynamic stabilization, four cycles of 5 min of coronary occlusion and subsequent 5-min periods of reperfusion (IP) were performed using the occluder with and without infusion of 5-HD (Research Biochemicals; 5 mg/kg iv) at 5 min before the first and 5 min after the last occlusion of the IP protocol [IP with 5-HD group (n = 7) and IP group (n = 8), respectively]. In seven other dogs, the LAD was occluded for 90 min after 45 min of hemodynamic stabilization and reperfused for 6 h with administration of 5-HD (5-HD group).

Protocol II: effect of intravenous diazoxide on infarct size. After a low or a high dose (40 or 400 μg·kg⁻¹·min⁻¹) of diazoxide (Sigma Chemical), the vehicle (saline with polyethylene glycol and ethanol at a final concentration <1%) or saline was infused into a systemic vein for 10 min. These amounts of diazoxide give the concentrations of ~8 and 80 μmol/l, respectively, in myocardial tissue. The LAD was then occluded for 90 min and subjected to 6 h of reperfusion [high-dose diazoxide group (n = 9), low-dose diazoxide group (n = 8), vehicle group (n = 8), and control group (n = 9), respectively].

Protocol III: effect of intracoronary 5-HD or glibenclamide on IP. To obtain an independent control for protocol III, the LAD was occluded for 90 min and reperfused for 6 h with and without the IP protocol [IP group (n = 9) and control group (n = 8), respectively]. In other dogs, 5-HD (0.5 mg·kg⁻¹·min⁻¹ ic, 30 mg/ml at an infusion rate of 0.0167 ml/kg·min⁻¹) or glibenclamide (5 μg·kg⁻¹·min⁻¹ ic, 300 μg/ml at the same infusion rate) was infused into the LAD for 5 min before IP, and infusion was continued for the first 60 min of reperfusion except during coronary occlusion [IP with 5-HD group (n = 9) and IP with glibenclamide group (n = 7), respectively]. 5-HD is a selective inhibitor of mitochondrial K<sub>ATP</sub> channels, and glibenclamide is a nonspecific inhibitor of K<sub>ATP</sub> channels. In 15 other dogs, the same dose of 5-HD or glibenclamide was infused into the LAD for 45 min without IP and for the first 60 min of reperfusion [5-HD group (n = 7) and glibenclamide group (n = 8), respectively]. 5-HD was dissolved in saline and glibenclamide was dissolved in DMSO at a final concentration <0.15%, which did not affect infarct size (36).

Protocol IV: effect of intracoronary diazoxide, cromakalim, or nicorandil on infarct size. To test whether opening of the mitochondrial K<sub>ATP</sub> channel was responsible for cardioprotection by IP, we administered diazoxide [200 μg·kg⁻¹·min⁻¹ ic, 12 mg/ml at an infusion rate of 0.0167 ml/kg·min⁻¹ (diazoxide group; n = 9)]. We also administered cromakalim (0.4 μg·kg⁻¹·min⁻¹ ic, 0.024 mg/ml at the same infusion rate) or nicorandil (4 μg·kg⁻¹·min⁻¹ ic, 0.24 mg/ml at the same infusion rate) into the LAD for four cycles of 5 min at 5-min intervals [cromakalim group (n = 8) and nicorandil group (n = 9), respectively], as reported previously (20). Cromakalim (Smith Kline Beecham), nicorandil (Chugai Pharmaceuticals), and diazoxide were dissolved in the same vehicle used in protocol II. In seven other dogs, we administered the vehicle alone into the LAD for four cycles of 5 min at 5-min intervals (vehicle group).

Protocol V: effect of 5-HD on cromakalim- or nicorandil-induced limitation of infarct size. In other dogs, cromakalim or nicorandil was administered as in protocol IV together with 5-HD (0.5 mg·kg⁻¹·min⁻¹ ic, 30 mg/ml at an infusion rate of 0.0167 ml/kg·min⁻¹) for 45 min before ischemia and for the first 60 min of reperfusion [cromakalim with 5-HD group (n = 8) and nicorandil with 5-HD group (n = 8), respectively].

Criteria for Exclusion and Measurement of Infarct Size and Myocardial Collateral Blood Flow

To ensure that all the animals included in the analysis of infarct size were healthy and were exposed to a similar extent of ischemia, we employed the exclusion criteria and methods of measuring infarct size and regional myocardial collateral blood flow described previously (21). The nonrisk areas were stained by systemic injection of Evans blue dye just before completion of the protocol, and the risk but noninfarcted areas were stained by triphenyltetrazolium chloride. Regional myocardial blood flow was determined by the microsphere technique, which uses nonradioactive microspheres (Sekisui Plastic, Tokyo, Japan) made of inert plastic labeled with different stable heavy elements. The mean diameter was 15 μm. Microspheres were suspended in isotonic saline with 0.01% Tween 80 to prevent aggregation. The microspheres were ultrasonicated for 5 min and then vortexed for 5 min immediately before injection. Approximately 2 ml of the microsphere suspension (4–8 × 10<sup>6</sup> spheres) were injected into the left atrium, and the suspension was flushed several times with warm (37°C) saline (5 ml). Microspheres were administered at 80 min after the onset of coronary occlusion. Just before microsphere administration, a reference blood flow sample was withdrawn from the femoral artery at a constant rate of 8 ml/min for 2 min.

The X-ray fluorescence of the stable heavy elements was measured by a wavelength dispersive spectrometer (model PW 1440, Phillips). When the microspheres are irradiated by a primary X-ray beam, the electrons fall back to a lower orbit and emit measurable energy with a characteristic X-ray fluorescence energy level for each element. Myocardial blood flow was calculated according to the formula:

\[ \text{time flow} = \left( \frac{\text{tissue counts}}{\text{reference flow}} \right) \times \left( \frac{\text{reference counts}}{\text{tissue counts}} \right) \]

and was expressed in milliliters per minute per gram wet weight. We measured the wet weight of the sampled myocardium.

Statistical Analysis

Results are expressed as means ± SE or as the number of animals or experiments. Statistical analysis was performed using ANOVA with Fisher’s post hoc test to determine significance at P < 0.05 for group pairs that showed significant differences. Analysis of covariance between regional collateral blood flow in the inner half of the left ventricular wall and infarct size was performed as described previously (21), with P < 0.05 indicating a significant difference.

RESULTS

Mortality and Exclusions

One hundred fifty-three dogs were assigned to 19 groups for assessment of infarct size (Table 1). Twenty-seven of these dogs developed ventricular fibrillation at least once. Among them, ventricular fibrillation sufficient to fulfill the exclusion criteria occurred in 6 dogs during 90 min of ischemia and in 15 dogs during reperfusion following subsequent ischemia. These 21 animals were excluded from the assessment of infarct size. Among the remaining 132 dogs, 10 were excluded from the data analysis because myocardial collateral blood flow was >15 ml·100 g⁻¹·min⁻¹. Therefore, 122
dogs completed the protocol satisfactorily and were used for data analysis.

**Hemodynamic Parameters, Risk Area, and Collateral Blood Flow**

When the changes of mean ABP and heart rate were compared between the 19 groups in protocols I–V (Fig. 1), there were no significant differences in mean ABP or heart rate among all of the groups. Figure 2 shows risk area and collateral flow in all 19 groups. Risk area and collateral flow were comparable in all groups.

**Infarct Size in Protocols I and II**

Figure 3 shows infarct size for each group in protocols I and II. IP decreased infarct size. The higher dose of diazoxide also reduced infarct size, but not as much as in the IP group, while the lower dose of diazoxide had no effect on infarct size, given that there was no statistical significance. Neither vehicle nor 5-HD affected infarct size. Figure 4 shows regression plots of the area at risk vs. collateral flow in protocols I and II. IP significantly reduced infarct size. Although diazoxide and 5-HD reduced infarct size, the action of the former was stronger than the latter. Furthermore, cromakalim and nicorandil mimicked the infarct size-limiting effect of IP, with this action also being completely blocked by glibenclamide and partially blocked by 5-HD.

**Infarct Size in Protocols III–V**

Figure 5 shows infarct size in all the groups from protocols III–V. IP markedly decreased infarct size. Glibenclamide completely abolished the infarct size-limiting effect of IP, and 5-HD partially did so, but infarct size did not increase to that in the control group. Cromakalim or nicorandil reduced infarct size to that in the IP group (Fig. 5), with this effect being blunted by 5-HD, as in the IP groups. Glibenclamide, 5-HD, or the vehicle alone did not affect infarct size. Diazoxide significantly reduced infarct size, but not as much as IP. Although glibenclamide and 5-HD blunted the infarct size-limiting effect at every level of collateral blood flow, the action of the former was stronger than the latter. Furthermore, cromakalim and nicorandil mimicked the infarct size-limiting effect of IP, with this action also being completely blocked by glibenclamide and partially blocked by 5-HD.

**DISCUSSION**

In the present study, we found evidence that mitochondrial and sarcolemmal K<sub>ATP</sub> channels contribute independently to the infarct size-limiting effect of IP in an anesthetized canine model. Some investigators have suggested the importance of opening of the sarcolemmal K<sub>ATP</sub> channels and others have suggested a role of mitochondrial K<sub>ATP</sub> channels in cardioprotection by IP. Our results indicate that both mechanisms are important for the cardioprotective effect of IP, at least in this canine model.
Role of Mitochondrial and Sarcolemmal $K_{ATP}$ Channels in IP

First, we administered $K_{ATP}$ channel openers to test the involvement of mitochondrial $K_{ATP}$ channels. We chose two doses of diazoxide (0.4 and 4.0 mg/kg iv) to achieve full activation of mitochondrial $K_{ATP}$ channels, because 1) cromakalim and diazoxide have an approximately equal ability to open mitochondrial $K_{ATP}$ channels (6) and 2) 0.04 mg/kg cromakalim has a cardioprotective effect on the rabbit heart in vivo (13). Diazoxide had a moderate dose-dependent and cardioprotective effect, but even the higher dose of diazoxide (~80 μmol/l in myocardial tissue) was less effective than IP. A study using 10 mg/kg iv diazoxide has been performed in rabbits (2), but this dose of diazoxide or 4.0 mg/kg iv cromakalim decreased the systemic blood pressure markedly in anesthetized dogs (data not shown). Since it was possible that the further activation of mitochondrial $K_{ATP}$ channels might completely reproduce the infarct size-limiting effect of IP, we performed intracoronary administration of $K_{ATP}$ channel openers to test the effect of high concentrations of diazoxide without systemic hemodynamic changes. The regional concentration of diazoxide in myocardial tissue reached as much as 300 μM, which should have opened mitochondrial $K_{ATP}$ channels fully in the canine heart, because 1) diazoxide opens $K_{ATP}$ channels reconstituted from rat liver or the purified mitochondrial fraction of beef heart with a dose that the agent can bind at 50% of maximal binding status ($K_{1/2}$) of 0.4 μM (T), 2) diazoxide causes reversible oxidation of flavoproteins in isolated rabbit myocardium with an
EC$_{50}$ of 27 μM (25), and 3) diazoxide decreases lactate dehydrogenase release from isolated rabbit hearts with ischemia-reperfusion at a dose of 30 μM (6). Since sufficient doses of cromakalim or nicorandil, which open mitochondrial and sarcolemmal KATP channels, completely reproduced the cardioprotective effect of IP, and doses of diazoxide sufficient to open mitochondrial KATP channels mimicked it by 50%, it seems that opening of mitochondrial and sarcolemmal KATP channels contributes equally to cardioprotection.

In this study, the regional concentration of 5-HD may have reached ~200 μM, and 100 μM 5-HD is reported to block mitochondrial KATP channels sufficiently. The present study showed that glibenclamide, a nonspecific blocker of KATP channels, abolished the infarct size-limiting effect of IP and 5-HD reduced it by 50%, confirming the synergistic contribution to IP of opening of the mitochondrial and sarcolemmal KATP channels.

However, the recent report (5) shows that 300 μmol/l diazoxide, which has little effect on native or recombinant cardiac SUR2A/Kir6.2 (identical to cardiac sarcolemmal K$_{ATP}$) channels, can affect the opening of these channels when intercellular ADP concentrations are elevated, as might occur during ischemia. This result suggests that the infarct size-limiting effect by diazoxide in the present study may be partially afforded by the direct effect of this agent on sarcolemmal K$_{ATP}$ channels. Furthermore, in the recent studies (5, 18), the protective effect of newly expressed cardiac SUR2A/Kir6.2 channels into a somatic cell line without native K$_{ATP}$ channels afforded by the existence of pinacidil was inhibited by 5-HD, suggesting a question...
on the specificity of 5-HD on mitochondrial \(K_{\text{ATP}}\) channels. On the other hand, the effect of diazoxide in protocols II and IV was similar, indicating that the lower dose of diazoxide, which may have much less effect on sarcolemmal \(K_{\text{ATP}}\) channels, can protect the myocardium at the same level, suggesting that there may also be a certain effect of mitochondrial \(K_{\text{ATP}}\) channels.

### Differences in the Role of Mitochondrial and Sarcolemmal \(K_{\text{ATP}}\) Channels Between the Present and Previous Studies

The findings in the present study were different from those in recent reports on rat and rabbit hearts or reconstituted \(K_{\text{ATP}}\) channels. Although diazoxide and cromakalim are reported to open mitochondrial \(K_{\text{ATP}}\) channels in the rat or rabbit heart with similar \(K_{1/2}\) or \(EC_{50}\) values (7, 25), there are reports that doses of cromakalim and nicorandil below the \(EC_{50}\) for opening of mitochondrial \(K_{\text{ATP}}\) channels could mimic the infarct

### Table 2. Linear regression model test in each group in Figs. 4 and 6

<table>
<thead>
<tr>
<th>Group</th>
<th>Formula</th>
<th>(R^2)</th>
<th>(P)</th>
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</thead>
<tbody>
<tr>
<td><strong>Fig. 4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>(y = 58.9 - 2.45x)</td>
<td>0.62</td>
<td>0.032</td>
</tr>
<tr>
<td>Vehicle</td>
<td>(y = 47.1 - 1.08x)</td>
<td>0.58</td>
<td>0.048</td>
</tr>
<tr>
<td>IP</td>
<td>(y = 12.8 - 0.85x^*)</td>
<td>0.59</td>
<td>0.045</td>
</tr>
<tr>
<td>High Dzx</td>
<td>(y = 26.8 - 1.06x^*)</td>
<td>0.58</td>
<td>0.047</td>
</tr>
<tr>
<td>Low Dzx</td>
<td>(y = 53.9 - 3.63x)</td>
<td>0.67</td>
<td>0.025</td>
</tr>
<tr>
<td>IP + 5-HD</td>
<td>(y = 36.5 - 2.83x^*)</td>
<td>0.57</td>
<td>0.049</td>
</tr>
<tr>
<td>5-HD</td>
<td>(y = 55.2 - 1.18x)</td>
<td>0.60</td>
<td>0.038</td>
</tr>
<tr>
<td><strong>Fig. 6</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>(y = 68.6 - 3.53x)</td>
<td>0.89</td>
<td>0.005</td>
</tr>
<tr>
<td>Vehicle</td>
<td>(y = 60.4 - 2.90x)</td>
<td>0.72</td>
<td>0.032</td>
</tr>
<tr>
<td>IP</td>
<td>(y = 13.4 - 1.71x^)</td>
<td>0.56</td>
<td>0.035</td>
</tr>
<tr>
<td>Dzx</td>
<td>(y = 34.1 - 1.06x^*)</td>
<td>0.58</td>
<td>0.029</td>
</tr>
<tr>
<td>Ncr</td>
<td>(y = 14.1 - 0.82x^)</td>
<td>0.67</td>
<td>0.049</td>
</tr>
<tr>
<td>Cro</td>
<td>(y = 13.0 - 0.43x^)</td>
<td>0.57</td>
<td>0.047</td>
</tr>
<tr>
<td>IP + 5-HD</td>
<td>(y = 29.7 - 0.99x^*)</td>
<td>0.59</td>
<td>0.049</td>
</tr>
<tr>
<td>IP + Glib</td>
<td>(y = 70.5 - 3.68x)</td>
<td>0.60</td>
<td>0.038</td>
</tr>
<tr>
<td>Ncr + 5-HD</td>
<td>(y = 36.1 - 1.40x^*)</td>
<td>0.57</td>
<td>0.048</td>
</tr>
<tr>
<td>Cro + 5-HD</td>
<td>(y = 33.1 - 1.62x^*)</td>
<td>0.61</td>
<td>0.039</td>
</tr>
<tr>
<td>Glib</td>
<td>(y = 81.4 - 4.30x)</td>
<td>0.88</td>
<td>0.016</td>
</tr>
<tr>
<td>5-HD</td>
<td>(y = 56.9 - 2.66x)</td>
<td>0.66</td>
<td>0.048</td>
</tr>
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\(^*P < 0.05, ^{**}P < 0.01 vs. control by analysis of covariance. \(P < 0.05\) supports the significant linearity of each line.
size-limiting effect of IP (10, 28), while diazoxide did not, in canine hearts. The previous data that emphasized the importance of opening of the mitochondrial K\textsubscript{ATP} channel in the infarct size-limiting effect of IP were obtained using rat or rabbit hearts, but some studies using rats and rabbits have countered these observations (11, 13). Therefore, the experimental conditions may alter the relative importance of the mitochondrial and sarcolemmal K\textsubscript{ATP} channels in IP. On the other hand, Auchampach et al. (1) reported that 5-HD almost completely blunted the effect of IP in mongrel dogs. There seem to be two critical differences between their study and ours. First, they perfused 5-HD into the coronary artery during coronary occlusion, which may have changed the natural collateral blood flow in the ischemic area. Second, the infarct size in their control group was much smaller than ours, because they did not exclude dogs with >0.15 ml·g\textsuperscript{-1}·min\textsuperscript{-1} of collateral flow during ischemia in regression plots, and this difference may have altered the nature and the causes of ischemia and reperfusion injury between the two studies. If the experiments with high collateral flow were excluded, the regression lines may become similar to ours. Indeed, there may be still a possibility that the difference in the procedure of IP or the duration of occlusion period may contribute, to some extent, to decrease the effect of mitochondrial K\textsubscript{ATP} channels, as suggested in a recent report (39).

Therefore, the use of different species or experimental conditions may alter the contribution of sarcolemmal and mitochondrial K\textsubscript{ATP} channels to the cardioprotective effect of IP.

**Mechanism of Involvement of Mitochondrial and Sarcolemmal K\textsubscript{ATP} Channels in IP**

How are these two kinds of K\textsubscript{ATP} channels involved independently in IP? Opening of mitochondrial K\textsubscript{ATP} channels is reported to alter the redox state of cardiomyocytes (25), prevent mitochondrial calcium overload (14), which can lead to apoptotic cell death, and reduce disruption of the actin cytoskeleton (2). Opening of sarcolemmal K\textsubscript{ATP} channels modulates Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in rabbit hearts (13), improves the no-reflow (22) phenomenon (35), and reduces neutrophil migration in humans (33). On the other hand, the cardioprotection by opening of sarcolemmal K\textsubscript{ATP} channels has been reported to be mediated by shortening of the action potential duration (4), preservation of high-energy phosphates (26), and prevention of calcium overload by decreasing the open probability of the voltage-dependent calcium channels.

What triggers opening of the mitochondrial and sarcolemmal K\textsubscript{ATP} channels? It has been reported that adenosine-induced protein kinase C (PKC) activation (15) and PKC-mediated induction of adenosine through augmentation of ecto-5'-nucleotidase (21) are important in the infarct size-limiting effect of IP. PKC is reported to modulate mitochondrial K\textsubscript{ATP} channels directly (36), while adenosine is reported to open sarcolemmal K\textsubscript{ATP} channels through A\textsubscript{1} receptor activation (10). Thus opening of mitochondrial and sarcolemmal K\textsubscript{ATP} channels in IP is modulated independently by two major trigger substances, PKC and adenosine.

In conclusion, it is clear that K\textsubscript{ATP} channels mediate the infarct size-limiting effect of IP and can be a major target for the treatment of ischemic heart disease. Further investigation of the cardioprotection by opening the mitochondrial and sarcolemmal K\textsubscript{ATP} channels is needed to allow safe and useful application for clinical cardiology.

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