Myocardial $K_{ATP}$ Channels in Preconditioning

Brian O’Rourke

Abstract—We are on the brink of harnessing the cell’s natural defenses against ischemia and reperfusion injury after years of research into the destructive and protective mechanisms involved. Since the discovery of ischemic preconditioning, the surface receptors and signal transduction pathways underlying this phenomenon have been clarified, but many questions remain about the downstream targets that ultimately protect the cell. ATP-sensitive $K^+$ ($K_{ATP}$) channels are thought to play a role in protection, but their mechanism of action has been unclear. Accumulating evidence now suggests that the location of the $K_{ATP}$ channels relevant to cytoprotection may be on the mitochondrial inner membrane instead of on the sarcolemma of the cardiac cell. This review discusses recent findings in preconditioning and protection. (Circ Res. 2000;87:845-855.)

Key Words: $K_{ATP}$ ■ protection ■ mitochondria ■ ischemia ■ apoptosis

Efforts to mitigate or prevent ischemic injury have traditionally focused on finding ways to block events associated with irreversible ischemic injury. More recently, the discovery of the endogenous cellular protective mechanism known as ischemic preconditioning (IPC) has raised hopes that natural pathways or target proteins could be activated to help cells stave off commitment to necrosis or apoptosis. IPC in the heart refers to the finding that brief periods of ischemia confer protection against infarction produced by a subsequent long ischemia.1–4 Both acute protection, in which the brief ischemia must be applied within a 1- to 2-hour window before the long ischemia, and second window protection,5,6 occurring 24 to 48 hours after the preconditioning ischemia, have been described.

As summarized in Figure 1, a variety of intracellular signaling pathways have been implicated in the protective mechanism of IPC. These include the activation of G protein–linked phospholipase C–coupled receptors, tyrosine kinase pathways, protein kinase C (PKC), and the generation of reactive oxygen species. Protection can be blocked at several steps in each cascade; however, redundancy is built in, so protection can be preserved through the activation of alternative pathways. The identification of a common end-effector for protection has been elusive.

Among the candidates as a mediator of protection is the ATP-sensitive potassium channel ($K_{ATP}$). This channel is normally inhibited by intracellular ATP and opens during periods of energy depletion.7–11 $K_{ATP}$ channels are found on a wide variety of tissues, and one of their most prominent functions is to modulate insulin release from pancreatic $\beta$ cells by setting the resting membrane potential ($E_r$) of the cell. An effect on $E_r$ also underlies the mechanism of $K_{ATP}$ action in vascular smooth muscle.12 In the heart, $K_{ATP}$ channels are present on the sarcolemma of cardiac myocytes, where they were first described,7–8 but their purpose remains unclear. The opening of surface $K_{ATP}$ (surface$K_{ATP}$) channels in cardiomyocytes has little effect on $E_r$, because it is already close to the equilibrium potential for $K^+$, but the outward current carried by $K_{ATP}$ shortens the action potential and, if large enough, can render the cell inexcitable. Thus, it has been suggested that suppression of excitability spares energy by reducing that required for active ion cycling because of membrane depolarization and Ca$^{2+}$ handling.13
positive feedback by altering upstream components such as intracellular reactive oxygen species (ROS) generation, converge ways confers cardioprotection. Multiple pathways, including PKC-dependent or tyrosine kinase–dependent signaling path-

protection induced by receptor stimulation. The kinase could mimic IPC and inhibitors could eliminate was implicated in the response, because direct activation of the kinase could mimic IPC and inhibitors could eliminate protection induced by receptor stimulation.

Role of K$_{\text{ATP}}$ Channels in Acute IPC

IPC was first described in 1986 by Murry et al., who showed that four 5-minute cycles of circumflex artery occlusion and reperfusion reduced infarct size produced by a long ischemia (40 minutes) from 30% to 7% of the area at risk. This acute protection is preserved for 1 to 2 hours after the preconditioning period. Although other endpoints, such as ventricular fibrillation, stunning, or functional recovery, are also influenced by preconditioning, infarct size limitation remains the gold standard for acute IPC studies. A leading mechanistic hypothesis, supported by findings that adenosine receptor antagonists abrogated and agonists mimicked protection, was that adenosine released locally from cells in the ischemic zone could activate receptors on cardiomyocytes in an autocrinoi manner, triggering a signaling cascade fortifying the cell against injury. Protection can also be conferred by stimulating other phospholipase C–linked receptors, including bradykinin, endothelin, or acetylcholine. The activation of PKC was implicated in the response, because direct activation of the kinase could mimic IPC and inhibitors could eliminate protection induced by receptor stimulation.

Figure 1. Mechanisms of preconditioning. Locally released adenosine, produced by the breakdown of adenine nucleotides during ischemia, or other agonists of G protein–coupled receptors lead to the activation of phospholipase C (or phospholipase D) and the generation of diacylglycerol (DAG), which activates and translocates PKC to target membranes. Phospholipase C also releases phosphatidyl inositol bisphosphate (PIP2), which is known to influence surfaceK$_{\text{ATP}}$ channel activity. Stimulation of PKC–dependent or tyrosine kinase–dependent signaling pathways confers cardioprotection. Multiple pathways, including intracellular reactive oxygen species (ROS) generation, converge on PKC activation. MitoK$_{\text{ATP}}$ channel opening may be a common downstream effector leading to protection but may also provide positive feedback by altering upstream components such as ROS or PKC. Tyr. kinase R. indicates tyrosine kinase receptor.

Another K$_{\text{ATP}}$ channel isoform, which is presumed to be ubiquitously distributed in all cells with mitochondria, is found on the mitochondrial inner membrane (mitoK$_{\text{ATP}}$). Recent evidence indicates that this channel may be both a trigger and effector of IPC. This review will compare the pharmacology of mitoK$_{\text{ATP}}$ with surfaceK$_{\text{ATP}}$ and discuss the evidence implicating mitoK$_{\text{ATP}}$ in cellular protection.

MitoK$_{\text{ATP}}$

Although the mitochondrial inner membrane was traditionally thought to be relatively impermeable to ions, K-selective cation transport has been widely observed in mitochondria, as supported by studies using light scattering to measure mitochondrial swelling or fluorescent dyes to measure K$^+$ influx. Suppression of K$^+$ flux by ATP and a sensitivity to K$^+$ channel openers and blockers led to the suggestion that a K$_{\text{ATP}}$ channel similar to surfaceK$_{\text{ATP}}$ existed on the inner mitochondrial membrane. This was strongly supported by patch-clamp studies of isolated mitoplasts, which revealed a channel with gating properties similar to K$_{\text{ATP}}$ but with a conductance ($\approx 10$ pS in 100 mmol/L K$^+$) smaller than the surface variety. The mitoK$_{\text{ATP}}$ channel was inhibited by 100 mmol/L ATP and blocked by glibenclamide or 4-aminopyridine. In reconstitution studies, mitoK$_{\text{ATP}}$ was shown to be competitively inhibited by ATP, ADP, and palmitoyl- or oleyl-CoA, and relief from inhibition was mediated by GTP and GDP. Modulator effects require the presence of Mg$^{2+}$. Although the excised patch studies indicated that the ATP inhibitory site was on the matrix face of the channel, other evidence suggests that it may be on the cytoplasmic (technically the intermembrane) face. This conclusion was based on the findings that the channel’s regulatory sites were all located on one side of the channel and that GTP and palmitoyl-CoA are maximally active only when applied to the external medium of intact mitochondria and are presumably not transported into the matrix.
Selectivity of K\textsubscript{ATP} Channel Openers and Inhibitors Toward Cardiac Mitochondrial K\textsubscript{ATP} (MitoK\textsubscript{ATP}) or Sarcolemmal K\textsubscript{ATP} (SarcK\textsubscript{ATP})

<table>
<thead>
<tr>
<th>Selectivity</th>
<th>MitoK\textsubscript{ATP}</th>
<th>MitoK\textsubscript{ATP} and SarcK\textsubscript{ATP}</th>
<th>SarcK\textsubscript{ATP}</th>
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<td>Openers</td>
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<td>Diazoxide†</td>
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<td>Pinacidil†</td>
<td>P-1075†</td>
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<td>Nicorandil*</td>
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<td>Cromakalim†</td>
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<td>BMS-180448†</td>
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<td>BMS-191095†</td>
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<td>Blockers</td>
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<td>5-Hydroxydecanoate*†</td>
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Compounds are listed that have been tested for activity in both mitochondrial and sarcolemmal K\textsubscript{ATP} functional assays. For all of these compounds, the effects on protection (or lack thereof) have been verified in isolated perfused hearts or intact animals (see text for details).

*Selectivity and effects on protection determined in adult rabbit myocytes.
†Selectivity determined in intact mitochondria or reconstituted sarcolemmal or mitochondrial proteins.
‡P-1075 was reported to be selective for sarcolemmal K\textsubscript{ATP} in isolated adult rabbit myocytes but activated mitochondrial K\textsubscript{ATP} in isolated rat mitochondria.

Studies of proteins reconstituted into proteoliposomes permitted a direct comparison of the pharmacology of mitochondrial K\textsubscript{ATP} and surface K\textsubscript{ATP} channels. Garlid et al\textsuperscript{24} showed that diazoxide was 1000 to 2000 times more potent in opening the mitochondrial K\textsubscript{ATP} channel compared with the sarcolemmal K\textsubscript{ATP} channel (K\textsubscript{1/2} ∼ 0.5 to 0.8 mmol/L for mitoK\textsubscript{ATP} versus 840 μmol/L for cardiac sarcolemmal K\textsubscript{ATP}). Furthermore, the effect of diazoxide could be blocked by 5-HD with a K\textsubscript{i} of 45 to 85 μmol/L. Glibenclamide (K\textsubscript{i} 1 to 6 μmol/L) and 5-HD block mitochondrial K\textsubscript{ATP} in a state-dependent manner, having no effect on channels activated by complete removal of ATP, but inhibiting channels activated by GTP or K\textsuperscript{+} channel openers in the presence of 5-HD.\textsuperscript{31}

Exploiting the selectivity of diazoxide for the mitochondrial isoform, Garlid et al\textsuperscript{24} compared the efficacy of diazoxide and cromakalim in protecting Langendorff-perfused rat hearts from ischemic contracture. Diazoxide significantly prolonged the time to ischemic contracture with a half-maximal effect at 8.8 μmol/L, whereas the K\textsubscript{1/2} for cromakalim was 11 μmol/L. Importantly, at equally protective doses of the 2 drugs, diazoxide produced markedly less action potential shortening. Both 5-HD (at 100 μmol/L) and glibenclamide abolished the protective effect of diazoxide. Garlid et al\textsuperscript{24} concluded that the protective effect of K\textsubscript{ATP} openers may be mediated by mitochondrial rather than sarcolemmal K\textsubscript{ATP} channels.

Contemporaneously, Liu et al\textsuperscript{22} made a similar connection between mitochondrial K\textsubscript{ATP} activation and cardioprotection in isolated cardiomyocytes. By developing a method for monitoring the opening of mitochondrial K\textsubscript{ATP} using the native autofluorescence of mitochondrial flavoproteins, the opening of mitochondrial K\textsubscript{ATP} by diazoxide was detected for the first time in intact cells as a reversible increase in flavoprotein oxidation. At concentrations up to 100 μmol/L, diazoxide dose-dependently increased mitochondrial matrix oxidation (K\textsubscript{1/2} 27 μmol/L) without activating sarcolemmal K\textsubscript{ATP} currents, and 5-HD inhibited the redox effect. The latter finding provides important support for the argument that mitochondrial K\textsubscript{ATP} channels are involved, because there is substantial evidence that 5-HD is selective for mitochondrial K\textsubscript{ATP} over sarcolemmal K\textsubscript{ATP}.\textsuperscript{15,24,33,34} The link between mitochondrial K\textsubscript{ATP} protection and cardiomyocytes was demonstrated by examining the extent of cell killing in response to simulated ischemia using a cell pelleting method.\textsuperscript{32} Diazoxide decreased cell killing to about one half of that in controls, and this protection was blocked by 5-HD. The latter result was similar to that of Armstrong et al.\textsuperscript{22} who showed that 5-HD inhibited preconditioning induced by 1 to 3 short pelleting episodes before the long ischemia in the same quiescent cell model.

Using the flavoprotein fluorescence method, the pharmacological profile of several K\textsubscript{ATP} openers and inhibitors have been characterized, and compounds have been found that specifically act on either the mitochondrial or sarcolemmal K\textsubscript{ATP} channels (Table). In isolated cell models of ischemia, protection is afforded by compounds capable of activating mitochondrial K\textsubscript{ATP}, and this protection can be inhibited by compounds effective at blocking sarcolemmal K\textsubscript{ATP}.\textsuperscript{35,36} Compounds selective for sarcolemmal channels have no such actions and serve as convincing evidence that sarcolemmal channels play much less of a role in protection. It should be noted that the specificities shown in the Table primarily apply to the case of intact cardiomyocytes or isolated mitochondria, usually determined with ATP present in the cytoplasmic solution, and may vary depending on the conditions of the experiment. For example, the pinacidil derivative P-1075 is quite selective for surface K\textsubscript{ATP} in the isolated cells\textsuperscript{35} but potently activates mitochondrial preparations (P. Paucek, personal communication, June 2000) and confers protection in intact hearts with an EC\textsubscript{50} of 57 nmol/L.\textsuperscript{37} The reason for this discrepancy is unknown.

Similarly, 5-HD was originally reported to block surface K\textsubscript{ATP} currents activated by high ADP (1 mmol/L), low pH of 6.6,\textsuperscript{38} and metabolic inhibition,\textsuperscript{39} so conclusions about selectivity need to be reexamined under many different conditions. Also, under conditions of high ADP or severe metabolic inhibition, surface K\textsubscript{ATP} channels are more readily opened by diazoxide;\textsuperscript{40} however, activation of heterologously expressed SUR2A/Kir6.2 channels by diazoxide is not inhibited by up to 500 μmol/L. Glibenclamide (K\textsubscript{i} 1 to 6 μmol/L) and 5-HD block mitochondrial K\textsubscript{ATP} in a state-dependent manner, having no effect on channels activated by complete removal of ATP, but inhibiting channels activated by GTP or K\textsuperscript{+} channel openers in the presence of 5-HD.\textsuperscript{31}

Acknowledging the caveat about pharmacological specificity mentioned above, there is remarkably good agreement between isolated cell data and results obtained in whole heart. Protection against infarction by diazoxide or low doses of cromakalim does not seem to be correlated with effects on coronary flow, action potential shortening, or ST segment elevation\textsuperscript{41} during ischemia. Almost universally, the protection is inhibited by 5-HD. Furthermore, HMR1883 (or its salt form HMR1098), an antagonist selective for surface K\textsubscript{ATP}, has no effect on IPC\textsuperscript{41}–46 but is effective in blocking reperfusion arrhythmias,\textsuperscript{43,47} presumably by reducing the dispersion of repolarization caused by the activation of surface K\textsubscript{ATP}. 5-HD,
on the other hand, does not influence ischemia-related electrical dispersion or arrhythmias. HMR1883 may also be more selective toward cardiomyocyte, rather than vascular, surface K\textsubscript{ATP} channels, because reperfusion hyperemia was blocked by glibenclamide but not HMR1883 in rat hearts.

A dichotomous recent result suggested that HMR1883 could block diazoxide, but not conventional IPC, in Rabbit hearts. Interestingly, diminution of ischemic ST segment elevation with successive occlusions was unaffected by doses of 5-HD known to block IPC, but abolished by HMR1883. Thus, this study confirmed the dissociation between surface K\textsubscript{ATP} activation and protection while at the same time supporting a surface K\textsubscript{ATP} role in diazoxide-induced protection (either that or the HMR1883 was not completely surface selective). These results will need to be reconciled with a report showing no effect of HMR1098 on diazoxide-induced protection in isolated myocytes.

Assuming that the primary action of 5-HD is on mitoK\textsubscript{ATP}, it is quite remarkable that this compound blocks protection induced by an endless variety of preconditioning protocols, including protection induced by IPC\textsuperscript{44,50,51}, adenosine\textsuperscript{52-56}, endothelin\textsuperscript{57}, nicorandil\textsuperscript{58}, opioids\textsuperscript{59}, acetylcholine\textsuperscript{60}, diazoxide\textsuperscript{62,44,61-64}, heat shock\textsuperscript{65-67}, Ca\textsuperscript{2+} preconditioning\textsuperscript{62}, HpETE\textsuperscript{68}, renal ischemia\textsuperscript{69}, monophosphoryl lipid A\textsuperscript{70,71}, phorbol myristic acid\textsuperscript{72}, BMS-180448\textsuperscript{73}, RP52891\textsuperscript{16} and volatile anesthetics\textsuperscript{74}. Although perfect isoform selectivity of K\textsubscript{ATP} openers and blockers has yet to be achieved, all of the pharmacological results taken together favor mitoK\textsubscript{ATP} rather than surface K\textsubscript{ATP} as the relevant effector of preconditioning. This conclusion will be bolstered by the future development of highly isomeric specific and potent drugs. In this regard, a new K\textsuperscript{+} channel opener, BMS-191095\textsuperscript{75} has recently been described that is extremely potent (K\textsubscript{D}, 83 nmol/L) and selective for mitoK\textsubscript{ATP}, has no effect on ischemic action potential shortening, will not open vascular or sarcolemmal K\textsubscript{ATP} channels, and is cardioprotective.

Another important question is the time frame for mitoK\textsubscript{ATP} channel opening necessary for protection. In a recent study in a rat model of IPC by Fryer et al\textsuperscript{44} 5-HD was applied either before or after the preconditioning ischemia or diazoxide application to examine whether mitoK\textsubscript{ATP} channel opening exerted its protective effect during the trigger or long ischemia phases or both. In controls, IPC reduced infarct size (normalized to area at risk) from 56% to 7%. When 5-HD was applied 5 minutes before IPC, protection was largely eliminated (infarct size was 40%), whereas in the absence of preconditioning, 5-HD had no significant effect on infarct size. Diazoxide given 15 minutes before the long ischemia reduced infarct size (to 36%), whereas 5-HD applied either before or after diazoxide exposure completely eliminated this protection. This suggests that mitoK\textsubscript{ATP} opening is important as both a preconditioning trigger and effector of protection during the long ischemia (discussed in the context of delayed preconditioning in the next section). Also noted in this study was a diminished effect of 5-HD with longer pretreatment periods, suggesting that 5-HD was being actively metabolized. This result highlights the difficulty of verifying the local concentration of a pharmacological agent at an effector site in vivo, particularly when the site is an intracellular one like the mitochondria and coronary flow is changing during the experiment.

A recent study by Pain et al\textsuperscript{77} supported the role of mitoK\textsubscript{ATP} as a trigger of IPC but challenged its role as an effector during the long ischemia in perfused rabbit hearts. They found that 5-HD (or glibenclamide) administered during a 5-minute preconditioning ischemia or a short diazoxide pretreatment could block the infarct limiting effect of IPC but was ineffective when given after the preconditioning period. Tyrosine kinase inhibition abrogated diazoxide-induced protection, whereas PKC inhibition did not. In addition, free radical scavengers applied during diazoxide pretreatment were capable of blocking protection, suggesting that mitoK\textsubscript{ATP} opening is an upstream trigger of radical production and tyrosine kinase activation. As discussed by Gross and Fryer,\textsuperscript{78} these findings will need to be reconciled with several earlier studies demonstrating an effect of 5-HD applied after the preconditioning period.

Although preconditioning in humans is more difficult to verify, several studies have noted a significant effect of K\textsubscript{ATP} channel blockers or openers either in vivo or in explanted human tissues.\textsuperscript{45,56,79-83} It is reassuring to find that the pharmacological profile described in animal models has been replicated in human myocardium. As reported recently by Ghosh et al\textsuperscript{45}, hypoxic preconditioning (assessed by prevention of creatine kinase loss) of human atrial tissue could be blocked by previous treatment with glibenclamide or 5-HD, but not HMR1883, and protection was mimicked by diazoxide.

**MitoK\textsubscript{ATP} in Delayed IPC**

In addition to acute IPC, which lasts for 1 to 2 hours, a second window of protection or delayed preconditioning phase reappearing 24 to 72 hours after the brief ischemic period has been described.\textsuperscript{1,6} Protection by delayed preconditioning is usually less effective than acute IPC and requires more cycles of brief ischemia for maximal activation. The activation of tyrosine kinases and PKC, the expression of heat shock protein 72 and inducible NOS, and the release of nitric oxide have been shown to be involved in delayed preconditioning.

Recent findings indicate that mitoK\textsubscript{ATP} channel opening may be a component of delayed protection. In a rabbit model of delayed preconditioning, protection at 24 hours (conferring by either four 5- or 10-minute ischemia/reperfusion cycles\textsuperscript{84} or by adenosine treatment\textsuperscript{85}) could be eliminated by treatment with glibenclamide or 5-HD given just before the long ischemia. As is consistently found in acute IPC, these blockers did not worsen the extent of injury in the absence of preconditioning, indicating that K\textsubscript{ATP} channels are probably involved in the IPC recruitable protection but do not limit infarct size under normal circumstances.

It is noteworthy that the selectivity of 5-HD for mitoK\textsubscript{ATP} was disputed in the report described above\textsuperscript{44} and in others\textsuperscript{85-87} on the basis of the observation that action potential shortening was significantly diminished by 5-HD. Similarly, early studies of 5-HD showed inhibition of whole-cell and single-channel K\textsubscript{ATP} currents by 5-HD.\textsuperscript{38,39,88} Although a direct effect of 5-HD on sarcolemmal K\textsubscript{ATP} may be possible under some
conditions, a result demonstrating an effect of 5-HD on action potential shortening or surfaceK<sub>ATP</sub> currents does not necessarily imply that the drug is acting on the surface channel. It is possible, and perhaps to be expected, that a drug acting on mitoK<sub>ATP</sub>, by influencing cellular energy metabolism, could have secondary effects on surfaceK<sub>ATP</sub>. Conversely, agents that are intended to alter subsarcolemmal energy balance, through tight coupling between sarcolemmal and mitochondrial membranes, could potentially influence mitoK<sub>ATP</sub> activity as well. This could provide an alternative explanation to the conclusions of Haruna et al., who recently showed that inhibition of Na<sup>+</sup>/K<sup>+</sup>-ATPase with digoxin could inhibit the activation of surfaceK<sub>ATP</sub> and abrogate preconditioning but could not inhibit K<sub>ATP</sub> channel opener-mediated protection.

These results were interpreted as evidence that selective surfaceK<sub>ATP</sub> channel inhibition could block protection. Even taking into account possible cross-reactivity of 5-HD with surfaceK<sub>ATP</sub>, its mitochondrial site of action is likely to be the relevant one for protection, because 5-HD can block protection without an effect on ischemic action potential shortening and when 5-HD does have an electrophysiological effect, there is still no correlation between the action potential shortening and protection. These findings have also been confirmed in a cellular model of IPC. Delayed protection (elicited by brief ischemia or adenosine) at 24 hours can be blocked by 5-HD given just before the long ischemia in a human cardiac cell line.

Also in support of mitoK<sub>ATP</sub> as an effector of delayed preconditioning is that a single dose of diazoxide, applied 24 hours before a long ischemia, is enough to afford significant protection in rats. This protection can be blocked by 5-HD, applied either before diazoxide exposure or 10 minutes before the long ischemia, again supporting the hypothesis that mitoK<sub>ATP</sub> channels are involved in both the trigger phase and in protection during the lethal ischemia. In another study in rabbits, diazoxide mimicked both early and delayed IPC, and 5-HD applied before the long ischemia blocked protection, as did L-NAME, leading to the conclusion that both mitoK<sub>ATP</sub> and nitric oxide (NO) were mediators of protection induced by diazoxide.

The NO pathway and mitoK<sub>ATP</sub> activation have been linked in a recent study by Sasaki et al., who showed that NO donors facilitate the activation of mitoK<sub>ATP</sub> opening by diazoxide and partially activate the channel directly. A similar facilitation of mitoK<sub>ATP</sub> opening by PKC activation was earlier noted by Sato et al. Thus, modulation of mitoK<sub>ATP</sub> could play a role in both the short- and long-term memory of IPC. Little is known about how signal transduction pathways modify mitochondrial targets, but a recent study by Wang et al. reported that one isoform of PKC (PKC-δ) is specifically translocated to the mitochondria after diazoxide treatment. How this finding fits in with the known participation of other PKC isoforms in protection has not been determined.

**MitoK<sub>ATP</sub> and Apoptosis**

Several studies have shown that apoptosis contributes to cardiac cell death after ischemia, and emerging evidence indicates that preconditioning may suppress apoptosis in addition to necrosis in intact hearts. Regarding the role of mitoK<sub>ATP</sub> in protecting against apoptosis, several recent studies show that diazoxide pretreatment decreases the appearance of apoptotic markers resulting from ischemia/reperfusion injury. The protection against apoptosis was also blocked by PKC inhibitors or 5-HD, reminiscent of the general mechanism of protection already discussed, and by the same reasoning implicating mitoK<sub>ATP</sub> in the inhibition of apoptosis.

A recent study by Nakamura et al. showed that IPC blunted the upregulation of Bax protein expression associated with ischemia/reperfusion without altering the levels of Bcl-2. The role of Bax in inducing or Bcl-2 in preventing cytochrome c release from the mitochondrial intermembrane space begs the question of whether mitoK<sub>ATP</sub> activation influences this early trigger of apoptosis. The only published evidence thus far paradoxically suggests that cytochrome c release may be induced by mitoK<sub>ATP</sub> opening. Holmuhamedov and colleagues reported that in Ca-loaded mitochondria, diazoxide treatment caused mitochondrial swelling, Ca<sup>2+</sup> release, and loss of cytochrome c. However, recent findings in cultured neonatal rat myocytes suggest the opposite. Apoptosis induced by H<sub>2</sub>O<sub>2</sub> treatment [assessed by cytochrome c translocation, caspase activation, mitochondrial membrane potential loss, and poly(ADP-ribose)polymerase cleavage] can be inhibited by diazoxide, and 5-HD blocks this protection.

**Mechanisms of Protection**

Now that substantial evidence has accumulated in support of mitoK<sub>ATP</sub> as a trigger and late effector of cardioprotection, attention is focussing on how the opening of a K<sup>+</sup> influx pathway on the mitochondrial inner membrane may be protective. At face value, it may seem paradoxical that the opening of an energy dissipating cation conductance on the mitochondrial inner membrane would be beneficial. Because tight coupling between proton pumping and ATP production requires a relatively impermeable inner membrane, mitoK<sub>ATP</sub> opening must lead to changes in mitochondrial function that supersede the loss of energy attributable to uncoupling. A least 3 main mechanistic hypotheses have been proposed to explain the protective effect of mitoK<sub>ATP</sub> channel opening (Figure 2). These hypotheses are not mutually exclusive and may all contribute to protection, but it is still undetermined whether they play a role in either acute or delayed preconditioning in vivo.

**Mitochondrial Swelling and Optimization of Respiration**

A well-described consequence of mitoK<sub>ATP</sub> opening in studies of isolated mitochondria is matrix swelling. According to the model of Garlid et al., the electrophoretic uptake of K<sup>+</sup>, in concert with the operation of a K<sup>+</sup>/H<sup>+</sup> antiporter, are the components of a volume regulatory K<sup>+</sup> cycle. K<sup>+</sup> influx is accompanied by the movement of diffusible weak acids to maintain electroneutrality and water movement attributable to osmotic forces. The net result is matrix swelling. Because the changes in matrix K<sup>+</sup> and H<sup>+</sup> concentrations are initially insufficient to directly activate K<sup>+</sup>/H<sup>+</sup> exchange, some initial
swelling is required before indirect activation of the antiporter (by Mg\(^{2+}\) or H\(^{+}\)) is invoked, and a higher steady-state matrix volume is maintained until the K\(^{+}\) influx ceases. In normally respiring mitochondria, the K\(^{+}\) fluxes are small relative to the rate of proton pumping, so mitochondrial membrane potential and ΔpH is largely maintained.

Matrix swelling by itself may improve the rate of oxidative metabolism, as previously suggested by Halestrap et al., who showed that swelling activated fatty acid oxidation, respiration, and ATP production. If swelling plays a role in improving mitochondrial function, it is worthwhile to consider other factors that may be involved in preventing excess swelling and loss of function. In an earlier model of mitochondrial volume homeostasis described by Garlid and Beavis, respiratory-driven cation influx through mitoK\(_{ATP}\) might also be accompanied by anion efflux through an inner membrane anion channel, or IMAC, in coordination with the operation of the K\(^{+}/H^{+}\) antipporter. IMAC was therefore suggested to be a safety valve that prevents excessive matrix swelling. In light of recent findings that chloride channel inhibitors may play a role in cardioprotection, it is intriguing to speculate that a balance between K\(^{+}\) influx and anion efflux may tune the mitochondrion to the optimal volume for preserving function during ischemia and reperfusion.

On the basis of thermodynamic considerations, it has also been theorized that optimal efficiency of oxidative phosphorylation is achieved when mitochondria are partially uncoupled, and this idea has been put forward as the purpose of mitochondrial uncoupling proteins. By analogy, optimization of respiration may also be the result of the partial uncoupling induced by mitoK\(_{ATP}\) opening. Similarly, Fryer et al showed that mitochondria isolated from the area at risk of preconditioned hearts had higher rates of ATP synthesis than those of hearts subjected to long ischemia alone and that this preservation of mitochondrial function could be partially inhibited by 5-HD, supporting improved ATP production as a common feature of IPC and K\(_{ATP}\) channel openers.

**Mitochondrial Ca\(^{2+}\) Handling**

A decrease in the extent of mitochondrial Ca\(^{2+}\) overload may be a consequence of opening mitoK\(_{ATP}\). Holmuhamedov et al demonstrated that the rate of Ca\(^{2+}\) uptake by isolated mitochondria in suspension (with 150 μmol/L Ca\(^{2+}\) in the bath) is dose-dependently suppressed by diazoxide or pinacidil. Both compounds also activated Ca\(^{2+}\) release from pre-loaded mitochondria through a mechanism that was apparently separate from the mitoK\(_{ATP}\) channel. The effect on Ca\(^{2+}\) release (but not uptake) was cyclosporin A-sensitive and accompanied by cytochrome c release, suggesting that the permeability transition pore may be involved. Although these investigators were unable to obtain consistent block of either response by 5-HD or glybenclamide in the isolated organelles, mitochondrial Ca\(^{2+}\) concentration was reduced by diazoxide in intact neonatal myocytes, and 5-HD inhibited this effect.

It was suggested that the mechanism of decreased Ca\(^{2+}\) uptake by mitoK\(_{ATP}\) opening was attributable to a decreased driving force for Ca\(^{2+}\) entry. From a baseline resting potential of −195 mV in the isolated mitochondria, the K\(_{ATP}\) openers depolarized ΔΨ by 15 to 20 mV, and this effect was not inhibited by cyclosporin A.

These interesting results raise several questions that will require additional investigation. Why was the K\(_{1/2}\) for inhibition of Ca\(^{2+}\) uptake 65 μmol/L for diazoxide, 100 times higher than the K\(_{1/2}\) for K\(^{+}\) uptake via mitoK\(_{ATP}\) as reported by Garlid et al.? What is the nature of the Ca\(^{2+}\) release mechanism? Why is there a permeability transition and cytochrome c release if diazoxide protects against apoptosis?

**Free Radicals and Redox State**

Reactive oxygen species (ROS) have long been implicated in the cellular damage associated with ischemia and reperfusion. On the other hand, ROS generation is thought to be a trigger of signaling pathways mediating preconditioning. Mitochondria not only set the redox balance of the cell, but also are a source of ROS production because of electron leakage from the electron transport chain. Recent work has suggested that the opening of mitoK\(_{ATP}\) channels may alter the rate of mitochondrial ROS production and contribute to cardioprotection. In models of hypoxic preconditioning in embryonic chick myocytes, IPC-, adenosine-, or acetylcholine-mediated protection was associated with an early increase in ROS production during the preconditioning period, as determined by the accumulation of 2',7'-dichlorofluorescein produced by oxidation of its precursor. Protection and ROS production were inhibited by 5-HD, the thiol reductant 2-mercaptooproinyl glycerine, or the mitochondrial site III inhibitor myxothiazol, indicating that mitochondria were the source of ROS production and that the...
opening of mitoK$_{ATP}$ channels could stimulate ROS accumulation. Vanden Hoek et al$^{106}$ showed that the chloride channel blocker diisothiocyanato-stilbene-2,2'‑disulfonate abrogated protection, and they proposed a model in which superoxide generated in the mitochondria is exported through anion channels to the cytoplasm, where it is dismutated to hydrogen peroxide. Interestingly, the generation of ROS during reperfusion, presumed to contribute to irreversible cellular injury, was attenuated by adenosine preconditioning or pinacidil applied just at the time of reperfusion. This protection was eliminated in the presence of 5-HD or an inhibitor of PKC.$^{113}$ Thus, mitoK$_{ATP}$ opening could either enhance or attenuate mitochondrial ROS production, depending on the phase of preconditioning, ischemia, or reperfusion. Using a ROS-sensing microprobe, Obata et al$^{114}$ also demonstrated that cromakalim or nicorandil increased hydroxyl radical production in rat myocardium. The effect was inhibited by 5-HD or glibenclamide. The link between mitoK$_{ATP}$ opening, ROS generation, and PKC remains incompletely defined at present. MitoK$_{ATP}$ opening seems to increase ROS production, which may in turn activate PKC, but the mitoK$_{ATP}$ channel is also modulated by these factors.$^{33,92}$ In a recent study by Wang et al.$^{93}$ diazoxide induced PKC translocation and protection in Langendorff-perfused rat hearts and these responses could be blocked by PKC inhibitors. In contrast, Miura et al$^{115}$ reported that the PKC inhibitor calphostin C could block adenosine-, but not diazoxide-mediated cardioprotection.

**Other Mechanisms**

Other effects of K$_{ATP}$ channel openers have been reported, which may also contribute to protection in the intact heart. Oe et al$^{116}$ reported that diazoxide or cromakalim attenuated resting and stimulated norepinephrine release in perfused guinea pig hearts, but not in human right atrium, with the effect being antagonized by glibenclamide. Glibenclamide by itself increased NE release in both preparations. Sakamoto et al$^{117}$ showed that 5-HD, like glibenclamide, blunts the early, but not late myocardial K$^+$ efflux from ischemic guinea pig hearts, indicating potential crosstalk between the mitochondrial and sarcoplasmal isoforms of the K$_{ATP}$ channel during ischemia (assuming mitochondrial selectivity of 5-HD).

Another possible mechanism involving mitoK$_{ATP}$ and the actin cytoskeleton was suggested by Baines et al.$^{118}$ who found that cytochalasin D, a disrupter of the cytoskeleton, could eliminate protection conferred by diazoxide, pinacidil, or IPC in isolated adult cardiomyocytes subjected to simulated pelleting ischemia. Furthermore, anisomysin, a p38/JNK activator, decreased osmotic fragility in a 5-HD‑sensitive manner.

**K$_{ATP}$ Isoforms in Heart**

Elucidation of the molecular structure of mitoK$_{ATP}$ is thus far limited by the lack of an identified channel clone. Present working models of surfaceK$_{ATP}$ channels suggest that they are composed of a tetramer of core inward rectifier K$^+$ channels (Kir6.x) surrounded by 4 sulfonylurea receptor subunits (SUR), which confer sensitivity not only to glibenclamide but also to K$_{ATP}$ channel openers.$^{9,10}$ There are 3 SUR isoforms encoded by 2 genes: SUR1 and the splice variants SUR2A and SUR2B. The predominating pancreatic isoform is thought to be SUR1 paired with Kir6.2, whereas the myocyte sarcoplasmal isoform has been identified as SUR2A/Kir6.2.$^{119,120}$ In smooth muscle, SUR2B/Kir6.1 is thought to be the small-conductance, diazoxide-sensitive, ATP-insensitive isoform observed in patch-clamp recordings.$^{121}$ Similarities between mitoK$_{ATP}$ and surfaceK$_{ATP}$ have led to the assumption that mitoK$_{ATP}$ will also consist of SUR and Kir components. This is reinforced by the tentative identification of a 63 kDa sulfonylurea binding protein purified from mitochondria and a putative pore forming channel subunit of 55 kDa reported by Grover and Garlid.$^4$

A study by Susuki et al$^{122}$ suggested that antibodies raised against a 12 amino acid stretch of Kir6.1 immunolocalize to mitochondria, implying that this isoform may be present on the inner membrane. In a recent test of whether Kir6.1 is an essential component of mitoK$_{ATP}$, dominant-negative adenoviruses, in which the pore signature sequence GFG was mutated to AFA in Kir6.1 and Kir6.2, were constructed to knock out current carried by SUR/Kir6.x channels.$^{123}$ The AFA mutation eliminates the ability of the channel to conduct K$^+$ but does not prevent it from coassembling with native K$_{ATP}$ channels. These constructs selectively suppressed surface currents carried by their wild-type counterparts when coinfected into A549 cells.$^{122}$ The 6.2AFA virus, but not the 6.1AFA virus, could also suppress native sarcoplasmal K$_{ATP}$ current in isolated rabbit cardiomyocytes. Notably, infection of adult myocytes with the dominant-negative Kir6.1AFA subunit had no effect on the mitochondrial redox response to diazoxide, indicating that it is unlikely that Kir6.1 is part of mitoK$_{ATP}$. However, this does not preclude the possibility that a Kir6.1-like subunit, with antigenic similarity to 6.1 but without the ability to heteromultimerize with its surface counterpart, could still contribute to the mitoK$_{ATP}$ channel structure. In this regard, recent work by Liu et al$^{123}$ indicates that among the combinations of known SUR and Kir subunits expressed heterologously as surface channels, only SUR1/Kir6.1 fits the pharmacological profile for mitoK$_{ATP}$ (as defined in intact rabbit myocytes; see the Table).

Isoform-specific localization of the binding sites for K$_{ATP}$ channel openers and inhibitors may provide some insight into the structure of mitoK$_{ATP}$. To fit the pharmacological profile of mitoK$_{ATP}$, one would look for a combination of SUR/Kir subunits that is readily opened by diazoxide, nicorandil, cromakalin, and pinacidil (and, perhaps, P1075) and is sensitive to inhibition by glibenclamide and 5-HD. In this context, recent evidence using chimeras of SUR1 (the pancreatic isoform potently activated by diazoxide) and SUR2A (insensitive to diazoxide), both coexpressed with Kir6.2, indicates that diazoxide sensitivity may be conferred by a region comprised of SUR1 transmembrane domains 6 to 11 interacting with its first nucleotide binding fold.$^{126}$ Cromakalin and pinacidil, which readily open SUR2A/Kir6.2 channels, interact with a region spanning transmembrane domains 12 to 17 of SUR2A,$^{126}$ and the inhibitory binding site of sulfonylureas is also in this area.$^{127}$ The critical residues for pinacidil binding were additionally narrowed by Uhde et al.$^{128}$
who showed that P1075 binding involved regions including amino acids 1059 to 1087 and 1218 to 1320 of SUR2.

The relatively small 42 amino acid carboxy-terminal divergence between the splice variants SUR2A and SUR2B contributes to major differences in K_{ATP} opener sensitivity. Not only is SUR2B sensitive to diazoxide, but nicorandil is more than 100 times more potent at opening this isoform as compared with SUR2A, whereas both are equally sensitive to pinacidil.130

Regarding the effects of different Kirs on the K_{ATP} pharmacology, Russ et al131 reported that glibenclamide binding to SUR2B is enhanced by coexpression of Kir6.1, although the K_{0} for binding was about 7 times lower than the K_{i} for channel inhibition. Koster and colleagues132,133 showed that mutations of Kir6.2 altered the nucleotide sensitivity of the channel and activation by phosphatidylinositol 4,5-bisphosphate. The N-terminus of Kir has been implicated in the coupling of sulfonamide-bound SUR to the stabilization of the channel-closed state.127 Conductance and gating of the channel is also conferred by the Kir subunit. In a study by Kondo et al134 using chimeras of Kir6.1 and Kir6.2, the extracellular linker domain between the membrane-spanning regions determined conductance (80 pS in Kir6.2 and 35 pS in Kir6.1 when coexpressed with SUR2A), whereas the N- and C-terminal regions were implicated in spontaneous opening in the absence of intracellular ATP (not present in Kir6.1).

Reconstruction of the known properties of mitoK_{ATP} from structure-function studies of surface channels may provide clues toward the molecular structure of mitoK_{ATP} but will require additional elucidation of the isoform-specific differences in 5-HD sensitivity, conductance, and regulation.

Summary

The hypothesis that the mitoK_{ATP} channel plays a major role in both acute and delayed preconditioning has been well supported by an accumulating body of evidence. Questions remain regarding the selectivity of some of the pharmacological tools used, and it is unwise to rely on only a single compound to justify a particular target, especially when intracellular conditions are changing markedly during ischemia. Still, when the results of many studies are taken together, protection is poorly correlated with drug action on surfaceK_{ATP} channels and well correlated with effects on mitoK_{ATP} channels. These questions will undoubtedly be resolved with a new generation of highly isoform-specific compounds.

If the mitoK_{ATP} hypothesis holds up to future scrutiny, the question remains as to what the role of surfaceK_{ATP} is in the cardioprotection. Because mitoK_{ATP} seems to mediate the extra protection associated with preconditioning but does not seem to affect injury in the absence of preconditioning, it is possible that surfaceK_{ATP} provides a baseline level of resistance to damage. Because surfaceK_{ATP} channel opening may also be arrhythmogenic on reperfusion, defining its true role in ischemia and reperfusion is critical.

The mechanism of mitoK_{ATP} protection may involve alterations in mitochondrial Ca^{2+} handling, the optimization of energy production, and modulation of ROS production during ischemia or reperfusion. It is not known which of these factors is important in vivo. Also to be resolved is the relationship between mitoK_{ATP} opening and protein kinase action. Recent findings indicate that mitoK_{ATP} channels are modulated by PKC and NO but may also trigger the translocation and activation of PKC or activate tyrosine kinases.

The molecular cloning of mitoK_{ATP} has not yet been achieved and has been hampered by uncertainty about the molecular determinants of intracellular targeting of transmembrane proteins. Until it is cloned, it is only possible to draw structural inferences about mitoK_{ATP} from known properties of surfaceK_{ATP}.

Despite many unanswered questions, recent experimental findings and drug developments support the feasibility of specifically recruiting the natural defenses of the cell and will likely lead to new therapeutic strategies in the near future.

References


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