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## Evidence for Mitochondrial K<sup>+</sup> Channels and Their Role in Cardioprotection

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### Abstract

Twenty years after the discovery of sarcolemmal ATP-sensitive K<sup>+</sup> channels and 12 years after the discovery of mitochondrial K<sub>ATP</sub> (mitoK<sub>ATP</sub>) channels, progress has been remarkable, but many questions remain. In the case of the former, detailed structural information is available, and it is well accepted that the channel couples bioenergetics to cellular electrical excitability; however, in the heart, a clear physiological or pathophysiological role has yet to be defined. For mitoK<sub>ATP</sub>, structural information is lacking, but there is abundant evidence linking the opening of the channel to protection against ischemia-reperfusion injury or apoptosis. This review updates recent progress in understanding the physiological role of mitoK<sub>ATP</sub> and highlights outstanding questions and controversies, with the intent of stimulating additional investigation on this topic.

### Keywords

ischemia; reperfusion; mitochondria; ATP-sensitive potassium channels; calcium-activated potassium channels

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Since their discovery in cardiac myocytes using single-channel patch-clamp techniques in the early 1980s,<sup>1</sup> surface membrane ATP-sensitive K<sup>+</sup> channels have been characterized extensively at the molecular level. Progress has been rapid, facilitated by the cloning of the major components of the heteromeric channel—the sulfonylurea receptor (SUR12 and SUR2<sup>3</sup>), an ABC transporter family member, and the pore-forming inward rectifier K<sup>+</sup> channel protein (Kir6.14 and Kir6.2<sup>5</sup>). ATP-sensitive K<sup>+</sup> channels play an important role in translating the metabolic status of a cell into a physiological effect, with perhaps the best examples being the initiation of insulin secretion in pancreatic  $\beta$  cells and modulation of vascular tone. The opening of cardiac sarcolemmal ATP-sensitive K<sup>+</sup> (sarcK<sub>ATP</sub>) channels by metabolic inhibition dramatically affects cardiac myocyte electrical excitability<sup>6</sup> and is responsible for ischemia-induced suppression of excitability,<sup>7</sup> but it is still not clear why this adaptation is important to the cell, organ, or organism. This issue has been revisited in several recent studies of Kir<sup>8–10</sup> or SUR<sup>11–13</sup> knockout mice.

Another door was opened in 1991, with the discovery of mitochondrial K<sub>ATP</sub> (mitoK<sub>ATP</sub>) channels in the liver mitochondrial inner membrane.<sup>14</sup> This finding was bolstered by evidence that a variety of K<sup>+</sup> channel openers and inhibitors influenced mitochondrial function,<sup>15–18</sup> culminating in the establishment of a link between the mitoK<sub>ATP</sub> channel and protection against ischemic injury in intact hearts<sup>18</sup> and isolated myocytes.<sup>19</sup> This hypothesis explained the paradoxical finding that the effects of K<sup>+</sup> channel openers on the cardiac action potential and

their ability to suppress ischemia-reperfusion injury were not correlated.<sup>20–22</sup> Since then, mitoK<sub>ATP</sub> has been implicated in cellular protection against metabolic stress in a variety of tissues, including liver, gut, brain, and kidney, and has been shown to be an essential component of the mechanism of ischemic preconditioning in the heart.<sup>21,23–25</sup>

Although progress has been made in delineating the mechanisms involved in mitoK<sub>ATP</sub>-mediated protection, the absence of a clear molecular definition of the protein has hampered structure-function studies of this intracellular channel. Nevertheless, the recent development of novel, more specific, mitochondrial K<sup>+</sup> channel openers and inhibitors lends support to the general paradigm that increased mitochondrial K<sup>+</sup> influx contributes to the cell's defense against ischemic injury.

Herein, a synthesis and critical review of available information on mitoK<sub>ATP</sub> is presented, with the hope of crystallizing important questions that need to be addressed as we move toward a more complete understanding of the functional role of mitochondrial ion channels in health and disease.

## Evidence Supporting Mitochondrial K<sub>ATP</sub> Channels

### Electrophysiological Recordings of Mitochondrial Channel Activity

Electrophysiological recordings, although technically challenging for intracellular ion channels, are perhaps the most concrete proof of the existence of mitoK<sub>ATP</sub> channels. Indeed, although the various other methods lend compelling support for a specific mitochondrial K<sup>+</sup> influx pathway, direct detection of single-channel activity in the mitochondrial inner membrane provided the foundation for subsequent interpretations. The electrophysiological evidence began with the original work of Inoue et al,<sup>14</sup> who directly patch-clamped mitoplasts (mitochondria stripped of the outer membrane) prepared from fused liver mitochondria. Single K<sup>+</sup> selective channels were identified and were inhibited by ATP (K<sub>i</sub>≈0.8 mmol/L) applied to the mitochondrial matrix face of the channel. They were also blocked by the K<sup>+</sup> channel inhibitor 4-aminopyridine. Channel properties resembled those of surface membrane K<sub>ATP</sub> channels, although with a much lower unitary conductance (10 pS in 100 mmol/L cytosolic K<sup>+</sup> and 33 mmol/L matrix K<sup>+</sup>). Importantly, the channels were inhibited by the sulfonyleurea glibenclamide (5 μmol/L), additional evidence that a K<sub>ATP</sub>-like channel was present.

There have been no additional publications demonstrating K<sub>ATP</sub> channel activity directly on the intact mitochondrial inner membrane since the original discovery. However, several independent groups have observed channels sensitive to K<sup>+</sup> channel openers and inhibitors in reconstituted membranes using highly purified mitochondrial inner membrane preparations. Paucek et al<sup>15</sup> reported the purification and reconstitution of a 54-kDa mitochondrial protein that conferred channel activity to a lipid bilayer with a saturating conductance of 30 pS (1 mol/L KCl), consistent with earlier studies showing K<sup>+</sup> channel activity attributed to a mitochondrial K<sup>+</sup> uniporter in a similar fraction.<sup>26,27</sup> It was subsequently found that nucleotide regulation of mitoK<sub>ATP</sub> in bilayers was polarized<sup>28</sup>; ATP added to the *trans* but not the *cis* chamber inhibited the channels (the *trans* chamber conventionally represents the matrix face of the bilayer; however, the orientation of protein insertion was shown to be specific to medium conditions). Based on the argument that GTP and palmitoyl CoA (assumed to be impermeable to the mitochondrial inner membrane) modulated the activity of mitoK<sub>ATP</sub> in intact mitochondria, these authors concluded that the regulatory site faced the cytosol (or, rather, the mitochondrial intermembrane space).

Zhang et al<sup>29</sup> observed K<sup>+</sup>-selective channel activity in planar lipid bilayers reconstituted with purified bovine mitochondrial membranes, having a conductance of 56 pS in 150 mmol/L KCl. These channels were dose-dependently inhibited by MgATP and activated by GTP when

applied only to the *trans* (matrix) side of the bilayer. They were blocked by 10 to 100  $\mu\text{mol/L}$  5-hydroxydecanoate (5-HD) or 10 to 100  $\mu\text{mol/L}$  glibenclamide and competitively activated by 10  $\mu\text{mol/L}$  diazoxide. The  $\text{K}^+$  channel blocker HMR1098, a subtype-selective  $\text{K}_{\text{ATP}}$  antagonist that preferentially blocks the sarc $\text{K}_{\text{ATP}}$  channel subtype (see pharmacology below), did not inhibit the channels. Interestingly, mito $\text{K}_{\text{ATP}}$  channel open probability was enhanced in the presence of superoxide, exogenously generated by a xanthine/xanthine oxidase reaction, and this activation was abolished by pretreatment with 5-HD or a thiol-reducing agent. Redox regulation of mito $\text{K}_{\text{ATP}}$  channel activity in bilayers was earlier demonstrated by Marinov and Mironova and colleagues,<sup>26,30,31</sup> who reported the opposite effect of the reducing agent dithiothreitol, ie, increased activity, but decreased selectivity.<sup>26</sup>  $\text{K}_{\text{ATP}}$  channels sensitive to diazoxide and 5-HD were also recently observed by Nakae et al<sup>32</sup> in reconstitution experiments with purified heart mitochondrial inner membranes. The  $\text{K}^+$  channels were activated by the anesthetic isoflurane, known to confer protection against infarction,<sup>33</sup> and inhibited by application of ATP to the *cis* (cytosolic) face of the bilayer. Consistent with other studies, the channels were inhibited by 5-HD (100  $\mu\text{mol/L}$ ) but not by HMR1098.

### Mitochondrial $\text{K}^+$ Uptake

A second line of evidence supporting a specific ATP-sensitive  $\text{K}^+$  channel in the mitochondrial inner membrane is the measurement of  $\text{K}^+$  uptake into proteoliposomes reconstituted with purified mitochondrial protein fractions. This method has been used extensively by Garlid and colleagues<sup>15,18,28,34,35</sup> to characterize the channel's regulation by  $\text{Mg}^{2+}$ , ATP and ADP, GTP and GDP, and acyl-CoA esters, as well as its sensitivity to pharmacological openers and inhibitors (see the online data supplement, available at <http://circres.ahajournals.org>, for more detail). In a key study, Garlid et al<sup>36</sup> reported a striking distinction between the pharmacology of sarc $\text{K}_{\text{ATP}}$  and mito $\text{K}_{\text{ATP}}$ ; diazoxide had a potency for the mito $\text{K}_{\text{ATP}}$  channel ( $\text{K}_{1/2}$ , 0.4  $\mu\text{mol/L}$ ) roughly 2000-fold higher than for the sarcolemmal channel (855  $\mu\text{mol/L}$ ). Cromakalim and two of its highly potent congeners, EMD60480 and EMD57970 ( $\text{K}_{1/2}$ , <10 nmol/L), activated mito $\text{K}_{\text{ATP}}$  in the ATP-inhibited state, but with no particular selectivity for the mitochondrial isoform.

### Mitochondrial Swelling

To avoid rupture of the mitochondrial outer membrane due to swelling, as well as to limit unnecessary energy wastage, mitochondrial cation flux is tightly regulated. The mitochondrial  $\text{K}^+$  cycle, counterbalancing mitochondrial uniporter activity with  $\text{K}^+/\text{H}^+$  exchange, is an important component in mitochondrial volume homeostasis.<sup>37,38</sup> With the opening of a mitochondrial  $\text{K}^+$  channel,  $\text{K}^+$  influx, accompanied by the movement of permeable anions and  $\text{H}_2\text{O}$ , can transiently exceed the extrusion capacity of the  $\text{K}^+/\text{H}^+$  exchanger. This results in an increase in steady-state matrix volume, assessed by the extent of light scattering at 520 nm. This assay has been extensively used to investigate the opening of mito $\text{K}_{\text{ATP}}$  in intact isolated mitochondria.<sup>16,39</sup> The advantage of this method compared with reconstitution studies is that the mitochondrial ion channels and the bioenergetic apparatus are intact and regulatory agents can be applied directly to the mitochondria. Limitations of the method include the need to work under hypoosmotic conditions (eg, 115 mOsm) to maintain linearity of the response,<sup>39,40</sup> the use of unphysiological ion gradients, nucleotide concentrations, or substrates, and the elimination of normal cytoskeletal architecture and the influence of neighboring organelles or membranes. Nevertheless, in addition to confirming the results of reconstitution experiments, this method has proven useful for characterizing the regulation of mito $\text{K}_{\text{ATP}}$ . For example, Jaburek et al<sup>39</sup> demonstrated that pharmacological inhibition of mi-to $\text{K}_{\text{ATP}}$  depends on how the channels are activated. Mitochondrial swelling activated by removal of ATP (but with  $\text{Mg}^{2+}$  present) was completely insensitive to 5-HD or glibenclamide; however, swelling activated by diazoxide, cromakalim, or GTP in the presence of ATP and  $\text{Mg}^{2+}$  was inhibited by glibenclamide (low  $\mu\text{mol/L}$ ) or by 5-HD (45 to 85  $\mu\text{mol/L}$ ).  $\text{K}^+$  channel openers induced

swelling at concentrations roughly five times higher than those required to activate  $K^+$  uptake into proteoliposomes; the  $K_{1/2}$  for cromakalim, diazoxide, and EMD60480 was 6.3  $\mu\text{mol/L}$ , 2.3  $\mu\text{mol/L}$ , and 5.4  $\text{nmol/L}$ , respectively, in 0.1  $\text{mmol/L}$  ATP plus 1  $\text{mmol/L}$   $\text{Mg}^{2+}$ .<sup>36</sup>

### Flavoprotein Oxidation

The autofluorescence of NADH and flavoproteins in the mitochondrial matrix provides a built-in sensor of mitochondrial redox state in intact cells and tissues.<sup>41,42</sup> In isolated cardiac myocytes excited with 480 nm light, the major component of fluorescence (500- to 550-nm emission band) arises from FAD tightly associated with Krebs cycle dehydrogenases (eg, isocitrate dehydrogenase,  $\alpha$ -ketoglutarate dehydrogenase, and pyruvate dehydrogenase) in equilibrium with the  $\text{NAD}^+/\text{NADH}$  redox couple. Under the right conditions, flavoprotein fluorescence can therefore be used to detect changes in the rate of mitochondrial oxidative phosphorylation and the minimum and maximum oxidation states of mitochondria can be calibrated in situ using an inhibitor of electron transport (cyanide) and an uncoupler (eg, 2,4-dinitrophenol), respectively. A study by Liu et al<sup>19</sup> and subsequent studies<sup>43–47</sup> used native flavoprotein fluorescence to assay the opening of  $\text{mitoK}_{\text{ATP}}$  in intact cardiac myocytes. The opening of a mitochondrial  $K^+$  channel dissipates energy that would normally be used by the  $\text{F}_1\text{F}_0$  ATPase to produce ATP; hence,  $\text{mitoK}_{\text{ATP}}$  opening results in mild uncoupling of mitochondrial energy production. This can be detected as a net change in the redox balance of the mitochondria and an increase in flavoprotein fluorescence if the stimulation of electron flow (NADH oxidation) exceeds the rate of production of NADH. The optimal conditions for measuring a flavoprotein response were found by culturing myocytes overnight and applying  $K^+$  channel openers in the absence of external substrates.<sup>19</sup> Under these conditions, the  $K^+$  channel opener diazoxide induced a reproducible and reversible net oxidation of the flavoprotein pool to a maximal level of 40% to 50% of the fully uncoupled state without any effect on  $\text{sarcK}_{\text{ATP}}$  currents measured simultaneously. The  $K_{1/2}$  for this response was 27  $\mu\text{mol/L}$  (Figure 1),  $\approx 10$ -fold higher than that found for diazoxide-mediated swelling in isolated mitochondria but approximately equal to its potency for cardio-protection in whole hearts,<sup>18</sup> intact cells,<sup>19</sup> or whole-animal studies.<sup>48</sup> The difference in diazoxide sensitivity in intact cells compared with isolated mitochondria can be explained by the 10- to 80-fold higher concentration ( $\approx 8$   $\text{mmol/L}$ ) of ATP in the cytosol of intact myocytes (the potency of  $K^+$  channel openers strongly depends on the ATP concentration) and the additional sarcolemmal barrier to diazoxide diffusion in the myocyte.

In subsequent studies, flavoprotein oxidation has been used to determine the pharmacology and regulation of  $\text{mitoK}_{\text{ATP}}$  in cardiac myocytes.<sup>43,44,46,47,49–51</sup> Regarding the selectivity of  $K^+$  channel openers and inhibitors for the mitochondrial versus sarcolemmal isoform of the  $\text{K}_{\text{ATP}}$  channel, the results, with two exceptions (the opener P-1075 did not open  $\text{mitoK}_{\text{ATP}}$  and HMR1098 did not inhibit the diazoxide response in intact cells), are in good agreement with those obtained using isolated mitochondria.

Assaying the mitochondrial flavoprotein oxidation state has provided the only convenient measure of mitochondrial channel opening in intact cells and permits one to study the response of  $\text{sarcK}_{\text{ATP}}$  channels simultaneously; however, this method, as with the others mentioned above, has some limitations. The mitochondrial redox potential is a steady-state variable that is the product of the net balance between NADH production by the Krebs cycle and the rate of NADH oxidation. The redox balance therefore does not report the overall rate of oxidative phosphorylation and it could either remain constant or differ over a range of respiratory rates. For example, when cardiac workload increases, there is often no net change in  $\text{NAD}^+/\text{NADH}$  due to the simultaneous stimulation of upstream and downstream flows. Furthermore, in most cases, mitochondrial membrane potential ( $\Delta\Psi_{\text{m}}$ ) is well compensated by enhanced proton pumping, so that  $\Delta\Psi_{\text{m}}$  does not change dramatically despite partial oxidation of the redox pool.

In fact, diazoxide-induced FP oxidation is accompanied by only a small depolarization of  $\Delta\Psi_m$  in intact myocytes under normoxic conditions.<sup>52</sup> Measuring oxygen consumption as an index of electron flow helps to resolve the extent of mitochondrial uncoupling induced by mitoK<sub>ATP</sub> openers, as discussed below.

### Effects on Respiration and $\Delta\Psi_m$

The opening of a mitochondrial ion channel will partially dissipate the energy stored as the proton motive force ( $\mu_H = \Delta\Psi_m - RT/F\Delta pH$ ) by contributing inward current and, in the case of a K<sup>+</sup> channel, by expending a portion of the proton gradient to eject K<sup>+</sup> via the K<sup>+</sup>/H<sup>+</sup> exchanger. This partial uncoupling (energy dissipation not coupled to ATP production) will result in a compensatory increase in proton pumping and oxygen consumption to maintain  $\Delta\Psi_m$  and oxidative phosphorylation. Because the relative change in K<sup>+</sup> influx due to K<sub>ATP</sub> channel opening has been estimated to be relatively small ( $\approx 30 \text{ nmol K}^+ \text{ min}^{-1} \text{ mg}^{-1}$ ),<sup>53</sup> the subtle influence of mitoK<sub>ATP</sub> opening on respiration can often be masked by other nonspecific actions of K<sup>+</sup> channel openers on respiration. For example, the most widely investigated mitoK<sub>ATP</sub> opening compound, diazoxide, inhibits succinate-supported respiration in isolated mitochondria at a concentration range that overlaps with the high end of the cardio-protective dose range (discussed below). This decrease in respiration has been attributed to an effect of diazoxide on succinate dehydrogenase (SDH) and is not evident when NADH-linked substrates are used. The latter conditions can thus be used to detect the uncoupling effect of mitoK<sub>ATP</sub> opening. K<sup>+</sup> channel inhibitor-sensitive stimulation of mitochondrial oxygen consumption usually amounts to an increase of  $\approx 20\%$  in liver or heart mitochondria<sup>53</sup> and up to 50% in brain mitochondria,<sup>34,54</sup> presumably due to a higher channel density in the brain.

In intact C2C12 skeletal myoblast and human-derived Girardi cell lines, Minners et al<sup>55</sup> reported that pharmacological (diazoxide or adenosine) or ischemic preconditioning results in mild uncoupling, evidenced by stimulation of cellular oxygen consumption, a small decrease in  $\Delta\Psi_m$ , and depression of cellular ATP levels. These effects, as well as the protection against ischemic damage, were attenuated by 5-HD, suggesting a link between the mechanism of protection and the uncoupling effects.

Similar effects of K<sup>+</sup> channel openers on mitochondrial respiration,  $\Delta\Psi_m$ , and NADH were recently reported in a comprehensive study by Debska et al<sup>54</sup> using isolated rat skeletal muscle mitochondria and intact L6 skeletal myoblast cells. In both preparations, diazoxide or nicorandil stimulated the rate of respiration in a saturable, concentration-dependent manner, with a K<sub>1/2</sub> of  $\approx 20 \text{ }\mu\text{mol/L}$ . Consistent with other studies,<sup>52,53,56</sup> the K<sup>+</sup>-specific depolarization of  $\Delta\Psi_m$ , determined by subtracting off a small nonspecific effect of the openers in K<sup>+</sup>-free medium, was diminutive; approaching 8 mV for 100  $\mu\text{mol/L}$  diazoxide (4 mV for nicorandil). Glibenclamide completely inhibited the depolarization, whereas 5-HD partially inhibited the response. Like the effects on respiration and  $\Delta\Psi_m$ , a K<sup>+</sup>-specific and saturable oxidation of the NADH redox pool was observed for diazoxide and nicorandil, with K<sub>1/2</sub>  $< 50 \text{ }\mu\text{mol/L}$  (similar to FP oxidation in cardiomyocytes). The swelling response of the mitochondria followed a similar concentration-response profile.

Although the effects of the prototype mitoK<sub>ATP</sub> opener diazoxide on respiration and  $\Delta\Psi_m$  have been characterized by several investigators, the use of this drug is complicated by channel-independent actions on mitochondrial metabolism. Unfortunately, more potent openers (eg, BMS-191095)<sup>22</sup> of mitoK<sub>ATP</sub> have not been studied as intensively but could provide needed confirmation in the future. In the meantime, certain questions should be kept in mind when trying to determine if a compound's effect is due to mitoK<sub>ATP</sub> opening. Does the effect occur in the right concentration range (ie, saturating at  $< 100 \text{ }\mu\text{mol/L}$ )? Is the effect inhibited by 5-HD, glibenclamide, or (preferably) both? Is the effect consistent with the pharmacology for

structurally distinct mitoK<sub>ATP</sub> openers and inhibitors that have been shown to confer or block protection against ischemia-reperfusion injury?

## Inconsistencies in the Mitochondrial K<sub>ATP</sub> Channel Story

### Nonspecific Effects of K<sup>+</sup> Channel Openers

The antihypertensive and diabetogenic properties of diazoxide predate the discovery of ATP-sensitive K<sup>+</sup> channels. Thus, early studies of diazoxide's effect on energy metabolism were designed to explain its role in insulin secretion and glucose homeostasis. In 1969, Schäfer et al.<sup>57</sup> reported that diazoxide inhibited succinate oxidation in isolated rat liver mitochondria or beef heart submitochondrial particles. The effect was highly specific for succinate, and the rate of NADH oxidation was unaffected or slightly stimulated ( $\approx 20\%$ ) by diazoxide ( $\beta$ -hydroxybutyrate substrate). Inhibition of succinate-supported respiration by diazoxide was not saturable up to 1 mmol/L and was attributed to reduced succinate uptake, with consequent suppression of SDH (complex II) activity. Subsequently,<sup>58</sup> a mild uncoupling effect of diazoxide (400  $\mu\text{mol/L}$ ) on state 4 respiration, ranging from 37% to 52%, was noted for all substrates (glutamate,  $\alpha$ -ketoglutarate, and  $\beta$ -hydroxybutyrate) except succinate (respiration decreased by 22%). Retrospective interpretation of these findings is complicated by the lack of comparison with K<sup>+</sup>-free medium and differing states of polarization of the mitochondrial membrane in different experiments. However, it is clear from these early studies that (1) high concentrations of diazoxide strongly inhibit succinate-supported mitochondrial respiration but not NADH-linked substrate oxidation,<sup>57–60</sup> (2) the inhibitory effect on SDH can obscure the mild uncoupling effect of diazoxide,<sup>61</sup> (3) the concentration-response curve for SDH inhibition overlaps with the high end of the diazoxide effect on mitoK<sub>ATP</sub> channel opening but does not saturate until the millimolar range (Figure 1),<sup>58</sup> and (4) glibenclamide does not inhibit SDH inhibition by diazoxide but does inhibit the uncoupling effect.<sup>61</sup>

Grimmsmann and Rustenbeck<sup>59</sup> later explored the effects of high concentrations of diazoxide on metabolism in liver mitochondria and intact pancreatic B-cells. Diazoxide (0.5 mmol/L) slightly improved state 4 mitochondrial ATP production in succinate or tetramethylphenylenediamine (TMPD) plus ascorbate but inhibited energy production from  $\alpha$ -ketoisocaproate. When respiration was stimulated with 400  $\mu\text{mol/L}$  ADP, ATP production by succinate or  $\alpha$ -ketoisocaproate, but not TMPD plus ascorbate, was inhibited by diazoxide (0.5 mmol/L), consistent with an inhibitory effect on SDH. The K<sup>+</sup> channel openers pinacidil and levcromakalim had no effect on succinate-supported oxidative phosphorylation, whereas they inhibited or stimulated  $\alpha$ -ketoisocaproate metabolism, respectively. In intact cells, diazoxide concentrations from 0.1 to 1 mmol/L depolarized  $\Delta\Psi_m$ , as did pinacidil (0.5 mmol/L), whereas levcromakalim (0.5 mmol/L) had no effect. In agreement with earlier studies,<sup>62</sup> neither tolbutamide nor glibenclamide could reverse the inhibition of respiration by the K<sup>+</sup> channel openers.

Recent studies have confirmed the finding that diazoxide<sup>60,63,64</sup> inhibits succinate-supported mitochondrial respiration, and some have postulated that inhibition of complex II may be involved in the mechanism of ischemic preconditioning. Ovide-Bordeaux et al.,<sup>64</sup> using saponin-permeabilized left ventricular subendocardial fibers, found that 100  $\mu\text{mol/L}$  diazoxide had no effect on mitochondrial respiration in the presence of glutamate plus malate but inhibited succinate-supported respiration by  $\approx 22\%$ . Glibenclamide did not block this effect. In addition, these investigators found no effect of diazoxide on the ADP sensitivity of respiration. Reproducing the results of the studies mentioned above, Hanley et al.<sup>63</sup> reported that diazoxide (10 to 100  $\mu\text{mol/L}$ ) inhibited succinate-, but not NADH-supported, respiration in beef heart submitochondrial particles in a nonsaturable manner. Pinacidil had no effect on succinate oxidation but partially inhibited NADH oxidation, probably as a result of inhibition of complex I.<sup>65</sup> Glibenclamide and 5-HD were not tested in this study, but evidence was presented that 5-

HD could be activated to 5-HD-CoA by acyl CoA synthetase, the first step toward entry into the  $\beta$  oxidation pathway.

In a study investigating the effects of ischemic or pharmacological preconditioning on mitochondrial parameters, Lim et al<sup>60</sup> found parallel increases in mitochondrial matrix volume and succinate oxidation in mitochondria isolated from hearts subjected to 30 minutes of ischemia and 30 minutes of reperfusion. Diazoxide (50  $\mu$ mol/L) similarly increased matrix volume, but inhibited succinate-supported respiration by  $\approx$ 30%, while having no effect on palmitoyl carnitine or glutamate plus malate oxidation. Although activation of 5-HD to 5-HD-CoA was confirmed in this study, 5-HD was not metabolized by the fatty acid  $\beta$ -oxidation pathway in heart mitochondria, and it partially inhibited the oxidation of all substrates at concentrations from 300  $\mu$ mol/L to 2 mmol/L. Importantly, 5-HD did not reverse the impairment of succinate oxidation by diazoxide. Contrary to these findings, a subsequent study from the same group reported that an increase in mitochondrial matrix volume by diazoxide could not be detected in isolated mitochondria, claiming this as evidence against the existence of mitoK<sub>ATP</sub> channels.<sup>66</sup>

Taken together, these results demonstrate that it is unlikely that inhibition of SDH underlies the protection afforded by K<sup>+</sup> channel openers for the following reasons: (1) the inhibition of respiration by diazoxide only applies to succinate-supported respiration and not physiologically relevant substrates, (2) inhibition of SDH by diazoxide does not saturate and does not parallel the dose-response for protection, (3) SDH inhibition is not reversed by glibenclamide or tolbutamide, and (4) many potent K<sup>+</sup> channel openers that have strong protective effects do not inhibit SDH activity.

Nevertheless, the diazoxide interaction with SDH begs the question of whether this protein is in any way involved in the activation of mitoK<sub>ATP</sub>. Studies in heart<sup>67</sup> and brain<sup>68</sup> have suggested that enhanced tolerance to ischemia conferred by the SDH inhibitor 3-nitropropionic acid might involve mitoK<sub>ATP</sub> opening, as evidenced by attenuation of the protection by 5-HD. Recent experiments suggest that 3-nitropropionic acid activates mitoK<sub>ATP</sub> channels reconstituted from a highly purified mitochondrial membrane preparation,<sup>69</sup> and this effect was inhibited by 5-HD or glibenclamide, implying that SDH modulates mitoK<sub>ATP</sub> as part of a macromolecular complex.

### Lack of K<sup>+</sup> Selectivity of Mitochondrial Response to Diazoxide

Suppression of pathological reactive oxygen species (ROS) production during ischemia/reperfusion is one mechanism by which K<sup>+</sup> channel openers seem to be acting to protect against cell injury. A recent study by Ozcan et al<sup>70</sup> found that on reoxygenation after 5 minutes of anoxia, the levels of ROS produced by isolated mitochondria were decreased by diazoxide or nicorandil. This reduction in ROS production was 5-HD sensitive and was associated with better preservation of oxidative phosphorylation and maintenance of the structural integrity of the mitochondria. Interestingly, the suppression of ROS production was evident even in K<sup>+</sup>-free medium (sucrose substitution), suggesting that K<sup>+</sup>-selective channels were not required for the beneficial effect. Furthermore, malonate, an inhibitor of SDH, also reduced ROS production. Again, a link was suggested between diazoxide-mediated SDH inhibition and mitochondrial preservation. Because this study used pyruvate plus malate to support respiration, which is not inhibited by diazoxide, it is unclear what role SDH inhibition could be playing in the response. Follow-up studies using mitochondrial K<sup>+</sup> channel openers that do not inhibit SDH (eg, pinacidil or cromakalim) and sensitivity of the response to glibenclamide would be useful to resolve the discrepancies raised by these findings.

## Pharmacology of mitoK<sub>ATP</sub> Channels

The amphipathic nature of compounds that interact with intracellular targets introduces possible nonspecific actions on metabolism that complicate interpretation of results. In the case of mitoK<sub>ATP</sub>, classification as a selective K<sup>+</sup> channel resembling an isoform of the ATP-sensitive K<sup>+</sup> channel has been inferred from the effects of a wide variety of K<sup>+</sup> channel openers or blockers. Misinterpreting a nonspecific effect of any particular compound can only be avoided by the use of multiple agents having dissimilar structures but the singular common property of a demonstrated action on K<sup>+</sup> channels.

In this context, the number and variety of K<sup>+</sup> channel openers that activate mitoK<sub>ATP</sub> provide some reassurance that the target is indeed an inner-membrane K<sup>+</sup> channel. These are compiled in the Table. In terms of isoform selectivity, the findings in cardiomyocytes and isolated mitochondria can be summarized as follows: diazoxide, nicorandil, and the highly potent BMS-191095 open mitoK<sub>ATP</sub> channels with minimal effects on the cardiac sarcK<sub>ATP</sub> isoform. A variety of other K<sup>+</sup> channel openers (eg, cromakalim or its stereoselective form levcromakalim, EMD60480, EMD57970, pinacidil, RP66471, minoxidil sulfate, and KRN2391), some with submicromolar potencies, also activate mitoK<sub>ATP</sub> but do not discriminate between the two K<sub>ATP</sub> channel isoforms. With respect to mitoK<sub>ATP</sub> inhibition, glibenclamide blocks both isoforms, whereas HMR1098 is generally found to be selective for the sarcolemmal channel (however, see the studies by Birincioglu et al,<sup>71</sup> Tsuchida et al,<sup>72</sup> and Krenz et al<sup>73</sup>). 5-HD selectively inhibits the mitochondrial isoform with little effect on sarcK<sub>ATP</sub>.<sup>51</sup>

Several new compounds have also been reported to interact with mitoK<sub>ATP</sub>, including sildenafil,<sup>74</sup> levosimendan,<sup>75</sup> YM934,<sup>76</sup> and MCC-134.<sup>47</sup> MCC-134, an aprikahm analogue known to inhibit pancreatic K<sub>ATP</sub> channels but open smooth muscle K<sub>ATP</sub> channels, recently has been shown to inhibit mitoK<sub>ATP</sub> while activating cardiac sarcK<sub>ATP</sub>.<sup>47</sup> Importantly, this compound prevented diazoxide-mediated protection against simulated ischemia but did not confer protection by itself, supporting the argument that mitoK<sub>ATP</sub> rather than sarcK<sub>ATP</sub> channels were responsible.

Confirming the general hypothesis that enhanced mitochondrial K<sup>+</sup> influx induces cardioprotection, recent single-channel and K<sup>+</sup> uptake studies demonstrate that mitochondrial Ca<sup>2+</sup>-activated K<sup>+</sup> channels are also present on the cardiac mitochondrial inner membrane.<sup>77</sup> NS-1619, a K<sub>Ca</sub> opener, was protective against infarction, and this effect was inhibited by the K<sub>Ca</sub> blocker paxilline. These findings support the development of a new class of protective K<sup>+</sup> channel openers targeted to the mitochondria.

Caution should be used when extrapolating results obtained in isolated myocytes or mitochondria to the intact heart or animal. For example, although diazoxide is not very potent for activation of cardiomyocyte K<sub>ATP</sub> channels, it is quite effective at activating smooth muscle or pancreatic K<sub>ATP</sub> isoforms. Thus, the infarct-limiting effects of this drug theoretically could be due to nonmitochondrial targets; however, in most models, such extrinsic factors have not been found to mediate protection.

The drug selectivity of the target may also be altered under ischemic conditions, either as a result of altered high-energy phosphate content or changes in pH. D'Hahan et al<sup>78</sup> have demonstrated that the sensitivity of sarcK<sub>ATP</sub> channels to K<sup>+</sup> channel openers may increase under simulated ischemic conditions (ie, high ADP), and the selective inhibitor 5-HD was originally characterized by its ability to block sarcK<sub>ATP</sub> currents activated by high ADP (1 mmol/L), low pH of 6.6,<sup>79</sup> or metabolic inhibition.<sup>80</sup> Conclusions about selectivity need to be reexamined under many different conditions. How and when mitoK<sub>ATP</sub> channels open during



ischemia is still undetermined, and limited information is available about the effects of ischemia on the efficacy of mitoK<sub>ATP</sub> channel openers.

## Links to Ischemic Preconditioning/Cardioprotection

### mitoK<sub>ATP</sub>: A Common Effector of Diverse Stimuli

In the years since it was proposed that the opening of mitoK<sub>ATP</sub> channels may be involved in the mechanism of protection against ischemic injury, there has been an explosion of interest in this topic. MitoK<sub>ATP</sub> has been implicated in the cardioprotective effects of a variety of stimuli (see the online data supplement) and is involved in the mechanism of both early and delayed preconditioning. In many of these studies, the conclusion that mitoK<sub>ATP</sub> is involved is based solely on inhibition of protection by 5-HD. Given the caveats described above, a direct link between protection and the mitochondrial channel should be regarded as incomplete and could be strengthened by additional pharmacological evidence (eg, sensitivity to glibenclamide and HMR1098).

### Trigger Versus Effector?

In the absence of a direct reporter of mitoK<sub>ATP</sub> activation in the intact heart, it is unclear when or how mitoK<sub>ATP</sub> channels open during the course of ischemia and reperfusion. It is assumed that physiological activation occurs as a result of impaired metabolism or in response to a potentiating stimulus such as NO,<sup>44</sup> protein kinase C (PKC) activation,<sup>51</sup> ROS,<sup>29</sup> or intracellular signaling pathways. Pharmacological inhibition with 5-HD or glibenclamide, applied either during a preconditioning stimulus or during the long ischemia, has been the principal means of examining this question. It should be recognized that inhibition of mitoK<sub>ATP</sub> does not seem to influence the extent of infarction in the absence of preconditioning. Thus, if mitoK<sub>ATP</sub> opens during a long ischemia, it does not seem to be conferring any protection. However, it is now well accepted that blocking mitoK<sub>ATP</sub> during the preconditioning ischemia inhibits protection against infarction. It is presently disputed whether 5-HD or glibenclamide, applied just before the long ischemia, abrogates protection.<sup>23,81</sup> Although several studies have shown that 5-HD or glibenclamide, applied after the preconditioning stimulus but before the index ischemia, failed to block protection,<sup>82</sup> others have found these inhibitors effective within the same time window<sup>83–85</sup> or when applied days after a delayed preconditioning stimulus.<sup>86–88</sup> Thus, it appears likely that mitoK<sub>ATP</sub> opening is both a trigger and an effector of the recruitable protection associated with preconditioning but does not provide a background level of protection for the first episode of ischemia.

### Role of sarcK<sub>ATP</sub>

With the wealth of recent evidence implicating mitoK<sub>ATP</sub> in protection against ischemic injury, one is left wondering what role sarcK<sub>ATP</sub> channels play in the process. These channels are the premier sensors of the energy state of the myocyte and, when activated, shorten or interrupt the action potential.<sup>6,89</sup> In times of metabolic stress, decreasing cellular electrical excitability could protect cells against injury; however, the performance of the heart would suffer and the risk of arrhythmias may increase.<sup>90</sup> Before the mitoK<sub>ATP</sub> hypothesis, activation of sarcK<sub>ATP</sub> channels and action potential shortening was viewed as the possible mechanism underlying the anti-ischemic effects of K<sup>+</sup> channel openers.<sup>21,91,92</sup> This idea was additionally supported by inhibition of protection by glibenclamide, but whereas both glibenclamide and 5-HD blocked the anti-ischemic effect of cromakalim, only glibenclamide inhibited action potential shortening.<sup>83,93</sup> Several subsequent studies also showed a lack of correlation between the extent of action potential shortening and the reduction of infarct size.<sup>20,94–96</sup> Doses of bimakalim,<sup>94</sup> cromakalim,<sup>95</sup> or BMS-180448<sup>20</sup> that had little or no effect on the action potential could still significantly reduce infarct size, demonstrating that sarcK<sub>ATP</sub> channel-mediated effects on the action potential were not obligatory for cardioprotection.

The role of sarcK<sub>ATP</sub> in ischemia-reperfusion injury has been revisited using transgenic mice in which Kir6.2, the pore-forming subunit of cardiac K<sub>ATP</sub> channels,<sup>97,98</sup> has been knocked out. The principal effect of knocking out Kir6.2 in the mouse is that hearts go into contracture within 5 minutes of exposure to ischemia and do not recover function during reperfusion after 20 minutes of ischemia. This differs markedly from wild-type controls, which partially recover from this protocol, but resembles the effect of blocking sarcK<sub>ATP</sub> channels with HMR1098. These results suggest that sarcK<sub>ATP</sub> channels protect mouse hearts from the pathological effects of early ischemia. It should be noted that this conclusion does not seem to apply to larger animals, because HMR1098 has little or no effect on contractility during ischemia in dogs<sup>90</sup> or human myocardium<sup>99</sup> and does not alter infarct size in rats<sup>100</sup> or rabbits.<sup>101</sup> Thus, mice seem to be exquisitely sensitive to block of sarcK<sub>ATP</sub> channels during ischemia, perhaps because they are normally operating at a high level of contractile performance with little cardiac reserve<sup>102</sup> and depend on sarcK<sub>ATP</sub> channels to modulate the action potential and cellular Ca<sup>2+</sup> in times of stress.<sup>10</sup> Unfortunately, this hypersensitivity to ischemia in the knockout mouse precludes firm conclusions regarding the role of mitoK<sub>ATP</sub> in cardioprotection. It has been argued that a failure to observe a preconditioning response in Kir6.2 knockout mice suggests that sarcolemmal, rather than mitochondrial, K<sub>ATP</sub> channels are responsible for protection<sup>8</sup>; however, it is hard to justify this conclusion given that the ischemic damage in the absence of preconditioning is much more severe than in wild-type mice (pressure-rate product in knockout mice after ischemia/reperfusion was about half that in wild-type mice). On the other hand, these results do provide renewed motivation to define the relative contributions of sarcolemmal and mitochondrial K<sub>ATP</sub> channels to early ischemic injury, as addressed pharmacologically in several studies.<sup>72,103–105</sup>

### Mechanisms of Protection

Investigation of the mechanism of protection by mitochondrial K<sup>+</sup> channel opening has spawned several hypotheses, which, in general, are not mutually exclusive and probably all contribute to preservation of mitochondrial and contractile function. Much of the data in support of a particular mechanism have been gleaned from isolated cell or mitochondrial studies, making it difficult to determine whether the conclusions are relevant in the context of the whole heart. Nevertheless, progress is being made toward validation of the influence of mitoK<sub>ATP</sub> opening on injury in the intact myocardium, and consensus is developing regarding several key hypotheses. There is good evidence that mitoK<sub>ATP</sub> activation increases ROS production under normoxic conditions, decreases ROS production during reperfusion, blunts mitochondrial Ca<sup>2+</sup> accumulation during ischemia, and improves mitochondrial energy production after ischemia (Figure 2). Precisely how these effects are brought to bear is a subject of active investigation.

### Mitochondrial Swelling and Improved Oxidative Phosphorylation

As discussed above, swelling of the mitochondria occurs as a consequence of activation of the mitochondrial K<sup>+</sup> cycle<sup>38</sup> and has been extensively used to characterize the regulation and pharmacology of mitoK<sub>ATP</sub> in isolated mitochondria. Expansion of the mitochondrial matrix improves fatty acid oxidation, respiration, and ATP production.<sup>106</sup> Thus, this mechanism has become one of several leading hypotheses to explain protection.

It has been argued that physiological swelling of the mitochondria would be impossible to detect in intact myocardium,<sup>60</sup> because a 25% increase in volume would result in a mere 3% increase in mitochondrial diameter, too small to detect within the error limits of electron microscopy (pathological swelling, on the other hand, can be detected and is inhibited by mitoK<sub>ATP</sub> opening<sup>107</sup>). By isolating mitochondria from perfused hearts under normoxic, preconditioned, ischemic, or reperfused conditions with or without treatment with diazoxide or diazoxide plus 5-HD, Lim et al<sup>60</sup> measured mitochondrial matrix volume using <sup>3</sup>H<sub>2</sub>O and

[<sup>14</sup>C]sucrose and found that two 5-minute cycles of preconditioning or diazoxide (50 μmol/L) increased matrix volume by 58% and 88%, respectively. Neither increase was inhibited by 5-HD, and it was found that 5-HD by itself significantly increased matrix volume. Mitochondrial function was also assessed in this study and depended on which substrates were used. In mitochondria isolated under preischemic conditions, IPC increased (by 30% to 40%) state 3 respiration of 2-oxoglutarate or succinate but not that of ascorbate plus TMPD. In preischemic mitochondria, diazoxide inhibited state 3 respiration of 2-oxoglutarate (by 22%) and slightly inhibited that of succinate but had no effect on ascorbate plus TMPD, glutamate plus malate, or palmitoyl carnitine plus malate oxidation. For mitochondria isolated at the end of 30-minute ischemia, state 3 respiration was inhibited in all groups relative to preischemic controls, regardless of substrate. Diazoxide significantly improved end-ischemic mitochondrial respiration (55% higher), but this effect was not inhibited by 5-HD, which paradoxically increased respiration by itself. Although the beneficial effects of IPC or diazoxide on cardiac hemodynamics were inhibited by 5-HD, none of the effects on isolated mitochondria were. Partial recovery of mitochondrial function was achieved by reperfusion; however, IPC or diazoxide treatment provided no additional benefit. Changes in matrix volume or respiration of these mitochondria, therefore, were uncorrelated with protection.

A similar approach was used by Fryer et al<sup>100</sup> for mitochondria isolated from preconditioned or diazoxide-treated hearts. Mitochondria from hearts exposed to ischemia-reperfusion had markedly suppressed ATP synthesis compared with nonischemic controls, and the depression was reversed by IPC. 5-HD, but not HMR1098, eliminated this protective effect. In contrast, despite its infarct-sparing effect, diazoxide treatment did not reverse the decline in mitochondrial ATP synthesis.

In chronically hypoxic rabbit hearts, postischemic recovery of left ventricular function is enhanced compared with normoxic control animals, and this extra protection is attenuated by glibenclamide or 5-HD,<sup>108</sup> which had no effect on recovery in controls. Mitochondria isolated from the chronically hypoxic hearts had improved basal ATP synthesis rates. Bimkalim treatment decreased ATP synthesis in control mitochondria in a 5-HD-sensitive or glibenclamide-sensitive manner, but in mitochondria from hypoxic hearts, this opener had no effect, although the inhibitors slowed basal ATP synthesis rates. These data suggest that mitoK<sub>ATP</sub> channels are open in the chronically hypoxic animals, providing enhanced resistance to ischemia.

Several other studies have reported better preservation of ATP production by mitochondria treated with K<sup>+</sup> channel openers,<sup>53,70,109</sup> although, again, this effect depends on the choice of substrate and whether ΔΨ<sub>m</sub> is maintained. Kowaltowski et al<sup>53</sup> have argued that improved function is the result of expansion of the mitochondrial matrix space, which may preserve the mitochondrial inner and outer membrane contact sites in the optimal orientation for ADP import.<sup>110</sup> In contrast, in skinned cardiac trabeculae, Ovide-Bordeaux et al<sup>64</sup> reported no effects of diazoxide (100 μmol/L) on the K<sub>m</sub> for ADP stimulation of respiration by diazoxide.

It is apparent that although mitochondrial swelling is a likely consequence of mitoK<sub>ATP</sub> channel opening and has been demonstrated for isolated mitochondria, linking this effect directly to mitochondrial preservation after ischemia-reperfusion is a challenging task.

### Suppression of Ca<sup>2+</sup> Overload

Several recent studies have confirmed the hypothesis, initially suggestion by Liu et al,<sup>19</sup> that mitochondrial Ca<sup>2+</sup> accumulation during ischemia and reperfusion may be attenuated by mitoK<sub>ATP</sub> opening. Holmuhamedov et al<sup>111</sup> reported that diazoxide and pinacidil decreased the rate and magnitude of Ca<sup>2+</sup> uptake into isolated mitochondria and that, in intact cardiomyocytes, this effect was inhibited by 5-HD. They attributed the effect to partial

depolarization (10 to 24 mV) of  $\Delta\Psi_m$ , which was shown to occur in response to pinacidil (100  $\mu\text{mol/L}$ ), cromakalim (25  $\mu\text{mol/L}$ ), or levcromakalim (20  $\mu\text{mol/L}$ ).<sup>56</sup> This small depolarization was  $\text{K}^+$ -dependent and inhibited by a  $\text{K}^+$  channel blocker. This group also reported a second effect of  $\text{K}^+$  channel openers, release of  $\text{Ca}^{2+}$  and intermembrane components such as cytochrome *c* from  $\text{Ca}^{2+}$ -loaded mitochondria. The latter finding is at odds with the antiapoptotic effects of mitoK<sub>ATP</sub>. Murata et al<sup>52</sup> demonstrated that mitochondrial  $\text{Ca}^{2+}$  accumulation during simulated ischemia was attenuated by mitoK<sub>ATP</sub> opening (Figure 1), and this mechanism reduced the magnitude of PTP-opening on reperfusion. Wang et al,<sup>112</sup> using either ischemic preconditioning or diazoxide treatment, also showed that mitoK<sub>ATP</sub> activation blunts mitochondrial  $\text{Ca}^{2+}$  accumulation during ischemia in intact hearts. Recently, Korge et al<sup>113</sup> found that diazoxide protected isolated mitochondria from anoxic injury in a 5-HD-sensitive manner. This effect was mimicked by phorbol myristate, implying that mitoK<sub>ATP</sub> could also be activated by PKC. The protective effect was associated with strong depolarization of  $\Delta\Psi_m$  under anoxic conditions and a consequent decrease in mitochondrial  $\text{Ca}^{2+}$  loading, which prevented a mitochondrial permeability transition on reoxygenation. These data are consistent with other reports indicating that mitoK<sub>ATP</sub> opening prevents apoptosis,<sup>88</sup> presumably by inhibiting the activation of the PTP.<sup>107,114–120</sup> In neurons, the antiapoptotic effect of mitoK<sub>ATP</sub> opening and a reduction in cortical infarct size were attributed to a shift in the balance between apoptotic and antiapoptotic proteins; diazoxide treatment suppressed Bax translocation and cytochrome *c* release and enhanced Bcl2 levels.

### Increased/Decreased ROS Production

ROS play an essential, but double-edged, role in mitoK<sub>ATP</sub>-mediated protection. ROS generation is a trigger of the preconditioning response,<sup>121</sup> and protection by  $\text{K}^+$  channel openers can be inhibited by ROS scavengers.<sup>122</sup> Several reports have measured increased mitochondrial ROS generation in response to preconditioning or mitoK<sub>ATP</sub> activation.<sup>122–124</sup> This mechanism activates protective, PKC-dependent, signaling pathways.<sup>121</sup> Another physiologically important free radical, NO, produced from cytosolic and mitochondrial NO synthases, plays an important role as a trigger of both early and delayed preconditioning<sup>125–127</sup> and is known to potentiate mitoK<sub>ATP</sub> opening.<sup>44</sup>

In contrast, it is well-known that ROS produced on reperfusion after a long ischemia can cause irreversible cell injury.<sup>128</sup> This postischemic burst of ROS is suppressed by pretreatment with mitoK<sub>ATP</sub> openers.<sup>70,129</sup> Hence, the consensus is that mitoK<sub>ATP</sub> facilitates the production of protective ROS during preconditioning but decreases injurious postischemic ROS production. The mechanistic details of this effect remain to be determined.

### Molecular Structure?

Determination of the structure of the pore-forming protein underlying mitoK<sub>ATP</sub> conductance will resolve many of the controversial issues raised in this review. Unfortunately, several early leads have not produced definitive results. In general,  $\text{K}^+$  uniport activity has been observed when purified mitochondrial proteins in the molecular weight range of 50 to 60 kDa have been reconstituted into proteoliposomes,<sup>27,130</sup> and a 54-kDa protein was tentatively identified as a component of mitoK<sub>ATP</sub>.<sup>15</sup> These data, together with the similar pharmacology of known plasma membrane isoforms of K<sub>ATP</sub> (particularly the combination of SUR1/Kir6.1<sup>131</sup>), suggest that mitoK<sub>ATP</sub> might be composed of an inward rectifier potassium channel subunit (Kir) in association with a sulfonylurea receptor (SUR). Binding of Kir6.1 antibodies to a 51-kDa protein in a mitochondrial membrane preparation and intact mitochondria<sup>132</sup> fortified this hypothesis. In contrast, dominant-negative knockout of Kir6.1 or Kir6.2 using adenoviruses did not affect mitoK<sub>ATP</sub> responses in intact myocytes.<sup>45</sup> More recently, transgenic knockout of Kir6.1, a component of vascular K<sub>ATP</sub> channels, resulted in a phenotype of sudden cardiac death associated with vasospasm.<sup>9</sup> The response of these mice to ischemic

injury has not yet been determined, but mitoK<sub>ATP</sub> opening was apparently not disrupted by elimination of this K<sub>ATP</sub> subunit.<sup>9</sup>

Regarding the possibility that SUR is present in mitochondrial membranes, low-affinity sulfonylurea binding sites were identified in purified mitochondrial preparations,<sup>133,134</sup> and a putative 63-kD sulfonylurea binding protein was reported by Grover and Garlid<sup>21</sup>; however, the molecular structure of this binding site remains unresolved. A recent study by Munoz et al<sup>11</sup> tested whether elimination of SUR1 influenced ischemic preconditioning in the brain. Double carotid occlusion for 20 minutes protected hippocampal neurons from damage induced by a subsequent 40 minutes of ischemia, as did treatment with diazoxide. The extent of protection by ischemic preconditioning or diazoxide did not differ between WT and knockout mice and was inhibited by 5-HD. Nevertheless, 5-HD exacerbated neurodegeneration in the absence of preconditioning, and the authors concluded that this result was consistent with the involvement of mitoK<sub>ATP</sub> channels. SUR1 was apparently not a required component of mitoK<sub>ATP</sub>.

## Conclusions

The role of the mitochondria in ischemic preconditioning and cell survival during ischemia-reperfusion continues to be a fertile area of investigation. Several lines of evidence, using a variety of techniques, support the hypothesis that K<sup>+</sup>-selective channels are present and confer protection against ischemic and apoptotic injury. These include direct electrophysiological recordings, K<sup>+</sup> flux assays, mitochondrial swelling, flavoprotein oxidation, effects on mitochondrial bioenergetics, and pharmacological responses. Pharmacological evidence is not simply based on a single compound or class of compounds but on a plethora of structurally dissimilar K<sup>+</sup> channel openers and a lesser number of available inhibitors that have as their sole common factor a demonstrated effect on K<sup>+</sup> channel activity. Any one piece of evidence cannot definitively prove (or disprove) the existence of mitoK<sub>ATP</sub>, but taken together, the argument in favor is quite strong.

Overall, the links between mitoK<sub>ATP</sub> and cardioprotection are robust; however, when individual studies are considered, using a single K<sup>+</sup> channel opener or inhibitor to support the primary argument renders the interpretation vulnerable. This is exemplified by the widespread use of diazoxide alone as the activator of mitoK<sub>ATP</sub>. There is ample evidence that this compound, at concentrations that overlap but do not reproduce the dose-response relationship for ischemic protection, inhibits SDH activity, but it is highly unlikely that SDH inhibition *per se* is responsible for protection. However, there is still the possibility that a diazoxide interaction with SDH may be important for inducing the opening of mitoK<sub>ATP</sub>, a topic that will require additional investigation.

Consensus is building that mitoK<sub>ATP</sub> opening evokes a response involving several different protective mechanisms, including matrix swelling, ROS modulation, and effects on mitochondrial Ca<sup>2+</sup> homeostasis. A unifying hypothesis has yet to be elaborated because of the complexity of control of mitochondrial oxidative phosphorylation and the dangers of extrapolating *in vitro* data to the conditions mitochondria experience during ischemia and reperfusion in the intact heart.

Finding the pore-forming proteins underlying the protective effects of K<sup>+</sup> channel openers will undoubtedly resolve many outstanding questions and convince skeptics, but, in the meantime, novel and selective pharmacological agents are already being developed, facilitating the translation of basic knowledge about mitoK<sub>ATP</sub> into clinical treatments for ischemia-related and apoptotic diseases.

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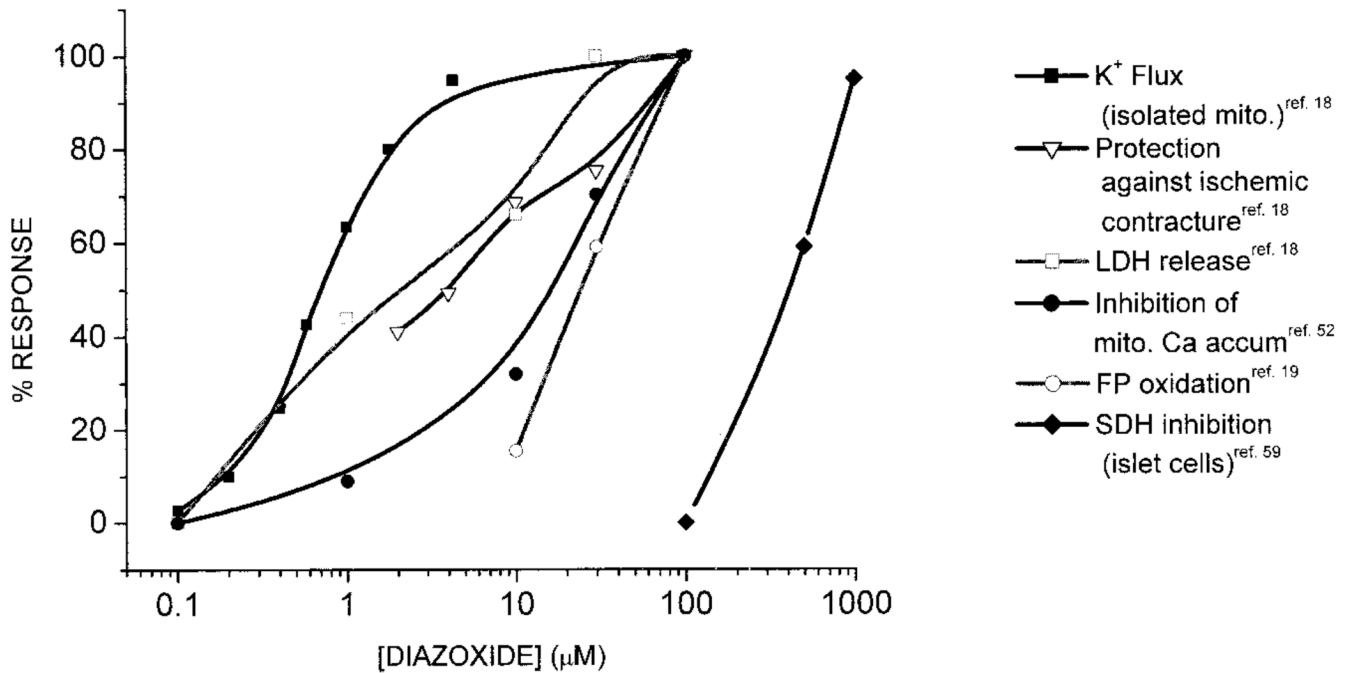
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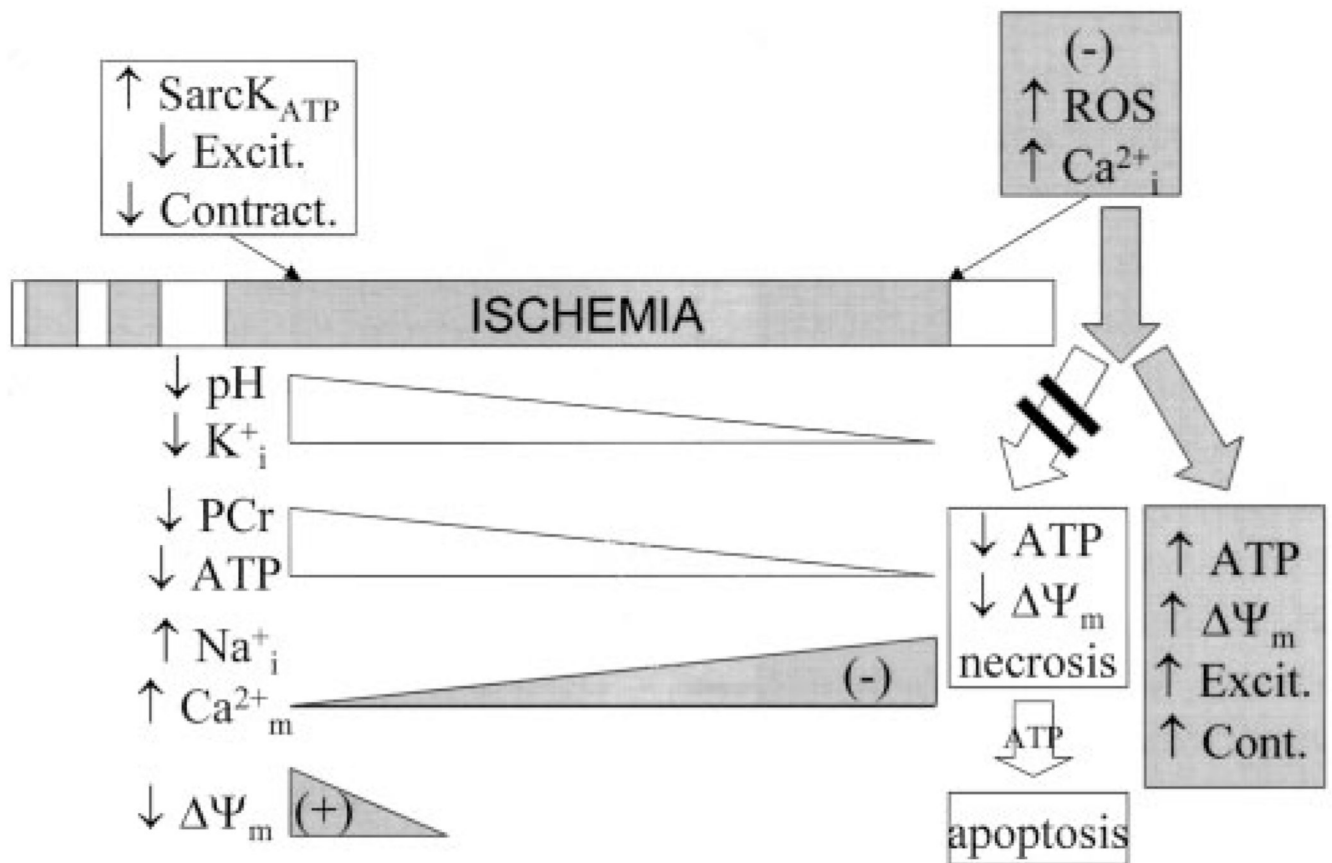
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**Figure 1.**

Concentration-dependent effects of diazoxide. Diazoxide potently activates mitoK<sub>ATP</sub> in isolated mitochondria and protects against ischemic contracture and LDH release with saturation near 50 μmol/L. Diazoxide inhibits mitochondrial Ca<sup>2+</sup> accumulation during simulated ischemia and oxidizes mitochondrial flavoproteins in isolated myocytes with similar potency ( $K_{1/2} \approx 25 \mu\text{mol/L}$ ). All of the above effects are inhibited by K<sub>ATP</sub> channel blockers. Depolarization of  $\Delta\psi_m$  attributed to SDH inhibition under normoxic conditions in pancreatic islet cells occurs in the 0.1- to 1-mmol/L concentration range and is not inhibited by K<sub>ATP</sub> channel blockers.



**Figure 2.** Mitigation of ischemic injury by preconditioning. Ischemic or pharmacological preconditioning diminishes accumulation of intracellular Na<sup>+</sup> and Ca<sup>2+</sup> ions during a long ischemia and blunts reperfusion-induced Ca<sup>2+</sup> overload and ROS generation. These actions favor improved mitochondrial energy generation and suppress necrotic and apoptotic cell death.

Selectivity of K<sup>+</sup> Channel Openers and Inhibitors Toward Cardiac Mitochondrial K<sub>ATP</sub> (mitoK<sub>ATP</sub>), Sarcolemmal K<sub>ATP</sub> (sarcK<sub>ATP</sub>), or Mitochondrial K<sub>Ca</sub> (mitoK<sub>Ca</sub>)

	mitoK <sub>ATP</sub>	sarcK <sub>ATP</sub>	mitoK <sub>Ca</sub>
<b>K<sup>+</sup> channel openers</b>			
Diazoxide	✓	<i>a</i>	
Nicorandil	✓	<i>b</i>	
BMS-180448	✓	<i>c</i>	
BMS-191095	✓		
Cromakalim	✓	✓	
EMD60480, 57970	✓	✓	
Pinacidil	✓	✓	
P-1060	✓	✓	
P-1075	<i>d</i>	✓	
Minoxidil sulfate	✓	✓	
KRN2391	✓	✓	
Sildenafil	✓	✓	
Isoflurane	✓	✓	
Aprikalim	✓	✓	
MCC-134		✓	
Levosimendan	✓	✓	
NS-1619			✓
<b>K<sup>+</sup> channel blockers</b>			
5-Hydroxydecanoate	✓	<i>e</i>	
MCC-134	✓		
Glibenclamide, glipizide, glisoxepide	✓	✓	
Glimepiride	<i>f</i>	✓	
HMR1098 (1883)	<i>g</i>	✓	
ChTx, IbTx			✓
Paxilline			✓

MitoK<sub>ATP</sub> activation has been reported for all K<sub>ATP</sub> channel openers listed except MCC-134. Mitochondrial selectivity reported for the openers diazoxide, BMS-191095, and BMS-180448 and for the inhibitors 5-hydroxydecanoate and MCC-134, whereas HMR1098 and glimepiride show selectivity for the sarcK<sub>ATP</sub> channel. Selectivity depends on the cell type examined and the conditions used:

<sup>a</sup> Diazoxide activates sarcK<sub>ATP</sub> at high concentrations or when ADP is high<sup>78</sup>;

<sup>b</sup> nicorandil activates sarcK<sub>ATP</sub> at high concentrations<sup>50</sup>;

<sup>c</sup> BMS-180448 is cardioprotective at concentrations that do not shorten the action potential<sup>20</sup>;

<sup>d</sup> P-1075 was selective for sarcK<sub>ATP</sub> in adult rabbit myocytes<sup>43</sup> but activated mitoK<sub>ATP</sub> in isolated rat mitochondria;

<sup>e</sup> 5-hydroxydecanoate inhibits sarcK<sub>ATP</sub> at low pH<sup>135</sup>;

<sup>f</sup> glimepiride blocks sarcK<sub>ATP</sub> but does not inhibit cardioprotection<sup>48</sup>;



<sup>g</sup>HMR1098 blocks sarcK<sub>A</sub>TP but does not block ischemic preconditioning<sup>101</sup>; however, HMR1098 blocked diazoxide-mediated protection.<sup>71</sup>