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Review

Mitochondrial potassium transport: the role of the mitochondrial ATP-sensitive K^+ channel in cardiac function and cardioprotection

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Abstract

Coronary artery disease and its sequelae—ischemia, myocardial infarction, and heart failure—are leading causes of morbidity and mortality in man. Considerable effort has been devoted toward improving functional recovery and reducing the extent of infarction after ischemic episodes. As a step in this direction, it was found that the heart was significantly protected against ischemia–reperfusion injury if it was first preconditioned by brief ischemia or by administering a potassium channel opener. Both of these preconditioning strategies were found to require opening of a K_{ATP} channel, and in 1997 we showed that this pivotal role was mediated by the mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}). This paper will review the evidence showing that opening mitoK_{ATP} is cardioprotective against ischemia–reperfusion injury and, moreover, that mitoK_{ATP} plays this role during all three phases of the natural history of ischemia–reperfusion injury preconditioning, ischemia, and reperfusion. We discuss two distinct mechanisms by which mitoK_{ATP} opening protects the heart-increased mitochondrial production of reactive oxygen species (ROS) during the preconditioning phase and regulation of intermembrane space (IMS) volume during the ischemic and reperfusion phases. It is likely that cardioprotection by ischemic preconditioning (IPC) and K_{ATP} channel openers (KCOs) arises from utilization of normal physiological processes. Accordingly, we summarize the results of new studies that focus on the role of mitoK_{ATP} in normal cardiomyocyte physiology. Here, we observe the same two mechanisms at work. In low-energy states, mitoK_{ATP} opening triggers increased mitochondrial ROS production, thereby amplifying a cell signaling pathway leading to gene transcription and cell growth. In high-energy states, mitoK_{ATP} opening prevents the matrix contraction that would otherwise occur during high rates of electron transport. MitoK_{ATP}-mediated volume regulation, in turn, prevents disruption of the structure–function of the IMS and facilitates efficient energy transfers between mitochondria and myofibrillar ATPases.

Keywords: Adenosine triphosphate; Mitochondrion; Heart; ATP-sensitive potassium channel; Ion channel gating; Myocardial infarction; Ischemic preconditioning; Myocardial

1. Introduction

The mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}) has been a major focus of our research, and we have reviewed its basic properties in the previous paper [1]. A surprising outcome of these studies was the discovery that mitoK_{ATP} is the receptor for K_{ATP} channel openers (KCOs) that protect the heart against ischemia–reperfusion injury [2]. This finding led to a flurry of scientific activity directed toward understanding the roles and mechanisms of mito-K_{ATP} in cardioprotection, and these will be reviewed here in some detail. Because cardioprotection employs normal physiological pathways and processes, we will also discuss the physiological roles of mitoK_{ATP} in heart. There has been some disagreement on the question of *when* mitoK_{ATP} opening is critical for cardioprotection, and we will review evidence that mitoK_{ATP} plays an important role during all three phases of preconditioning.

Opening mitoK_{ATP} appears to have two distinct consequences, depending on the underlying bioenergetic state. When the membrane potential ($\Delta\Psi$) is high, as in the normoxic, resting heart, K^+ influx via mitoK_{ATP} causes matrix alkalinization and a consequent rise in mitochondrial

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production of reactive oxygen species (ROS) [3]. When $\Delta\Psi$ is depressed, as occurs during ischemia or inotropy, mito-K_{ATP} opening adds a parallel K^+ conductance pathway to prevent contraction of the matrix and expansion of the intermembrane space (IMS) [4,5].

This review will discuss hypotheses for the mechanisms by which mitoK_{ATP} opening protects the heart. Despite its narrow scope, this review cannot do justice to the many excellent contributions that have been made in this field of research. Other reviews are available that cover other aspects of this rapidly growing field or present a different perspective [6–14].

2. Protecting the heart against ischemia–reperfusion injury

Preconditioning describes an experimental treatment of the normal heart that reduces myocardial damage from a subsequent ischemia–reperfusion event. Preconditioning involves a series of cell signaling events that take place prior to ischemia and that modify one or more cellular components that play a role as end-effectors of protection. Postconditioning describes treatments that are effective after reperfusion of the ischemic heart.

2.1. Modes of preconditioning and postconditioning

At least seven different modes of cardioprotection have been described. (1) Ischemic preconditioning (IPC) describes the phenomenon in which a brief period of ischemia protects the myocardium from damage induced by a subsequent, longer period of ischemia [15]. (2) Calcium preconditioning (CPC) describes the phenomenon in which a transient increase in intracellular calcium protects the heart against subsequent global ischemia [16–18]. (3) KCO preconditioning describes the phenomenon in which administration of a KCO prior to ischemia protects the heart [19]. The protection afforded by preconditioning is of brief duration, about 1 h. All three modes of preconditioning inhibit development of ischemic contracture, improve post-ischemic functional recovery after reperfusion, and reduce necrosis and apoptosis. Each mode of preconditioning mobilizes the cardioprotective signaling pathway discussed in Section 4.1.

Four additional modes of cardioprotection have been described: (4) Delayed preconditioning arises as a secondary event from the first three modes of preconditioning. Thus, acute protection arises after IPC and disappears after 1–2 h. Twelve hours later, with no further intervention, cardioprotection begins again, reaching a maximum at 24 h and lasting until 72 h after the preconditioning ischemia [20–22]. (5) Adaptive preconditioning (APC) is a long-lasting cardioprotection arising from chronic hypoxia, including the hypoxia of gestation and high altitude [23]. (6) Na^+/H^+ exchange inhibition by agents such as cariporide is also cardioprotective [24,25]. (7) Ischemic post-conditioning is a new finding reported by Zhao et al. [26]. The heart undergoes ischemia and reperfusion without pretreatment. Beginning shortly after reperfusion, the heart is subjected to three cycles of 30 s ischemia and 30 s reperfusion. Remarkably, these hearts were protected to the same extent with ischemic post-conditioning as they were with IPC.

2.2. Cardioprotection by KCOs

Gross et al. [27] observed cardioprotection by nicorandil before this agent was known to be a KCO. Grover et al. [19] were the first to evaluate KCOs for their ability to protect the heart against ischemia–reperfusion injury. They found cromakalim and pinacidil to be protective in the perfused rat heart model [19], and subsequently showed that other KCOs, including bimakalim, aprikalim, and P-1075, also protected isolated rat hearts [28–30]. Investigators have demonstrated cardioprotection by KCOs in isolated hearts from rats, rabbits, ferrets, and guinea pigs [31–36]. Armstrong et al. [37] found that KCOs protect isolated rabbit cardiomyocytes undergoing simulated ischemia, indicating that the effects are mediated at the level of the cardiomyocyte. Speechly-Dick et al. [38] and Puddu et al. [39] showed that cromakalim and bimakalim protected hypoxic human atrial trabecula, suggesting that K_{ATP} channels are a clinically relevant pharmacologic target. The protective effects of KCOs are abolished by K_{ATP} channel blockers.

2.3. K_{ATP} channels are required for protection by IPC and CPC

Preconditioning with KCOs mimics IPC, implicating K_{ATP} channels in the mechanism of protection by IPC. This was shown directly by Gross et al. [40,41], who were the first to demonstrate that the K_{ATP} channel blockers glibenclamide and 5-hydroxydecanoate (5-HD) block the cardioprotection of IPC. Further linking these two modes of protection, Yao and Gross [42] found that a subthreshold dose of bimakalim protected ischemic tissue when combined with a subthreshold ischemic-preconditioning protocol. K_{ATP} blockers have been shown to abolish IPC in rabbits, rats, pigs, and man [6]. Finally, K_{ATP} channels were shown to be involved in CPC by Kouchi et al. [43], who showed that protection by CPC is blocked by glibenclamide.

These studies made it clear that K_{ATP} channels play a central role in all three modes of acute preconditioning. It was initially believed that opening of sarcolemmal K_{ATP} channels (sarcK_{ATP}) was protective because it shortened action potential duration (APD), thereby reducing Ca^{2+} entry to the cytosol. Several features then arose that raised doubts about this mechanism. In particular, it was shown that cardioprotection was preserved in conditions where there was no APD shortening [44,45] (for reviews, see Refs. [7,10]).

3. MitoK_{ATP} opening in cardioprotection against ischemia–reperfusion injury

3.1. The central role of mitoK_{ATP} in cardioprotection

Until 1996, it was universally assumed that cardioprotection by IPC, CPC, and KCOs was afforded by opening sarcK_{ATP}. Our laboratory reported three findings that shifted the focus from sarcK_{ATP} to mitoK_{ATP} as the key receptor for cardioprotection. First, we showed that various KCOs open mitoK_{ATP} within their cardioprotective concentration. We suggested that mitoK_{ATP}, rather than sarcK_{ATP}, may be the key player in cardioprotection [46]. Second, we compared the sensitivities of K_{ATP} channels from cardiac sarcolemma and cardiac mitochondria and showed that cardiac sarcK_{ATP} is essentially insensitive to diazoxide and 5-HD, whereas mitoK_{ATP} is sensitive to both agents [46,47]. Fig. 1 contains two sets of dose–response curves for cromakalim and diazoxide. These agents open mitoK_{ATP} reconstituted from bovine heart mitochondria with $K_{1/2}$ values of 1.6 and 0.8 μM, respectively (Fig. 1A), and they opened cellK_{ATP} reconstituted from beef heart sarcolemmal membranes with $K_{1/2}$ values of 18 and 840 μM, respectively (Fig. 1B). It should be noted that 800 μM diazoxide is cytotoxic, because it both uncouples and inhibits mitochondrial respiration at such high concentrations [4,48].

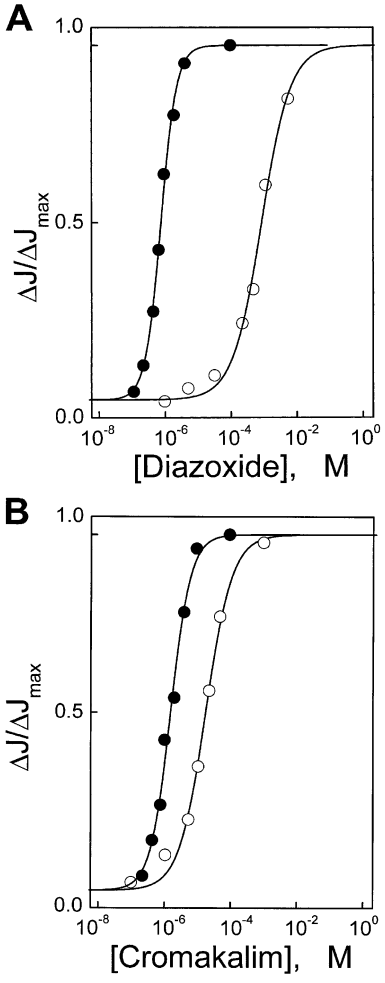

Fig. 1. Comparative potencies of cromakalim and diazoxide on K_{ATP} channels from cardiac mitochondria and sarcolemma. The relative flux, $\Delta J/\Delta J_{max}$, is plotted versus concentrations of diazoxide (Panel A) and cromakalim (Panel B). Each panel includes data from mitochondrial (●) and sarcolemmal (○) K_{ATP} channels from bovine heart. The K_{ATP} channels were extracted from their respective membranes using Triton-X100, partially purified, and reconstituted into liposomes. $K_{1/2}$ values for diazoxide were 0.8 and 840 μM for mitochondrial and sarcolemmal channels, respectively. $K_{1/2}$ values for cromakalim were 1.6 and 18 μM for mitochondrial and sarcolemmal channels, respectively. Data are taken from Garlid et al. [2].

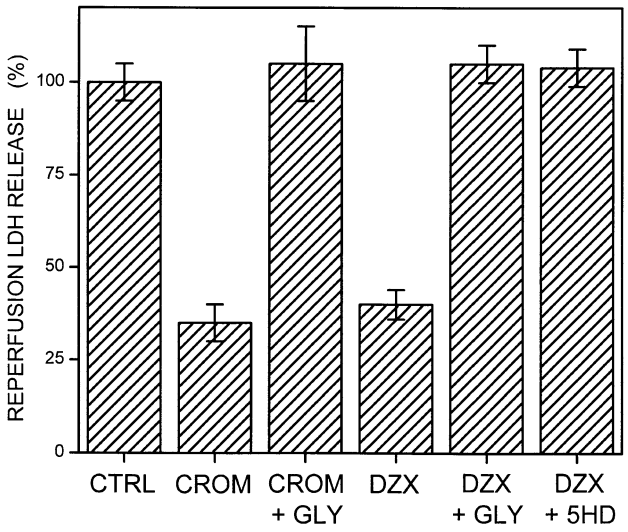

Fig. 2. Cardioprotection against ischemia–reperfusion injury by cromakalim and diazoxide. Shown are values of cumulative lactate dehydrogenase (LDH) release from hearts subjected to 25 min of global ischemia and 30 min of reperfusion. Values are normalized to LDH released in untreated hearts. LDH release is taken as a measure of cell damage due to ischemia–reperfusion. Data are taken from Garlid et al. [2].

Our third finding was based on an exploitation of these pharmacological differences in a study of cardiac ischemia–reperfusion injury [2]. We found that diazoxide and cromakalim were equally effective in protecting the heart from ischemia–reperfusion injury, as summarized in Fig. 2. Importantly, protection by the nonselective drug, cromakalim, was accompanied by a marked APD shortening, whereas the mitoK_{ATP}-selective agent, diazoxide, caused no APD shortening, showing that diazoxide was not acting via sarcK_{ATP}. Protection by both drugs was blocked by glibenclamide and 5-HD. From this set of results, we concluded that mitoK_{ATP} is the receptor for cardioprotective K_{ATP} openers, a hypothesis that is now widely accepted [9,49–52].

3.2. How is mitoK_{ATP} opened by the endogenous signaling pathways of IPC and CPC?

Cardioprotection by IPC and CPC is blocked by inhibitors of mitoK_{ATP}, such as 5-HD and glibenclamide. This indicates not only that mitoK_{ATP} is involved in these modes

of protection, but also that mitoK$_{ATP}$ must be opened by *endogenous* mechanisms. Since many protein kinases are activated during IPC, we hypothesize that mitoK$_{ATP}$ is modified in vivo by phosphorylation, leading to a sustained open state [1].

3.3. Is mitoK$_{ATP}$ involved in all modes of cardioprotection?

We have seen that mitoK$_{ATP}$ plays the key role in cardioprotection by all three modes of acute preconditioning—IPC, CPC, and KCO cardioprotection. What is known about the other four modes of protection described in Section 2.1? The evidence is reasonably clear that mitoK$_{ATP}$ is involved in delayed preconditioning [53,54] and APC [55,56]. Miura et al. [57] have concluded that mitoK$_{ATP}$ also plays a role in cardioprotection by Na^+/H^+ exchange inhibition. Experiments have not yet been performed to determine whether or not mitoK$_{ATP}$ is involved in ischemic post-conditioning.

3.4. During which phase is mitoK$_{ATP}$ opening crucial for cardioprotection?

Experimental ischemia–reperfusion studies can be divided into three phases, as shown diagrammatically in Fig. 3. The *preconditioning phase* begins 15–30 min prior to the test ischemia. The *ischemic* phase is typically of 30 min duration in the perfused rat heart. Increased time to ischemic contracture, which indicates significant conservation of ATP in the protected heart, is the first indication of protection during ischemia. Most assays for protection are performed during the *reperfusion phase*. Recovery of contractile function and reduced enzyme release due to sarcolemmal damage are detected early, and infarct size is commonly measured several hours after reperfusion. Additional assays include recovery of high-energy phosphates [29,58] and outer mitochondrial membrane permeability to cytochrome *c* and ADP, performed on saponin-skinned fibers [5,59].

In which of these phases does mitoK$_{ATP}$ opening play its protective role? There is good evidence that mitoK$_{ATP}$ is an intrinsic component of the signaling pathways that are activated during the preconditioning phase and that mitoK$_{ATP}$ opening also plays a critical role during the ischemic phase. We propose that mitoK$_{ATP}$ opening may also be important during the initial stages of reperfusion. Thus, mitoK$_{ATP}$ opening exerts effects that are temporally distinct as a trigger of cardioprotection (during the preconditioning phase) and as an end effector of cardioprotection (during the ischemic and reperfusion phases). As discussed later, these effects are also mechanistically distinct.

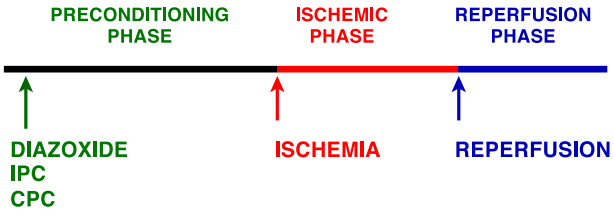

Fig. 3. The three phases of ischemia–reperfusion studies. MitoK$_{ATP}$ is proposed to play distinct roles in each phase of ischemia–reperfusion—preconditioning, ischemia, and reperfusion (see text).

3.4.1. MitoK$_{ATP}$ opening as a trigger of cardioprotection during the preconditioning phase

The role of mitoK$_{ATP}$ opening in the preconditioning signaling pathway is to increase mitochondrial production of ROS [3]. The finding that mitoK$_{ATP}$ opening leads to a moderate increase in mitochondrial ROS production in cardiomyocytes [3,60] has been confirmed in vascular smooth muscle cells [61] and perfused hearts [62,63]. It is entirely consistent with earlier studies showing that scavenging ROS blocks cardioprotection [51,64–66]. As discussed in Section 4.1.3., the role of ROS is to activate kinases within the cardioprotective signaling pathway.

3.4.2. MitoK$_{ATP}$ opening as an end-effector of cardioprotection during the ischemic phase

The target of cell signaling is an end effector of cardioprotection, one or more cellular elements, or processes that are altered to protect the heart during ischemia and reperfusion. We proposed that mitoK$_{ATP}$ is the end effector of cardioprotection [2], and many studies show that mitoK$_{ATP}$ is required to be open during the ischemic phase [52,67–70]. Results of a study by Tsuchida et al. [69] are particularly convincing in this regard. They show that diazoxide reduced infarct size even when administered after the onset of ischemia, provided that diazoxide was added before the development of necrosis.

In an important paper, Pain et al. [51] showed that mitoK$_{ATP}$ opening triggers the preconditioned state and that protection persisted despite wash-out of diazoxide. 5-HD blocked protection when added prior to 5 min of preconditioning ischemia or prior to diazoxide; however, it did not block protection when added 5 min prior to the index ischemia. The results of these studies led the authors to conclude that mitoK$_{ATP}$ acts to trigger protection but is not an end effector of protection. This conclusion conflicts with those of the preceding paragraph. However, there are two possible explanations for the failure of 5-HD to block protection when added after preconditioning ischemia or diazoxide. First, when mitoK$_{ATP}$ is opened by endogenous mechanisms, the open state may be less susceptible to inhibition by 5-HD. We have previously reported state-dependent inhibition of mitoK$_{ATP}$ by glibenclamide and 5-HD [47]. Indeed, Wang et al. [67] found that 5-HD did block protection when added 5 min prior to ischemia, but that this blockade required a higher dose. Secondly, there may be a pharmacokinetic limitation—5 min may not be sufficient for 5-HD to reach its intracellular target. For example, Kouchi et al. [43] found in thoracotomized rabbits that glibenclamide blocked the protection of IPC and CPC when administered 30 min, but not 5 min, prior to the preconditioning step. We have investigated this issue in

perfused rat hearts and find that the protection by four 5-min ischemic periods, each followed by 5 min reperfusion, was blocked by 300 μM 5-HD added 10 min prior to the test ischemia. Accordingly, we conclude that mitoK$_{ATP}$ is an end effector of cardioprotection.

The identity of the proximate end effect that causes ischemia–reperfusion injury is not entirely clear. One candidate is the mitochondrial permeability transition (MPT) [71], which opens upon reperfusion [72,73] and subsequently triggers damage to the sarcolemmal membrane [74] and causes cell death [75]. MPT is not a specific channel, but rather the consequence of oxidative damage to membrane proteins, notably the adenine nucleotide transporter. Although this field is controversial, the important work of Vercesi et al. (reviewed in Ref. [76]), indicates the following: (1) The primary role of *matrix* Ca^{2+} is to stimulate ROS production upon reperfusion. (2) Ca^{2+} cannot open MPT unless thiol oxidizing reagents (ROS) are present. (3) *Cytosolic* Ca^{2+} (which is elevated after ischemia–reperfusion) may play an additional role in promoting ROS oxidation of adenine nucleotide translocase (ANT) with formation of MPT. These conclusions imply that *mitochondrial protection* during ischemia is critical for cardioprotection, because MPT does not open in normal or protected mitochondria.

There is considerable evidence for preconditioning-induced mitochondrial protection during ischemia. IPC and KCO protection reduce the rate of ATP loss during ischemia in vivo [29,58,77]. Mitochondrial $\Delta\Psi$ is lower in protected hearts during ischemia, leading to reduced Ca^{2+} accumulation [78,79], which has been identified by several laboratories as being important for cardioprotection [49,80].

Perhaps the most important factor conferring protection is preservation of cellular adenine nucleotides, because this assures sufficient ADP for an appropriate response during reperfusion and avoidance of the compromised state leading to increased ROS. Indeed, Jennings et al. [81] have shown that adenine nucleotides are rapidly degraded during ischemia and that IPC retards the rate of degradation. As discussed in Section 4.2., we hypothesize that an open mitoK$_{ATP}$ preserves the structure–function of the IMS and maintains the low permeability of the outer membrane to adenine nucleotides, thereby preserving ADP for phosphorylation upon reperfusion.

3.4.3. MitoK$_{ATP}$ opening as an end-effector of cardioprotection during the reperfusion phase

Reoxygenation of the ischemic heart is associated with a burst of ROS that occurs within moments of reperfusion [82]. Bolli et al. [83] showed that the intensity of ROS generation is related to the severity of ischemia, that ROS contribute to the persistent contractile dysfunction (myocardial stunning) observed after brief ischemia, and that antioxidants given at reperfusion have a beneficial effect on stunning. In a nice study on chick cardiomyocytes undergoing hypoxia, Vanden Hoek et al. [84] also demonstrated a transient ROS burst upon reoxygenation. The ROS burst was blocked, and the cells were protected, by IPC. These protective effects were abolished by 5-HD. The ROS burst was also blocked by antioxidants or pinacidil given at the time of reperfusion. These findings indicate that IPC, which itself causes a moderate ROS increase during the preconditioning hypoxia, protects cells by reducing ROS generation at reperfusion, and that this involves a signaling pathway that includes mitoK$_{ATP}$. Vanden Hoek et al. [84] point out that the pharmacokinetics of pinacidil are more favorable in the cell culture system that they used, which may account in part for the protection observed when the drug was added during reperfusion. However, Mizumura et al. [85] observed reduction in infarct size when bimakalim was administered just before reperfusion, and Toyoda et al. [70] found that 5-HD added at reperfusion caused an increase in infarct size in IPC-treated hearts. These results suggest that mitoK$_{ATP}$ must be open during the reperfusion phase in order to achieve full protection. As discussed in Section 4.3., we hypothesize that an open mitoK$_{ATP}$ during reperfusion facilitates rapid energy conversion to phosphocreatine (PCr). Under these conditions, mitochondria will not produce a burst of ROS upon reperfusion, and the irreversible opening of the MPT will not occur.

3.5. The role of mitoK$_{ATP}$ opening in apoptosis

Apoptosis is a biochemical process that results in cell death. Many apoptotic processes originate in mitochondria, including the release of cytochrome *c*, which is a caspase-activating cofactor. Pro- and antiapoptotic members of the Bcl-2 family regulate cytochrome *c* release by unknown mechanisms (for excellent reviews, see Refs. [86–88]).

Anversa et al. [89] found that apoptosis accounted for a majority of cell death after myocardial infarction. Kang et al. [90] studied cell death in cardiomyocytes subjected to hypoxia and reoxygenation and found that necrotic cell death predominated during the hypoxic phase, whereas apoptosis predominated during the reoxygenation phase. Bcl-2 overexpression blocked the cytochrome *c* release and activation of caspase-3 and -9. Our laboratory has shown in isolated perfused rat hearts that ischemia–reperfusion causes an increase in outer membrane cytochrome *c* permeability without accompanying uncoupling. Both IPC and diazoxide preconditioning prevented the increase in cytochrome *c* permeability, suggesting a role for mitoK$_{ATP}$ in preventing apoptosis associated with ischemia–reperfusion injury [5,59].

Akao et al. [91] carried out a study in which cultured neonatal rat cardiomyocytes were exposed for 16 h to 200 μM H_2O_2. The cells exhibited apotosis by the TUNEL assay, and caspase-3 activation reached a peak in 8 h. Pretreatment with diazoxide caused these effects to be attenuated by about 50%, and diazoxide protection was abolished by 5-HD. Notably, the authors also found that diazoxide *decreased* the number of cells undergoing mitochondrial depolarization.

An interesting possibility is that protection against apoptosis is provided by the cardioprotective signaling cascade, which causes activation of many kinases. Outer membrane permeability to cyt *c* is controlled by Bcl-2 and Bax homologs [87,88]. It has been found in some cell types that cyt *c* permeability is prevented by phosphorylation of pro-apoptotic Bid [92].

3.6. The role of mitoK$_{ATP}$ opening in ischemic protection in other tissues

Preconditioning protects a variety of tissues against ischemia–reperfusion injury. Pang et al. [93,94] showed that K_{ATP} channels are involved in IPC of skeletal muscle. Several studies have shown that KCOs are protective in central nervous system models of ischemia–reperfusion [95–97]. MitoK_{ATP} may play a role in brain protection. We have shown that brain mitochondria contain mitoK_{ATP} and that its properties are similar to those of heart mitoK_{ATP} [98]. In an important study, Domoki et al. [97] showed that diazoxide protected against neuronal ischemia–reperfusion injury in neonatal pigs and that the protection was blocked by 5-HD.

4. Mechanisms of cardioprotection induced by mitoK$_{ATP}$ opening

Our views on the mechanisms by which mitoK_{ATP} opening protects the heart derive in large part from experiments studying the bioenergetic consequences of mitoK_{ATP} opening in isolated rat heart mitochondria, as discussed in Ref. [1]. MitoK_{ATP} opening causes a modest increase in K^+ influx that will have two different effects, depending on the underlying bioenergetic state of the cardiomyocyte:

(a) In the resting cell, mitochondrial $\Delta\Psi$ is high, and mitoK_{ATP} opening will cause net K^+ influx into the matrix with consequent swelling and alkalinization of the matrix. The net uptake of K^+ salts leads to increased ROS production.
(b) In the ischemic cell, mitochondria depolarize due to anoxia. In the reperfused cell, mitochondria also depolarize, due to high rates of electron transport. When $\Delta\Psi$ is low, mitoK_{ATP} opening adds a parallel K^+ conductance that *counteracts* the decrease in K^+ influx and matrix contraction that would otherwise occur [1,4,98]. MitoK_{ATP} opening therefore maintains constant volume of the mitochondrial matrix and IMS.

4.1. Preconditioning phase—the mechanism by which mitoK$_{ATP}$ opening triggers cardioprotection

4.1.1. Mitochondrial ROS production

Excessive levels of ROS, including superoxide radical, hydroxyl radical, and H_2O_2, are associated with cell damage, including ischemia–reperfusion injury and stunning. It is now clear, however, that moderate increases in ROS play an important second messenger role in a variety of signaling pathways essential for cell physiology [99–105]. In particular, elevated ROS levels in the cardiomyocyte are essential for activating the signaling pathways leading to cardioprotection against ischemia–reperfusion injury [51,66].

ROS levels in the cell are the result of a balance between scavenging enzymes and production. A recent paper identifies the flavin mononucleotide group (FMN) of Complex I as the physiologically important site of superoxide formation in brain mitochondria [106]; however, it appears that mitochondrial ROS originates primarily at Complex III from reaction of Coenzyme Q intermediates with molecular oxygen in heart mitochondria [107,108]. In either case, uncoupling of mitochondria will lead to decreased ROS production and lower ROS levels [108].

4.1.2. A proposed mechanism for increased mitochondrial ROS production

When mitoK_{ATP} is opened during the preconditioning phase, there ensues an increase in mitochondrial ROS (mitoROS) production. The bioenergetic setting is the quiescent heart, in which $\Delta\Psi$ is high. Under these conditions, mitoK_{ATP} opening will cause net K^+ influx, matrix swelling, and matrix alkalinization [1]. Matrix swelling appears not to be consequential in this phase, because it is modest in extent and does not lead to outer membrane rupture or uncoupling [4]. K^+ uptake is driven by electron transport-driven H^+ ejection, creating a gradient for uptake of Pi on the electroneutral Pi–H^+ symporter, the most rapid anion transporter in mitochondria. Pi uptake will be less than K^+ uptake, because Pi is present in much lower concentrations than K^+. For this reason, matrix pH always increases when matrix volume increases due to uptake of K^+ and Pi [1]. It thus appears that a moderate increase in matrix pH and ROS production are the primary consequences of mitoK_{ATP} opening in the quiescent heart.

Unpublished experiments on isolated heart mitochondria indicate that matrix alkalinization and ROS production are causally related. For example, adding 20 mM ammonium chloride to the medium, which alkalinizes the matrix due to nonionic equilibration of ammonia, results in a 50% increase in mitoROS production. It follows that net K^+ influx, which also alkalinizes the matrix, should increase mitoROS. When respiring mitochondria were titrated with low concentrations of valinomycin, ROS production increased with a peak activity at about 0.8 pmol Val/mg. At higher concentrations, ROS production decreased due to uncoupling. The amount of K^+ flux catalyzed by this level of valinomycin is very small, as demonstrated by the observation that respiration was stimulated to a minor extent, about 3–5 ng atom O/min mg. Indeed, we found that the K^+ influx induced by 0.8 pmol/mg valinomycin is approximately the same as that induced by mitoK_{ATP} opening. This demonstration was based on a comparison of the effects of

valinomycin and diazoxide on respiration and on steady state matrix volume. These preliminary data are consistent with the view that a modest K^+ influx alkalinizes the matrix, which in turn causes increased ROS production.

4.1.3. The preconditioning signal amplification loop

A cardioprotective signaling pathway is mobilized by all three modes of preconditioning and has been extensively studied and reviewed by Downey et al. [109–111]. Preconditioning involves three major components: activation of membrane receptors and protein kinases, opening of mito-K_{ATP}, and subsequent release of ROS from mitochondria. For example, the signaling pathway of IPC begins with the activation of G protein-coupled receptors induced by the agonists that are released locally during the preconditioning ischemia, including adenosine, acetylcholine, bradykinin, catecholamines, angiotensin II, and opioids [66,109]. Receptor activation triggers a signaling cascade, resulting in stimulation of a variety of protein kinases such as protein tyrosine kinases (PTK) [112], protein kinase C (PKC) [113–115], and mitogen-activated protein kinases (MAPK) [116].

PKC plays an important role in both IPC [113–115] and CPC [16–18]. There is evidence that a threshold quantity of various stimuli is necessary in order to activate PKC to a level sufficient for cardioprotection [117]. Studies using PKC isoform-specific agonist and antagonist peptides provide convincing evidence that ϵPKC activation is required for cardioprotection [118,119]. Interestingly, Chen et al. [120] propose that ϵPKC activation confers protection, whereas δPKC activation blocks protection.

Downey et al. [121,122] have focused on the important question of the mechanism responsible for the memory exhibited by IPC—that is, what holds the preconditioning signal for about 1 h between the short ischemia and the index ischemia? Inhibitors of PKC and PTK were found to block protection only when they are present during the index ischemia, suggesting that the memory occurs prior to protein phosphorylation. This group has suggested that the memory resides in the translocation of PKC to its RACK (Receptor of Activated C Kinase), that this translocation occurs as a consequence of the preconditioning ischemia, and that kinase activity is initiated during the index ischemia [123].

Involvement of PTKs including Src in IPC has also been well documented (16–18). Src family kinases are 52–62 kDa membrane-associated nonreceptor tyrosine kinases (RTKs) that are key regulators of various signal transduction pathways [124–127]. Src, for example, plays an important role not only in the signal transduction pathways of growth factors, but also in coupling stimulation of other receptors and cellular stress to RTKs [124–127]. Furthermore, there is strong evidence that activation of Src contributes significantly to hypertrophic growth in cardiac myocytes [128,129]. Recently, Ping et al. [130–132] have demonstrated that IPC activated Src in the rabbit heart and that inhibition of Src blocked IPC. Significantly, they showed that IPC as well as nitric-oxide-induced late preconditioning induced the formation of a signaling module consisting of ϵPKC, Src, and several other proteins. Activation of ϵPKC was essential for the formation of this signaling module and activation of Src [132]. These studies not only confirmed the role of Src in IPC, but also linked Src to PKC activation. However, it is still not clear how extracellular stimuli transduce the signal to the PKC–Src complex, and how the PKC–Src signal is transmitted to mitoK_{ATP}. Korge et al. [133] report indirect evidence that phorbol 12-myristate 13-acetate, a PKC activator, opens mitoK_{ATP} in isolated rat heart mitochondria.

Na/K-ATPase hydrolyzes ATP to maintain the transmembrane gradients of Na^+ and K^+ found in most mammalian cells and is inhibited specifically by cardiac glycosides such as ouabain [134–136]. Recently, we showed that in addition to pumping ions, the Na/K-ATPase interacts with neighboring membrane proteins and organized cytosolic cascades of signaling complexes to send messages to various intracellular organelles [137]. The signaling pathways that are rapidly elicited by the interaction of ouabain with the enzyme were initiated by Src binding to the Na/K-ATPase, which resulted in Src activation and transactivation of the EGFR. The downstream events related to these signals include activation of Shc, phospholipase C-gamma/PKC isozymes, Ras/p42/44 MAPKs, p38, increases in intracellular concentrations of ROS, $[Ca^{2+}]_i$ and contractility, induction of some of the early response protooncogenes, and activation of transcription factors AP-1 and NF-κB [60,105,138–141]. Interplays among these pathways eventually result in changes in the expression of a number of cardiac growth-related genes and stimulation of protein synthesis and myocyte hypertrophy [137].

Interestingly, as shown in Fig. 4, ouabain-induced ROS production originated from mitochondria and required opening of mitochondrial K_{ATP} since preincubation of cardiac myocytes with 5-HD blocked ouabain-induced ROS production [60,140]. In addition, inhibition of Src by either herbimycin A or PP2 completely blocked ouabain-induced

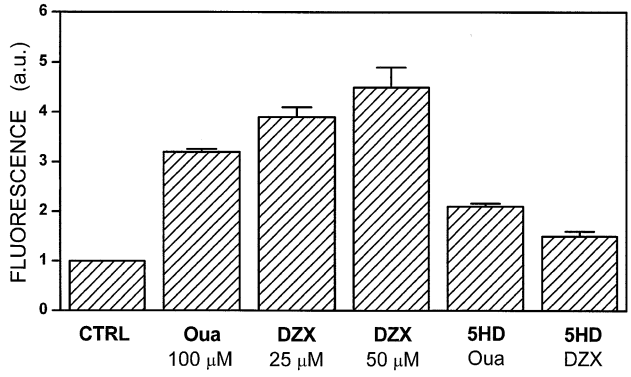

Fig. 4. Effects of ouabain (Oua), diazoxide (DZX), and 5-HD on ROS production in cardiac myocytes. Ros production in isolated cardiomyocytes was measured from the fluorescence of intracellular DCF (2′,7′-dichlorofluorescein). See also Tian et al. [60].

ROS production. Moreover, ouabain-induced ROS production also required Ras activation [140]. Because ouabain activated Raf in cardiac myocytes, we propose that extracellular ouabain may transmit its signal to mitoK$_{ATP}$ through the Src/Ras/Raf cascade (as diagrammed in Fig. 5) since Raf isoforms have been localized in mitochondria [142,143], where they play a role in regulation of apoptosis [144]. Because the above ouabain-activated pathways bear a strong resemblance to those activated during IPC, we recently tested if ouabain can protect cardiac myocytes as well as perfused heart from ischemia-induced cell death. These studies demonstrated that nontoxic concentrations of ouabain exhibited a similar protection against ischemia as IPC in both the perfused heart model (P. Dos Santos, unpublished studies) and isolated cardiomyocyte model (D. Van Winkle, unpublished studies). In the latter model, inhibition of Src by PP2 or herbimycin A completely abolished ouabain-induced protection. Pretreatment of myocytes with 5-HD also caused a significant repression of ouabain-induced protection. Clearly, these findings are significant. First, they confirm the role of Src in IPC, identify the steps that lead to the activation of PKC and Src, and make a connection between Src activation and opening mitoK$_{ATP}$. Second, we established a clean in vitro model for identification of missing linkers between activation of Src and mitochondrial production of ROS via opening of mitoK$_{ATP}$. Finally, since digitalis glycosides are still widely used in the treatment of congestive heart failure, these findings have clinical implications.

The involvement of mitoK$_{ATP}$ in IPC has been extensively discussed in the previous sections. However, the ability of KCOs such as diazoxide to trigger the cardioprotective signaling pathway [3,51] is intriguing because it implies that mitoROS activate a key kinase in the neighborhood of mitochondria. Under these conditions, mitoK$_{ATP}$ opening appears to be *upstream* of the above-discussed kinases. During IPC, cell signaling acts to open mitoK$_{ATP}$ and increase ROS production, indicating that mitoK$_{ATP}$ opening is *downstream* from the kinases. This apparent temporal contradiction can be resolved by postulating that mitoK$_{ATP}$ is a component of a signal amplification loop designed to assure full opening of mitoK$_{ATP}$. As shown in Fig. 5, there is no distinction between upstream and downstream elements in this part of the pathway, and several turns of the cycle may be required to achieve the end result of mitoK$_{ATP}$ phosphorylation to assure that the channel is open during the test ischemia. Now, the question is whether KCO-induced ROS are able to activate the PKC/Src module and initiate the signaling loop as outlined in Fig. 5. In this regard, we recently found that exposure of cardiac myocytes to diazoxide was sufficient to cause 25% inhibition of Na/K-ATPase in a time-dependent manner in cardiac myocytes (unpublished data). These data suggest that diazoxide-induced ROS may transduce its signal in the same manner as oaubain, through the Na/K-ATPase to the PKC/Src module, followed by initiation of the signal amplification loop.

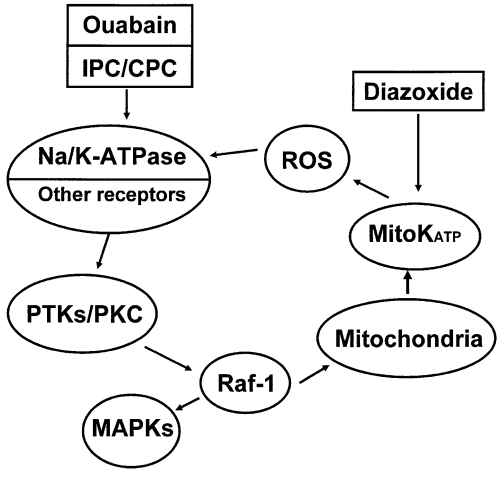

Fig. 5. The mitoK$_{ATP}$ signal amplification loop. The figure illustrates a hypothetical positive feedback cycle within the cardioprotective signaling pathway. The three major modes of ischemic protection are IPC (ischemic preconditioning) CPC (Ca^{2+} preconditioning), and pharmacological preconditioning by diazoxide. Ouabain has also been shown to be cardioprotective (unpublished data). The proposed end result of the loop is to phosphorylate mitoK$_{ATP}$, putting it in a long-lived open state.

4.2. Ischemic phase—the mechanism by which an open mitoK$_{ATP}$ protects the heart during ischemia

4.2.1. Outer mitochondrial membrane permeability to adenine nucleotides

Rostovtseva and Colombini [145] have shown that the voltage-dependent anion channel (VDAC) controls outer membrane permeability to ADP and ATP. In heart, VDAC is normally in a low-conductance state that is poorly permeable to nucleotides, and energy transfers between mitochondria and cytosol are mediated instead by creatine and creatine phosphate [146]. Octamers of mitochondrial creatine kinase (Mi−CK) bridge the IMS between outer membrane VDAC and the inner membrane ATP/ADP translocator (ANT) [147,148], and it has been shown that Mi-CK binds to VDAC [149]. We hypothesize that binding of octameric Mi-CK to VDAC confers a low outer membrane conductance to nucleotides and that this binding requires a narrow intermembrane distance. During ischemia, $\Delta\Psi$ will decrease, and the balance of the K^+ cycle will shift to efflux and matrix contraction. The resulting expansion of the IMS will cause Mi-CK to dissociate from VDAC, leading to a high outer membrane conductance to ATP and ADP. If mitoK$_{ATP}$ is open during ischemia, normal matrix and IMS volumes will be maintained, and the outer membrane will retain its low permeability to nucleotides. This model is depicted in Fig. 6 (Ischemia).

4.2.2. Preservation of adenine nucleotides and reduced $\Delta\Psi$ during ischemia

To explore this hypothesis in greater depth, we carried out a series of measurements of ATP hydrolysis by mito-

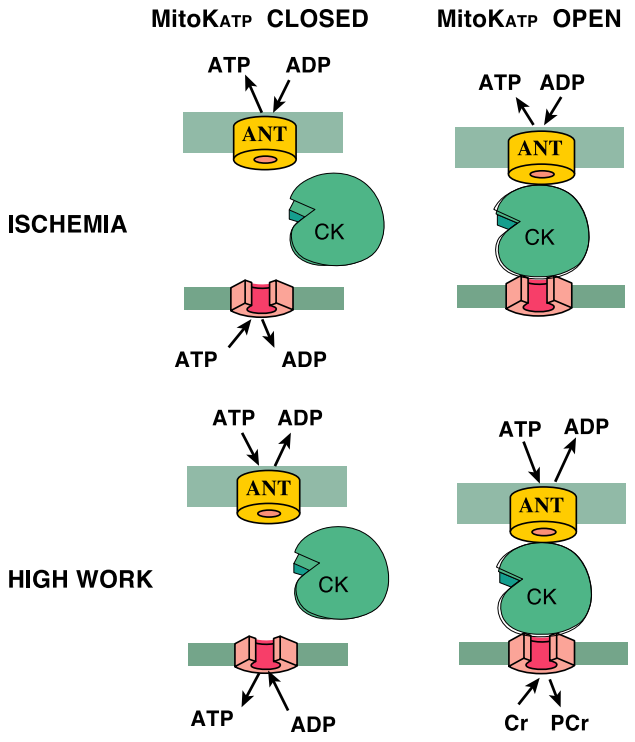

Fig. 6. MitoK_{ATP} regulation of VDAC permeability to nucleotides during ischemia and during high rates of ATP production. $\Delta\Psi$ is supported by ATP hydrolysis during ischemia and by electron transport during the high work state. Both of these stresses will cause a decrease in $\Delta\Psi$, resulting in reduced uptake of K^+ salts and water, contraction of the matrix, and expansion of the IMS. This structural change can be prevented by a compensatory increase in K^+ conductance mediated by opening mitoK_{ATP}. If mitoK_{ATP} is blocked or does not open, the IMS expansion results in increased outer membrane permeability to ATP and ADP. During ischemia, this means that all of cellular ATP is available for hydrolysis by mitochondria, with consequent degradative loss of adenine nucleotides, and, ultimately, unavailability of ADP for rephosphorylation upon reperfusion. During the high work state, increased VDAC permeability to ADP and ATP constitutes a diversionary leak in the system away from the more efficient metabolic channeling through mitochondrial creatine kinase ("CK"). Consequently, If mitoK_{ATP} is blocked, mitochondria cannot supply ATP at the high rates required, and the heart cannot respond to inotropic stress.

chondria in which respiration was inhibited (simulated ischemia). Following is a brief summary of the results of these studies, which are described in Dos Santos et al. [5]: About 55% of total ATP was hydrolyzed in 90 s in untreated mitochondria, and a combination of diazoxide and mild osmotic swelling reduced this value to 15%. After the outer membrane was ruptured by matrix swelling, ATP hydrolysis returned to control values (55%) and was no longer sensitive to changes in matrix volume. These findings support the hypothesis that IMS volume regulates ATP hydrolysis and show (1) that opening mitoK_{ATP} reduces ATP hydrolysis; (2) that this effect is due to small changes (about 15%) in mitochondrial volume; and (3) that an intact outer membrane is necessary for the effect. Ozcan et al. [68] have also shown reduced ATP hydrolysis in isolated mitochondria treated with diazoxide.

The mechanism of reduced ATP hydrolysis requires further scrutiny. Clearly, the rate of ATP hydrolysis in these experiments depends on the rate of ion leak across the *inner* membrane, and any effect of outer membrane permeability to ATP can only be indirect. Ion leak is determined by $\Delta\Psi$, which in turn is in equilibrium with the mitochondrial phosphorylation potential, ΔG_P. As $|\Delta G_P|$ falls, due to consumption of ATP, $\Delta\Psi$ will decrease and ion leaks will decrease. When VDAC is open during ischemia (mitoK_{ATP} closed, matrix contracted, IMS expanded), nucleotide equilibration across the outer membrane will make all of the ATP available to support ATP hydrolysis. The rate of decrease in $\Delta\Psi$ will be slow during in vitro experiments, because total ATP is very high (infinite bath). When VDAC is closed during ischemia (mitoK_{ATP} open, matrix expanded, IMS contracted), nucleotides cannot equilibrate rapidly across the outer membrane. In this condition, the primary supply of ATP is limited to the IMS, and ΔG_P is no longer in equilibrium across the outer membrane. $|\Delta G_P|_{mito}$ and $\Delta\Psi$ will decrease much more rapidly, ion leaks will decrease correspondingly, and ATP hydrolysis will be much slower, as observed. Confirming this view, we observed that $\Delta\Psi$ declined much more rapidly when ATP hydrolysis was inhibited by mild matrix swelling, as shown in Dos Santos et al. [5]. Thus, the reduced ATP hydrolysis derives from segregation of ATP and ΔG_P between the mitochondrial and external compartments. This segregation depends on VDAC permeability, which, in turn, depends on the volume of the IMS. It should be emphasized that these findings exclude uncoupling as a cause of the more rapid depolarization of $\Delta\Psi$, because uncoupling would increase ATP hydrolysis, whereas we observed decreased ATP hydrolysis.

These findings support a plausible mechanism by which mitoK_{ATP} opening during ischemia, through its effect on IMS volume, (1) reduces the rate of ATP loss; (2) reduces the rate of adenine nucleotide degradation so that ADP is available for phosphorylation upon reperfusion; and (3) reduces $\Delta\Psi$ and Ca^{2+} accumulation, preventing Ca^{2+} overload. These effects preserve mitochondria so that they can return upon reperfusion to their normal function of providing adequate ATP supply to cytosolic ATPases.

4.3. Reperfusion phase—the mechanism by which mitoK_{ATP} opening preserves energy transfer and improves functional recovery at reperfusion

4.3.1. Energy transfers between mitochondria and cytosol in heart

Energy transfer from mitochondria to myofibrils and SERC-ATPase is mediated by two parallel pathways—creatine/creatine phosphate (Cr/CrP) and ATP/ADP. Cr/CrP is the more efficient of the two and inhibition of this pathway prevents the heart from working in the upper 50% of its range [150–152]. About 67% of the energy produc-

tion in heart has been found to arise from the CK system [153]. Tian and Ingwall [154] and Saks et al. [151] showed that inhibition of CK in heart causes a profound decrease in rate-pressure product in response to Ca^{2+}. Thus, when the myocyte must rely solely on ADP diffusion to mitochondria, it can no longer maintain high rates of ATP synthesis and consumption [151,152,155].

In the Cr/CrP system, myofibrillar creatine kinase (MM-CK) converts ADP to creatine, which diffuses to the mitochondrial IMS. Octamers of Mi-CK bridge the IMS between outer membrane VDAC and inner membrane ATP/ADP translocator (ANT_ [147]. The Mi-CK reaction is very rapid. Cr combines with ATP as it leaves the matrix, and the ADP formed is simultaneously taken up by ANT for rephosphorylation. In this way, Cr input is immediately followed by CrP output, so that ATP and ADP are only involved at the source (ATP synthase) and sink (myofibril ATPase) of the cycle [151,152,155,156]. VDAC has a low permeability to ADP and ATP in vivo, and the amplifying effect of the Mi-CK system permits very high rates of respiration, even at low ADP concentrations [157]. These features permit $|\Delta G_P|$ to be maintained at high levels in the face of high ATPase activity. If, however, the architecture of the IMS is perturbed by detachment of Mi-CK from the mitochondrial membranes, this control is lost [158].

The efficiency of this energy transfer system requires adenine nucleotide compartmentation, which, in turn, requires low permeability of the outer mitochondrial membrane to adenine nucleotides. As in the preceding section, we hypothesize that binding of octameric Mi-CK to VDAC is responsible for maintaining low adenine nucleotide permeability and that this binding requires a narrow intermembrane distance. During reperfusion, $\Delta\Psi$ will be low due to high rates of electron transport, and the balance of the K^+ cycle will shift to efflux and matrix contraction. The resulting expansion of the IMS will cause Mi-CK to dissociate from VDAC, leading to a high outer membrane conductance to ATP and ADP. If mitoK_{ATP} is open during ischemia, normal matrix and IMS volumes will be maintained, the outer membrane will retain its low permeability to nucleotides, and the mitochondria can restore energy levels using the more efficient metabolic channeling via Mi-CK. This model is depicted in Fig. 6 (High Work).

It should be noted in this regard that Saks et al. [146,157] and Toleikis et al. [159,160] have observed in saponin-skinned fibers that the normally high $K_{1/2}$ (ADP) is sharply reduced by controlled proteolysis. Saks et al. has concluded that outer membrane nucleotide permeability is controlled by intracellular attachments to cytoskeleton. The extent to which this proteolysis occurs in vivo is not known, but such a modification may occur during chronic ischemia or apoptosis. We do not view these two distinct mechanisms of regulating VDAC permeability as being mutually exclusive: volume regulation occurs on short time scales and regulation by proteolysis occurs on long time scales.

4.3.2. Preservation of nucleotide compartmentation by opening mitoK_{ATP}

We examined outer membrane nucleotide permeability in permeabilized fibers taken at the end of ischemia–reperfusion [5,59]. Thirty-minute ischemia caused a decrease in the $K_{1/2}$ (ADP) for respiration, which reflects an increased outer membrane permeability to ADP. IPC and diazoxide prevented this ischemia-induced change in mitochondrial function, and 5-HD blocked the protective effects of IPC and diazoxide. We also examined whether small changes in matrix and IMS volumes affect ADP permeability in the permeabilized fibers from normal perfused rat hearts. In this preparation, we found that the normally high $K_{1/2}$ (ADP) [59] was reduced by 70% by the addition of a very low concentration (8 nM) of nigericin, which had no effect on respiration per se, but was sufficient to contract the mitochondrial matrix [5]. These studies support the hypothesis that VDAC permeability to ADP in situ is controlled by IMS volume. We note that the ischemia-induced change in the $K_{1/2}$ (ADP) reflects an alteration of energy transfer from the mitochondrial matrix to ATP-utilizing sites in the cytosol that may contribute to the poor functional recovery of ischemic hearts.

We next examined the effects of small changes in matrix and IMS volumes on $K_{1/2}$ (ADP) in isolated rat heart mitochondria. We observed that isolated mitochondria exhibit a low $K_{1/2}$ (ADP) (100 μM) when incubated in isoosmotic media, even in the presence of polyethylene glycol to reduce IMS volume [161]. Recall, however, that the matrix of isolated mitochondria is artifactually contracted due to loss of K^+ salts during isolation [1,5]. When matrix volume was increased by a combination of diazoxide and osmotic swelling, $K_{1/2}$ (ADP) increased to 275–325 μM. When matrix swelling was sufficient to break the outer membrane, the volume effect disappeared and $K_{1/2}$ (ADP) became independent of matrix volume [5,162]. These findings show (1) that opening mitoK_{ATP} affects nucleotide compartmentation; (2) that this effect is due to small changes in mitochondrial volume; and (3) that an intact outer membrane is necessary for the effect. Note that these conclusions parallel those drawn for ATP hydrolysis in the previous section.

If the myocyte survives ischemia, it is subjected to new stresses upon reperfusion. PCr has been consumed, ATP is low, and Na^+ and Ca^{2+} are elevated. In order to correct these imbalances, mitochondria must be able to supply ATP at a high $|\Delta G_P|$ to the cytosol. If oxidative phosphorylation is sluggish, reintroduction of oxygen will lead instead to high rates of potentially deleterious mitoROS production. When mitoK_{ATP} is open during reperfusion, the association of Mi-CK with VDAC will be preserved, preventing leaks of ATP and ADP through VDAC and permitting direct energy transfers through the creatine–PCr system. This more efficient system may be required to support energy transfers at high rates at the time of reperfusion [151,152].

5. The physiological roles of mitoK$_{ATP}$ in heart

Man, with his susceptibility to coronary artery disease, is subject to repeated bouts of ischemia. For this reason, it is conceivable that cardioprotection by IPC may have evolved as a specific protective mechanism. However, IPC appears to exist in all mammalian species, including those that are not subject to ischemic heart disease. Therefore, it seems unlikely that preconditioning evolved specifically to increase resistance to ischemia and more likely that protection is a beneficial consequence of triggering normal physiological responses. Accordingly, it is important to develop an understanding of how mitoK$_{ATP}$ participates in normal cardiac physiology. This subject is being actively investigated by our laboratories, and we will summarize our preliminary findings.

5.1. The role of mitoK$_{ATP}$ in physiological cell signaling

New results indicate that mitoK$_{ATP}$ plays a role in normal cell signaling processes leading to cell growth. Xie et al. [105,138,140] have shown that binding of ouabain to Na/K-ATPase activates multiple signal transduction pathways in cardiac myocytes, including increased mitochondrial production of ROS, rise in $[Ca^{2+}]_i$, and activation of PKC and MAP Kinases. Furthermore, interplays among these pathways in cardiac myocytes leads to hypertrophic growth and changes in the expression of multiple growth-related genes. Mitochondrial ROS were shown to be essential second messengers within this signaling pathway, and recent work has shown that the ouabain signaling pathway is blocked not only by antioxidants but also by 5-HD (Fig. 4) [60], thereby linking mitoK$_{ATP}$ to cell growth and hypertrophy. Some elements of the ouabain signaling pathway overlap with those involved in the signaling pathway of preconditioning, including Src and PKC [139,141], and we have preliminary evidence that pretreatment with ouabain is cardioprotective (Dos Santos, Van Winkle, Laclau, Garlid, and Xie, unpublished results).

5.2. The role of mitoK$_{ATP}$ opening in positive inotropy–energy transfers between mitochondria and cytosol

When the cardiomyocyte is undergoing high rates of ATP production and consumption, electron transport is high and $\Delta\Psi$ decreases—as with any battery system, drawing high currents will decrease the output voltage. Exactly as hypothesized for cells undergoing ischemia–reperfusion, mitoK$_{ATP}$ opening adds a parallel K^+ conductance that *counteracts* the decrease in K^+ influx and matrix contraction that would otherwise occur, resulting in constant volume and maintenance of a constant uptake of K^+ salts [4,98]. Because there is no net change in volume, there is no increase in ROS production during the high work state.

Upon consideration of the mechanisms described in Sections 4.2 and 4.3, we propose that endogenous signals open mitoK$_{ATP}$ during high work states of the heart. This preserves the structure–function of the IMS and, in particular, the association of Mi-CK with VDAC. MitoK$_{ATP}$-dependent volume regulation will thus prevent excessive leaks of ATP and ADP through VDAC, and energy transfers will occur primarily through the creatine–PCr system. This more efficient system is thought to be required to support energy transfers at high rates [151,152]. This mechanism is modeled in Fig. 6 (High Work).

We have explored this hypothesis in human atrial fibers [39] and perfused rat hearts. Our preliminary results show that increased work states in heart cannot proceed if mitoK$_{ATP}$ is blocked. We examined the role of mitoK$_{ATP}$ in the response to calcium-, ouabain-, and dobutamine-induced positive inotropic stress in Langendorff perfused rat hearts and correlated these results with an examination of mitochondrial function in permeabilized fibers from the same hearts. To test the hypothesis that mitoK$_{ATP}$ opening is required for the positive inotropic response, we examined the effects of two mitoK$_{ATP}$ inhibitors, 5-HD and tetraphenylphosphonium cation (TPP^+) [1]. In support of the hypothesis, these mitoK$_{ATP}$ blockers prevented the inotropic response (Dos Santos and Garlid, unpublished results). Moreover, this effect was reflected in an increased permeability of VDAC to ADP, as predicted. Thus, the $K_{1/2}$ (ADP) was sharply reduced by both 5-HD and TPP^+. These findings support the views of Saks et al. [151], in which metabolic channeling through mitochondrial creatine kinase is essential for the high work state. Thus, the system can only operate efficiently if leaks of ADP and ATP across the outer membrane are minimized, in this case by volume regulation of the IMS. These results indicate for the first time a physiological role for mitoK$_{ATP}$ in inotropy and, by extension, in heart failure.

5.3. The role of mitoK$_{ATP}$ opening in positive inotropy-volume regulation of electron transport

Lehninger and Kennedy [163] observed in 1948 that substrate oxidation is affected by matrix volume, and Nicholls et al. [164] showed that the effect is independent of the means used to change volume. Volume activation of electron transport has subsequently been demonstrated in liver, heart and, brown adipose tissue mitochondria [165–167]. The effect of volume on fatty acid (FA) oxidation is so pronounced that rates are nearly zero when the matrix is contracted. Volume-activation appears to play an important role in hormone-stimulated gluconeogenesis and FA oxidation, and stimulation of FA oxidation can be mimicked in hepatocytes by increasing matrix volume with low doses of valinomycin [167,168]. Activated electron transport is needed during states of high ATP demand, but these states are associated with a decrease in $\Delta\Psi$. MitoK$_{ATP}$ opening preserves the activated state that would otherwise be lost due to matrix contraction.

6. The pharmacology of mitoK$_{ATP}$

6.1. The pharmacological approach to mitoK$_{ATP}$ function

Pharmacological agents have proved historically to be the most powerful tools for identifying cardiac receptors and characterizing their cellular function. MitoK$_{ATP}$ is no exception in this regard: The discovery that mitoK$_{ATP}$ may be the pivotal receptor for the cardioprotective effects of KCOs was based on the selective pharmacology of diazoxide and 5-HD [2]. Site selectivity is an important aspect of the pharmacological approach, and is discussed further in Section 6.2.

The true power of the pharmacological approach resides in the fact that mitoK$_{ATP}$ openers and blockers are chemically diverse. Chemical diversity allows us to address the question of multiple sites of drug action, because differences in chemical structures render unlikely the possibility that the cardioprotective actions of all agents are due to non-mitoK$_{ATP}$ interactions within the cell. MitoK$_{ATP}$ openers and blockers are hydrophobic, and it is generally the case that hydrophobic molecules will inhibit various respiratory chain complexes at high concentrations. Some agents will also possess intrinsic uncoupling activity. Thus, pinacidil and diazoxide have been shown to be mild inhibitors of respiration and to have intrinsic protonophoretic (uncoupling) properties as a function of concentration [4,48]. However, these effects occur at doses much higher than those required to open mitoK$_{ATP}$ [4].

The power of the pharmacological approach is often overlooked, as for example, in the reports of Halestrap [169] and Daut [170,171] and their coworkers, which are discussed in more detail in Section 6.3. These papers focus primarily on diazoxide, which misses the point of the pharmacological approach. It is reminiscent of arguments during the 1960s that the mechanism of action of uncouplers was due to their ability to inhibit respiration, and not due to their protonophoretic activity. Mitchell [172] pointed out that the hydrophobic sites of the electron transport chain are famously sensitive to a wide variety of hydrophobic agents. He then wrote: ''It would seem that inhibitory and competitive effects of hydrophobic substances on the ... reactions of oxidative phosphorylation may often require no more than trivial explanations'' [172]. The same statement may be applied to the claims that diazoxide and pinacidil act by inhibiting elements of the respiratory chain.

Stereospecificity is another powerful tool that is being explored in ongoing experiments. For example, Grover et al. [29] showed that the cardioprotective effects of cromakalim are steroselective. We have subsequently observed that the active enantiomer, levcromakalim, opens reconstituted mitoK$_{ATP}$ with $K_{1/2}$ of 1.6 μM, whereas the inactive enantiomer had no effect on mitoK$_{ATP}$. These findings are intrinsically important in linking mitoK$_{ATP}$ to cardioprotection. They also suggest a straightforward approach to the question of whether protection is caused by respiratory inhibition, because active and inactive enantiomers are likely to be equipotent in their non-mitoK$_{ATP}$ effects on mitochondria, including inhibition and uncoupling.

6.2. Pharmacological studies to distinguish between mitoK$_{ATP}$ and sarcK$_{ATP}$ in perfused hearts

The hypothesis that mitoK$_{ATP}$, and not sarcK$_{ATP}$, is the receptor for cardioprotective KCOs has been examined using the site-specific agents, diazoxide, and BMS191095. Thus, diazoxide was found to act on mitoK$_{ATP}$, but not sarcK$_{ATP}$ [2]. The experimental KCO, BMS 191095, is cardioprotective without affecting sarcK$_{ATP}$ or vascular smooth muscle K$_{ATP}$ channels [173]. BMS191095 opened mitoK$_{ATP}$ with $K_{1/2}$ of 83 nM and is a good alternative to diazoxide for experimental studies. It is expected that additional site-selective openers and blockers will be identified in the future.

In our studies, we have found that *all* known cardioprotective KCOs open mitoK$_{ATP}$ within their cardioprotective dose ranges (Paucek and Garlid, unpublished data). For example, nicorandil is cardioprotective [174] and opens mitoK$_{ATP}$ with $K_{1/2} = 5$ μM. P1075, a potent cyanoguanidine, is cardioprotective in isolated, perfused rat heart [175,176] and rabbit heart [177], with protection being blocked by 5-HD and glibenclamide. P1075 opens mitoK$_{ATP}$ from rat and rabbit heart mitochondria with $K_{1/2}$ of about 70 nM [176,177]. These agents, along with cromakalim and other members of its class, are nonselective and open both mitoK$_{ATP}$ and sarcK$_{ATP}$.

How can we determine the cardioprotective receptor site for nonselective KCOs? The ideal approach is to combine a nonselective KCO with a sarcK$_{ATP}$-selective inhibitor, such as HMR1098 [178]. Thus, Fryer et al. [179] showed that HMR1098 does not block IPC, confirming the hypothesis that IPC requires mitoK$_{ATP}$ opening. Moreover, Das and Sarkar [180] have recently shown that HMR1098 does not block protection by cromakalim or nicorandil, indicating that these two nonselective agents are protecting the heart via mitoK$_{ATP}$.

These findings suggest a general approach to identifying the receptor for cardioprotection by nonselective KCOs; however, perfused heart studies are not sufficient for such studies. It is also necessary to test each combination on mitoK$_{ATP}$ activity in vitro. A good example of this necessity is given by our experience with diazoxide. We were surprised to find that diazoxide protection is blocked by HMR1098 [61]. New studies on cardiac mitoK$_{ATP}$ have revealed the reason for this apparent paradox: HMR1098 selectively inhibits the diazoxide-opened mitoKATP ($K_i \sim 3$ μM), but has no effect on the cromakalim- or nicorandil-opened mitoK$_{ATP}$ (Costa and Garlid, unpublished data). This appears to be another example in which the ability of blockers to inhibit depend on the specific open state of mitoKATP, as discussed in Jaburek et al. [47]. The interesting anomaly with diazoxide does not detract from the power

of HMR1098 plus nonselective KCO to further test the mitoK$_{ATP}$ hypothesis of cardioprotection.

6.3. Non-mitoK$_{ATP}$ actions of diazoxide, pinacidil, and 5-HD

The laboratories of Halestrap [169] and Daut [170,171] repeated studies carried out by Schafer et al. [181] and Kowaltowski et al. [4], who showed that diazoxide inhibits SDH at high concentrations. It was reported that 100 μM diazoxide inhibited coupled respiration in submitochondrial particles (SMPs) by about 45%. However, this assay is not a pure measure of enzyme inhibition, because the coupling membrane imposes restraints such that state 4 respiration is very sensitive to inhibitors. A more accurate assay is carried out on uncoupled or state 3 rates, where these restraints are removed. Such experiments were performed on rat heart mitochondria, in which we found that 1 mM diazoxide, the highest concentration tested, inhibited uncoupled respiration by less than 15% [4]. Thus, diazoxide is a very weak inhibitor of SDH.

Halestrap and Daut concluded that cardioprotection by diazoxide is mediated by respiratory chain inhibition [169–171]. No evidence whatsoever is provided for this questionable conclusion. The authors fail to address the question of whether a small inhibition of cardiac SDH would have any significant effect on the heart. As discussed in the preceding section, they fail to take into account the chemical diversity of KCOs: if cardioprotection is caused by SDH inhibition, then all cardioprotective KCOs should inhibit SDH. This is already known to be untrue. The authors extrapolate their results from in vitro studies on mitochondria or SMPs without considering the pharmacokinetics of diazoxide in the heart. In intact tissue, diazoxide will bind extensively to proteins [182,183] and undergo retarded diffusion to the site of action. For example, the response of the flavoprotein fluorescence signal to diazoxide exhibits a very long lag time of 10–12 min [49], and this latency is eliminated in saponin-permeabilized cells, with higher diazoxide sensitivity (Brian O'Rourke, personal communication). Proper studies should compare inhibition and mitoK$_{ATP}$ opening in the same experimental model. When such studies were performed in our laboratory, the $K_{1/2}$ for mitoK$_{ATP}$ opening by diazoxide in isolated mitochondria was 2.3 μM, whereas mild inhibitory effects were seen at concentrations greater than 100 μM [162].

The same two laboratories report that 5-HD is converted to 5-HD-CoA by mitochondria in the presence of CoA and ATP [169] or in the test tube when fatty acyl CoA synthetase, ATP, and CoA were added [170,171]. Metabolism of 5-HD is expected and already known from previous work. Moritani et al. [184] reported in 1994 that 5-HD has a very short (7 min) biological half-life in dogs, and Munch-Ellingsen et al. [185] found that 5-HD blocked protection by IPC in rabbits when given 2 min before IPC, but had no effect at the same dose when given 8 min before IPC. As the authors suggested, this short half-life was probably due to metabolism. Halestrap [169] and Daut [170,171] and their coworkers speculate that 5-HD may block cardioprotection by interfering with cardiac metabolism. There is no basis for such a conclusion. No reasons were given to explain why drug metabolism should be of concern in heart studies, nor is metabolism of 5-HD expected to have any effects on the heart or on IPC. Moreover, 5-HD metabolism cannot explain why glibenclamide [2] and HMR1098 [61] blocked diazoxide protection.

It is important to point out that 5-HD has been shown to inhibit mitoKATP, both in isolated mitochondria and in proteolipsomes containing purified, reconstituted mitoKATP [47]. These experiments were carried out under conditions where there was no possibility of forming the acyl CoA derivative of 5-HD. 5-HD is a particularly useful drug for use in whole hearts for two reasons. It appears to be innocuous in that it has no effects on the control heart, and it appears to reach the intracellular site more readily than sulfonylureas such as glibenclamide.

7. Alternative mechanism of protection: uncoupling of oxidative phosphorylation

7.1. Does mitoK$_{ATP}$ opening cause uncoupling in the normoxic cardiomyocyte?

It has been suggested by several workers that mitoK$_{ATP}$ opening protects the heart by uncoupling due to increased K^+ cycling. Uncoupling is thought to be protective by virtue of the fact that decreased $\Delta\Psi$ would reduce mitochondrial Ca^{2+} uptake and Ca^{2+} overload during ischemia [49,78,80,186–188]. Because this issue is important and controversial, we will discuss the evidence in some detail.

7.1.1. If K^+ flux through mitoK$_{ATP}$ were sufficient to uncouple respiration, the outer membrane would rupture

It is important to recall that the K^+/H^+ antiporter can only respond to *changes* in matrix volume [1]. Therefore, net K^+ influx due to mitoK$_{ATP}$ opening will cause a shift to a higher steady state matrix volume [4,46,98]. The IMS is normally small in vivo, and K^+ influx sufficient to cause significant uncoupling may cause matrix swelling sufficient to break through the outer membrane. We examined this question directly by measuring the effects of valinomycin on respiration and matrix swelling (unpublished). First, we found a dose of valinomycin that is roughly equivalent to mitoK$_{ATP}$ opening; that is, a dose that caused the same increase in matrix volume and respiration as were observed after opening mitoK$_{ATP}$. This concentration is 0.6–0.9 pmol Val/mg protein. We then increased [valinomycin] and observed increased swelling and respiration, as expected. At about 3 pmol Val/mg protein, we observed a transition in the light scattering trace that is indicative of outer membrane rupture, as described in Dos Santos et al. [5]. (Note that

these studies were performed on mitochondria with a contracted matrix due to the isolation artifact [5]. Less K^+ influx would be required to rupture the outer membrane under in vivo conditions.) Outer membrane rupture was confirmed by the finding that addition of cytochrome *c*, which had no effect on state 2 respiration below 3 pmol Val/mg protein, increasingly stimulated respiration above that concentration. 3 pmol Val/mg protein stimulated respiration by only 70 ng atom O min^{-1} mg^{-1} at 25 °C, a very modest fraction of the FCCP-uncoupled rate (800 ng atom O min^{-1} mg^{-1}). We conclude that mitochondrial respiration cannot be significantly uncoupled by increased K^+ flux without, at the same time, causing rupture of the outer membrane.

7.1.2. K^+ flux through mitoK$_{ATP}$ is too low to uncouple respiration

In experimental studies, diazoxide is added to the normoxic perfused heart, in which the mitochondria have a high membrane potential ($\Delta\Psi$). Opening mitoK$_{ATP}$ under these conditions will increase futile K^+ cycling and dissipate energy; however, the degree of uncoupling and extent of depolarization will depend entirely on the magnitude of the added K^+ flux. We measured the effects of ATP, ATP + diazoxide, and ATP + diazoxide + 5-HD on respiration in both rat heart [4] and rat brain [98] mitochondria. Based on these studies, the magnitude of K^+ flux through mitoK$_{ATP}$ is 24–30 nmol K^+ min^{-1} mg^{-1} at 25 °C in rat heart mitochondria. Kopustinskiene [183] carried out similar measurements in rat heart mitochondria utilizing site I substrates at 37 °C. She observed a difference in State 2 respiration of about 5 ng atom O mg^{-1} min^{-1} as the consequence of mitoK$_{ATP}$ opening. Assuming a value of 10 for the H^+/O stoichiometry, this implies a rate of 50 nmol K^+ mg^{-1} min^{-1}, which is in very good agreement with the value we obtained at 25 °C [4]. A respiratory stimulation of 5 ng atom O min^{-1} mg^{-1} is a very low rate indeed, considering that rat heart mitochondria can respire in state 3 at over 1000 ng atom O min^{-1} mg^{-1}. Respiratory stimulation of this magnitude will cause an insignificant decrease in $\Delta\Psi$. These measurements provide direct evidence against significant uncoupling as a consequence of mitoK$_{ATP}$ opening; however, they do rely on extrapolation from mitochondria in vitro to mitochondria in situ.

7.1.3. MitoK$_{ATP}$ opening does not uncouple respiration in vivo

There is ample experimental evidence that mitoK$_{ATP}$ opening does not uncouple respiration in vivo. In an important paper, Ovide-Bordeaux et al. [189] confirmed that diazoxide had no detectable bioenergetic effects in permeabilized cardiac fibers from normoxic hearts. Lawrence et al. [190] observed diazoxide cardioprotection in cardiomyocytes with no detectable change in TMRE fluorescence, as a measure of mitochondrial $\Delta\Psi$. Similar results were reported by Carroll et al. [191] using JC1 fluorescence. Grover et al. [29,192] showed that KCOs in pharmacological doses have no effect on the cardiac efficiency of oxygen utilization in the intact heart, thus excluding significant uncoupling as a consequence of KCO administration.

7.1.4. Uncoupling is inconsistent with ROS production

The finding that mitoK$_{ATP}$ opening in vivo causes increased mitochondrial production of ROS [3,51,60–62] is incompatible with uncoupling, because mitochondrial ROS production is decreased by uncoupling [108].

7.1.5. Data showing uncoupling by diazoxide and pinacidil are independent of mitoK$_{ATP}$

The hypothesis that mitoK$_{ATP}$ opening protects by uncoupling [49] appeared to be supported by findings with KCOs in isolated mitochondria [193–195]. However, these findings arose from mitoK$_{ATP}$-independent actions of the drugs. First, the uncoupling cannot have arisen from mitoK$_{ATP}$ opening because the experiments were carried out under conditions in which mitoK$_{ATP}$ was already open [46]. Second, these studies [193–195] employed KCOs in massive doses, far in excess of concentrations required to open mitoK$_{ATP}$. We confirmed that diazoxide and pinacidil at these doses caused uncoupling and consequent inhibition of Ca^{2+} uptake in mitochondria; however, we showed directly that these drugs possess intrinsic uncoupling activity that only becomes apparent at excessive concentrations [4]. That the uncoupling did not arise from mitoK$_{ATP}$ opening was demonstrated directly by showing that an equal uncoupling effect was observed in TEA^+ or Li^+ media [4]. This result has been confirmed by Kopustinskiene et al. [48,196], who showed that high concentrations of diazoxide and pinacidil uncouple respiration and that this effect is independent of K^+ flux or mitoK$_{ATP}$. Interestingly, these authors found that intrinsic uncoupling by these agents was blocked by carboxyatractyloside, indicating that the uncoupling is mediated by drug transport via the ANT, perhaps in a manner analogous to fatty acid transport by ANT [197].

The preceding evidence may be summarized as follows: maximal rates of K^+ flux through mitoK$_{ATP}$ are too low to cause significant uncoupling under normal conditions. Moreover, if K^+ influx did occur at rates sufficient to uncouple respiration, mitochondria would lyse upon addition of a KCO in vivo.

7.2. Does mitoK$_{ATP}$ opening cause uncoupling during ischemia?

The rationale for uncoupling by mitoK$_{ATP}$ opening is that it would protect ischemic tissue against excessive mitochondrial Ca^{2+} accumulation by reducing the driving force for mitochondrial Ca^{2+} uptake [49,80,195]. We agree that mitoK$_{ATP}$ opening causes depolarization and reduced Ca^{2+} overload during ischemia. However, we have shown that these effects do not involve uncoupling, because they are accompanied by *reduced* rates of ATP hydrolysis. Uncoupling would increase ATP hydrolysis [5]. The proposed

mechanism for this effect involves regulation of IMS volume by mitoK$_{ATP}$ opening during ischemia, as discussed in Section 4.2.2.

7.3. Flavoprotein fluorescence as a measure of mitoK$_{ATP}$ opening in cardiomyocytes

Liu et al. [49] introduced a valuable new technique for detecting mitoK$_{ATP}$ opening in intact rabbit ventricular myocytes. They measured flavoprotein fluorescence, as an index of mitochondrial redox state, and found that diazoxide induced reversible flavoprotein oxidation to rather high levels, when compared to the response to dinitrophenol, a protonophoretic uncoupler. They concluded that changes in flavoprotein fluorescence can be used to detect mitoK$_{ATP}$ activity in situ. The authors also proposed that mitoK$_{ATP}$ opening may protect the heart by uncoupling. Although the latter conclusion may be incorrect, the flavoprotein fluorescence technique is nevertheless a promising approach to the difficult problem of detecting mitoK$_{ATP}$ activity in situ. Marban et al. have published numerous papers using this approach [49,91,186,198–208].

Given that mitoK$_{ATP}$ cannot cause significant uncoupling in the normoxic cardiomyocyte in vivo, what is the origin of the flavoprotein redox potential signal? This question has been clarified by Brian O'Rourke (personal communication). As predicted from the results in Section 7.1., the percentage of freshly isolated myocytes showing a net redox change was small. However, nearly all cells responded when they were first cultured overnight in the absence of added substrates. Under these conditions, it is expected that the redox potential will be affected even by the mild uncoupling caused by mitoK$_{ATP}$ opening. Thus, the assay conditions are designed to amplify the small signal due to mitoK$_{ATP}$ opening, and thereby to evaluate various agents for their effects on mitoK$_{ATP}$. This is a reasonable experimental approach. On the other hand, the results probably should not be extrapolated to physiological conditions that were obtained in vivo.

One discrepancy that remains to be resolved is that the cyanoguanidine KCO, P1075, has been reported to be inactive with respect to mitoK$_{ATP}$ on the basis of flavoprotein fluorescence measurements [204]. To the contrary, P1075 is a potent opener of rat and rabbit heart mitoK$_{ATP}$ ($K_{1/2}$ = 68 nM), and it is an equally potent cardioprotective agent in the perfused rat and rabbit heart models [175–177].

8. The roles of mitoK$_{ATP}$ in heart—overview

Mitochondria are recognized to be important in both cardiac bioenergetics and cardioprotection against ischemia–reperfusion injury. Our broad hypothesis is that cardioprotection utilizes the normal physiological functions of mitoK$_{ATP}$, and our work indicates that mitoK$_{ATP}$ opening has two different effects on the heart that depend on the underlying bioenergetic state at the time the channel is opened. When $\Delta\Psi$ is high, as in the resting heart, mitoK$_{ATP}$ opening leads to increased mitochondrial ROS production, and the ROS in turn activate kinases within a positive signal amplification loop (Fig. 4) leading to gene transcription and cell growth. This signaling pathway is also activated by IPC and leads to phosphorylation of mitoK$_{ATP}$. The resulting sustained mitoK$_{ATP}$ opening is cardioprotective against ischemia–reperfusion injury. When $\Delta\Psi$ is reduced, as occurs during inotropic stress or ischemia, mitoK$_{ATP}$ opening provides matrix volume homeostasis. The additional K^+ influx through mitoK$_{ATP}$ compensates for the lower driving force and maintains matrix and IMS volumes. This is essential for maintaining an activated electron transport system and a low-conductance state of outer membrane VDAC. The consequences are nucleotide preservation during ischemia and efficient energy transfers during the high work state and during reperfusion after ischemia. These working hypotheses will be rigorously examined in future work.

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