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Review

Mitochondrial potassium and chloride channels★✪

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Channels selective for potassium or chloride ions are present in inner mitochondrial membranes. They probably play an important role in mitochondrial events such as the formation of ΔpH and regulation of mitochondrial volume changes. Mitochondrial potassium and chloride channels could also be the targets for pharmacologically active compounds such as potassium channel openers and antidiabetic sulfonylureas. This review describes the properties, pharmacology, and current observations concerning the functional role of mitochondrial potassium and chloride channels.

Ion channels exist in all intracellular membranes analysed by electrophysiological techniques (for review, see Szewczyk, 1998). Over the last ten years, it has been well established that mitochondrial membranes contain ion channels, such as porin (VDAC), and cation

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Abbreviations: Ca^{2+}-PD, Ca^{2+} paradox; mitoK_{ATP} channel, mitochondrial ATP-regulated potassium channel; CLIC, chloride intracellular channel; K_{ATP} channel, plasma membrane ATP-regulated potassium channel; 5-HD, 5-hydroxydecanoic acid; PKC, protein kinase C; KCOs, potassium channel openers; IMAC, inner membrane anion channel; PMA, phorbol-12-myristate-13-acetate; PTP, permeability transition pore; SNAP, *S*-nitro-*N*-acetyl-DL-penicillamine; TMRE, tetramethylrhodamine ethyl ester; TNFα, tumor necrosis factor α.

and anion selective channels (Bernardi, 1999). Mitochondrial ion channels seem to play an important role in such cellular events as exocytosis (Giovannuci *et al.*, 1999) and synaptic transmission (Jonas *et al.*, 1999). Recently, mitochondrial potassium channel openers attract attention due to their possible role in ischemic preconditioning in heart (Garlid *et al.*, 1997; Liu *et al.*, 1998; Baines *et al.*, 1999a; Gross & Fryer, 1999).

MITOCHONDRIAL ATP-REGULATED POTASSIUM CHANNEL

In 1991, the mitochondrial ATP-regulated potassium channel (mitoK_{ATP} channel) was identified by patch-clamp single-channel recordings in the inner membrane of rat liver mitochondria (Inoue *et al.*, 1991). Its activity was later reconstituted into liposomes containing partially purified proteins from the inner membrane of beef heart mitochondria (Paucek *et al.*, 1992). This channel is blocked not only by ATP but also by antidiabetic sulfonylureas and 5-hydroxydecanoic acid (5-HD) (Jaburek *et al.*, 1998) and is activated by some potassium channel openers (KCOs) such as diazoxide (Garlid *et al.*, 1996). According to the present knowledge, the mitoK_{ATP} channel may have a dual physiological function (Szewczyk, 1998). First, it can maintain potassium homeostasis within mitochondria and thus control mitochondrial volume (Halestrap, 1994). Second, potassium uptake upon mitochondrial energization may partly compensate the electric charge transfer produced by proton pumping and thus enable the formation of a pH gradient along with transmembrane electric potential (Czyż *et al.*, 1995).

The molecular identity of mitoK_{ATP} channels is unknown (for review, see Szewczyk & Marban, 1999). Several observations on the pharmacological profile and immunofluorescence may suggest that the mitoK_{ATP} channel belongs to the inward rectifier K^+ channel family (Suzuki *et al.*, 1997). Using immunofluorescence and immunogoldstaining, it has been shown that the inward rectifier K^+ channel, Kir6.1, is present in rat skeletal muscle and liver mitochondria (Suzuki *et al.*, 1997). Recently, Kir6.1 was also localised in rat brain by *in situ* hybridization and immunohistochemistry (Zhou *et al.*, 1999). The mRNA of Kir6.1 was ubiquitously expressed in various neurons and glial cells. Interestingly, under electron microscope, the immunoreactive products were specifically restricted to the mitochondria (Zhou *et al.*, 1999).

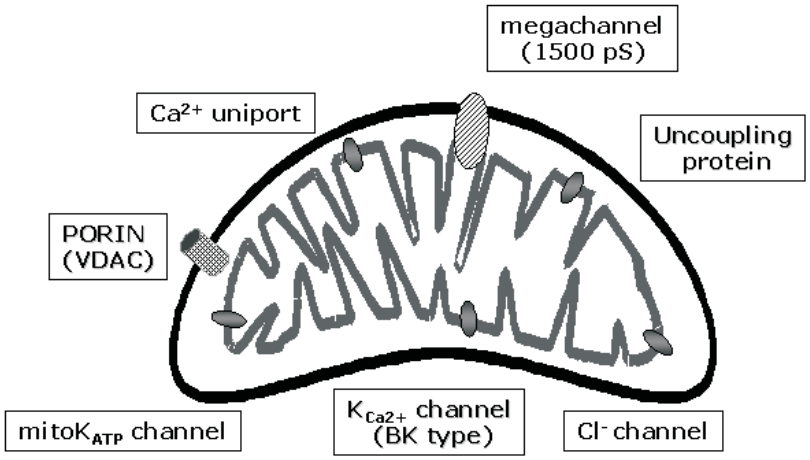

Figure 1. Mitochondrial ion channels.

Similar ATP-regulated K^+ channels are present in the plasma membrane of pancreatic B-cells, smooth, skeletal and cardiac muscle cells and of neurons (Lazdunski, 1994). The pore subunit of these channels, Kir6.1 or Kir6.2, together with the antidiabetic sulfonylureas receptor (SUR), constitutes the functional plasma membrane ATP-regulated K^+ channel (K_{ATP} channel) (Aguilar-Bryan *et al.*, 1995; Inagaki *et al.*, 1995). Plasma membrane K_{ATP} channels are specifically activated by drugs known as potassium channel openers (KCOs) (Edwards & Weston, 1993).

Garlid *et al.* (1996) demonstrated that the heart and liver mitoK_{ATP} channel share some pharmacological properties with the plasma membrane K_{ATP} channel, while possessing a distinct pharmacological profile. The outstanding pharmacological signature of mitoK_{ATP} channels is their sensitivity to the opening by diazoxide, exceeding the sensitivity of cardiac plasma membrane K_{ATP} channels by about 1 000-fold. This observation was crucial for further establishing the functional role of mitoK_{ATP} channels in cardiomyocytes.

Studies on the mitoK_{ATP} channel can be divided into two periods. The first, starting in 1991, could be named "The mitoK_{ATP} channel as an interesting object". This first part was dedicated to the biochemical characterisation of the mitoK_{ATP} channel and searching for its physiological function in cellular bioenergetics. The second period, starting in 1997, could be named "The mitoK_{ATP} channel as an important cellular effector". This part concerns studies providing evidence for the involvement of the mitoK_{ATP} channel in cardioprotection. Both periods are linked by the discovery of Garlid's group that the potassium channel opener, diazoxide, and potassium channel inhibitor, 5-HD, act potently on the heart mitoK_{ATP} channel but not on the heart plasma membrane K_{ATP} channel. Since this observation, a variety of reports have been published suggesting that the mitoK_{ATP} channel plays an important role in cardioprotection. Here, the current knowledge about the mitoK_{ATP} channel will be briefly reported.

MITOCHONDRIAL ATP-REGULATED POTASSIUM CHANNEL IN CARDIOPROTECTION

Applying KCOs, such as diazoxide, and the mitoK_{ATP} channel inhibitor 5-HD, it was shown that the mitoK_{ATP} channel could be an effector of ischemic preconditioning in heart (for review see Gross & Fryer, 1999).

Lethal ischemic injury to the heart can be dramatically blunted by brief periods of ischemia known as "ischemic preconditioning". Despite intensive investigation, the molecular mechanism of preconditioning remains poorly understood. Nevertheless, K_{ATP} channels are clearly involved: KCOs mimic preconditioning in the absence of ischemia, while K_{ATP} channel blockers such as glibenclamide and 5-HD abolish the beneficial effects of preconditioning ischemia. The initial hypothesis to explain these observations involved plasma membrane K_{ATP} channels. Recently, in order to compare cardioprotective potency, diazoxide and cromakalim were given to isolated rat hearts subjected to global ischemia and reperfusion (Garlid *et al.*, 1997). Diazoxide and cromakalim increased the time of onset of contracture with similar potency and improved postischemic functional recovery in a glibenclamide-sensitive manner. In addition, 5-HD completely abolished the protective effect of diazoxide. A conclusion of this study was that diazoxide protected ischemic hearts by opening mitoK_{ATP} channels rather than plasma membrane K_{ATP} channels. In a similar approach, Liu *et al.* (1998) indexed mitoK_{ATP} and surface K_{ATP} channel activity simultaneously in cardiac myocytes. This approach enabled the function of mitoK_{ATP} channels to be assayed in intact cells. MitoK_{ATP} channel activity was indexed by measuring flavoprotein fluorescence, an endogenous reporter of the mitochondrial re-

dox state. Opening of mitoK_{ATP} channels dissipates the mitochondrial membrane potential established by the respiratory chain. This dissipation accelerates electron transfer by the respiratory chain, and leads to net oxidation of mitochondria that can be monitored by recording the fluorescence of FAD-linked enzymes in mitochondria. Low concentrations of the KCO diazoxide have been reported to activate mitoK_{ATP} channels while cardiac plasma membrane K_{ATP} channels are quite resistant to this drug. It was shown that diazoxide induced reversible oxidation of the mitochondrial flavoproteins but did not activate plasma membrane K_{ATP} channels. The subcellular site of diazoxide action was localized to mitochondria by confocal imaging of flavoprotein fluorescence and mitochondria stained with fluorescent probe tetramethylrhodamine ethyl ester (TMRE). It was also shown that the flavoprotein fluorescence affected by diazoxide colocalizes precisely with the staining pattern for TMRE in the same cardiomyocyte. These studies also established that 5-HD is a specific blocker of the mitoK_{ATP} channel, at least in heart muscle cells. In a cellular model of cardiomyocyte ischemia, diazoxide prevented cell death to the same degree as preconditioning – an effect which was blocked by 5-HD (Liu *et al.*, 1998).

Initial observations of the cardioprotective action of diazoxide were further confirmed and developed by a series of reports:

- Isolated mitochondria from rat heart were used to examine the effect of KCOs on mitochondrial membrane potential, respiration, ATP generation, Ca^{2+} transport and matrix volume (Holmuhamedov *et al.*, 1998). KCOs such as pinacidil, cromakalim and levcromakalim induced membrane depolarisation by about 25 mV. Removal of extramitochondrial K^+ or application of K_{ATP} channel blockers abolished this effect. KCOs-induced membrane depolarisation was associated with an increase in the rate of mitochondrial respiration and decrease in the rate of mitochondrial ATP synthesis. Furthermore, treatment with KCOs released Ca^{2+} from mitochondria preloaded with Ca^{2+}; the effect was also dependent on extramitochondrial K^+ concentration and sensitive to mitoK_{ATP} inhibition. In addition, the KCOs cromakalim and pinacidil increased the matrix volume and released the mitochondrial proteins cytochrome *c* and adenylate kinase. These observations provided evidence for role of the mitoK_{ATP} channel in regulating functions vital for cardiac mitochondria. Also nicorandil, a potent cardioprotective agent, was also shown to act by opening the mitoK_{ATP} channel in cardiac myocytes (Sato *et al.*, 2000).
- It is known that protein kinase C (PKC) plays a key role in the induction and maintenance of preconditioning. Measuring mitochondrial matrix redox potential as an index of mitoK_{ATP} channel activity in rabbit ventricular myocytes, it was shown that diazoxide partially oxidised the matrix redox potential. Exposure of myocytes to the PKC activator phorbol-12-myristate-13-acetate (PMA) potentiated and accelerated the effect of diazoxide. These effects of PMA were blocked by the mitoK_{ATP} channel blocker 5-HD, suggesting that the activity of the mitoK_{ATP} channel could be regulated by PKC in intact heart cells (Sato *et al.,* 1998). Other data suggested that activation and translocation of the PKC δ-isoform to mitochondria appears to be important for the protection mediated by the mitoK_{ATP} channel (Wang *et al.,* 1999). Administration of 5-HD, an effective blocker of the mitoK_{ATP} channel, chelerythrine or calphostin C, both inhibitors of PKC completely abolished the beneficial effects of diazoxide on ischemia/reperfusion injury during diazoxide pretreatment (Wang *et al.*, 1999). PKC-α was translocated to the sarcolemma, whereas PKC-δ was translocated to mitochondria

and the intercalated disc and PKC-ε was translocated to the intercalated disc of the diazoxide-pretreated hearts. Colocalization studies for the mitochondrial distribution of TMRE and PKC isoforms by immunoconfocal microscopy revealed that a PKC-δ antibody specifically stained the mitochondria. Additionally, it was shown that the ATP concentration was significantly increased in diazoxide-treated hearts. It was concluded that activation and translocation of PKC to mitochondria appear to be important for the protection mediated by the mitoK_{ATP} channel (Wang *et al.*, 1999).

- Another study intended to approach the role of the mitoK_{ATP} channel and the sequence of signal transduction with PKC and adenosine A1 receptor (Miura & Tsuchida, 1999). These results suggested that mitoK_{ATP} channels are downstream of PKC in the mechanism of infarct-size limitation by A1-receptor activation and that the anti-infarct tolerance afforded by the opening of mitoK_{ATP} channels is associated with the preservation of mitochondrial function during ischemia/reperfusion.
- Opioids have been previously shown to confer short-term cardioprotection against prolonged ischemic insult. Recently, studies were performed to determine whether opioids can induce a delayed or "second window" of cardioprotection and to assess the potential involvement of the mitoK_{ATP} channel in this process (Fryer *et al.*, 1999; Liang & Gross, 1999). These reports suggested that stimulation of the δ_1-opioid receptor before ischemic insult produces a delayed cardioprotective effect that is possibly the result of mitoK_{ATP} channel activation.
- Recently, it has been shown that ischemic preconditioning depends on the interaction between the actin cytoskeleton and mitoK_{ATP} channel (Baines *et al.*, 1999b). For the evaluation of the participation of these proposed end effectors, rabbit hearts underwent regional ischemia and reperfusion. It was shown that diazoxide administered before ischemia was protective. Similarly, anisomycin, a p38/JNK activator, reduced infarct size but protection from both diazoxide and anisomycin was abolished by 5-HD, an inhibitor of the mitoK_{ATP} channel. Interestingly, the protection by preconditioning, diazoxide, or pinacidil could be abolished by disruption of the cytoskeleton by cytochalasin D. These data suggest that both the mitoK_{ATP} channel and the cytoskeletal protein actin are important in protecting hearts by preconditioning.
- Ischemic tolerance of the heart can also be increased by long-term exposure of animals to chronic hypoxia associated with natural or stimulated high altitude. Recently, it has been concluded that long-term adaptation of rats to high altitude hypoxia decreases the susceptibility of their hearts to ischemic arrythmias (Asemu *et al.*, 1999). The mitoK_{ATP} channel, rather than the plasma K_{ATP} channel, appeared to be involved in this protective mechanism (Asemu *et al.*, 1999).
- The role of PKC in the mitoK_{ATP} channel-mediated protection against Ca^{2+} overload injury of the rat myocardium was shown (Wang & Ashraf, 1999). Massive cell damage occurs in the heart within seconds when it is perfused with a medium devoid of Ca^{2+} followed by perfusion with a solution that contains Ca^{2+}. This phenomenon has been called the Ca^{2+} paradox (Ca^{2+} PD). A mild stress induced by brief Ca^{2+} depletion and repletion, called Ca^{2+} preconditioning, has been shown to protect the myocardium from Ca^{2+} PD injury or subsequent sustained ischemia and reperfusion. It has been tested if the protection by diazoxide reduces the Ca^{2+} paradox, whether diazoxide mimics the effects of Ca^{2+} preconditioning, and whether diazoxide reduces Ca^{2+} paradox injury *via* the PKC signalling pathway. The salutary effects of diazoxide on Ca^{2+} PD injury were similar to those in hearts that underwent Ca^{2+} preconditioning or pretreatment with PMA before Ca^{2+} PD. The

addition of 5-HD or chelerythrine during diazoxide pretreatment completely abolished the beneficial effects of diazoxide. PKC-δ was translocated to the mitochondria, intercalated disks and nuclei of myocytes in diazoxide-pretreated hearts, and PKC-α and PKC-ε were translocated to the sarcolemma and intercalated disks, respectively. This study suggested that the mitoK_{ATP} channel activity is mediated by the PKC signalling pathway.

- It has been observed that plasma membrane K_{ATP} channel inhibitors administered before ischemic preconditioning did not significantly attenuate cardioprotection (Fryer *et al.*, 2000). On the contrary, pretreatment with 5-HD before ischemic preconditioning partially abolished cardioprotection. Moreover, it has been shown that rats subjected to ischemia-reperfusion synthesise much less ATP than control animals and ischemic preconditioning significantly increases ATP synthesis when 5-HD is administered before ischemic preconditioning (Fryer *et al.*, 2000). These data are consistent with the notion that inhibition of mitoK_{ATP} channels attenuate ischemic preconditioning by reducing ischemic preconditioning-induced protection of mitochondrial function.
- Evidence for mitoK_{ATP} channels as effectors of human myocardial preconditioning was shown (Ghosh *et al.*, 2000).
- Preservation of mitochondrial function by diazoxide during sustained ischemia in rat heart was observed (Iwai *et al.*, 2000). It has been shown that hypoxia induces a decrease in the mitochondrial oxygen consumption rate of myocardial skinned bundles to approximately 40% of the pre-hypoxic value. In contrast, treatment of the bundles with diazoxide preserves the normal mitochondrial oxygen consumption rate during hypoxia. This effect was abolished by the combined treatment with either glibenclamide or 5-HD (Iwai *et al.*, 2000).
- Activation of the mitoK_{ATP} channel by nitric oxide was shown (Sasaki *et al.*, 2000). The NO donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) oxidised the mitochondrial matrix dose-dependently without activating the plasma membrane K_{ATP} channel. SNAP-induced oxidation was blocked by 5-HD and by a NO scavenger. The conclusion of this study was that NO directly activates mitoK_{ATP} channels and potentates the ability of diazoxide to open these channels. It has been shown that diazoxide induces both early and delayed anti-ischemic effects *via* the opening of mitoK_{ATP} channels, which is nitric oxide-dependent (Ockaili *et al.*, 1999).

Very recently, the effects of KCOs on intracellular Ca^{2+} concentration and mitochondrial potential ($\Delta\Psi_m$) in cultured rat hippocampal neurons have been studied (Jakob *et al.*, 2000). Using Western blots and immunochemistry it was shown that pretreatment with the KCOs cromakalim and diazoxide increased the expression level of proteins involved in apoptosis. These results also suggest that mitoK_{ATP} channels are present in hippocampal neurons and may confer neuroprotection by altering Bcl2 and Bcl-XL expression levels.

MITOCHONDRIAL LARGE CONDUCTANCE POTASSIUM CHANNEL

In 1999, a large conductance BK-type potassium channel was identified by patch-clamp techniques in the inner mitochondrial membrane of the human glioma cell line LN229 (Siemen *et al.*, 1999). This channel shows similarities to the BK-type $K_{(Ca)}$ channel of the plasma membrane found in several tissues (Latorre *et al.*, 1989) and in cell granules of giant green alga *Chara australis* (Laver & Walker, 1991).

Conductance of the mitochondrial BK-type $K_{(Ca)}$ channel is 295 pS and it shows a linear dependence of single channel current from

voltage in 150 mM KCl solutions on either side of the inner mitochondrial membrane. This channel is activated by Ca^{2+} (EC_{50} = 0.9 μM at 60 mV) and the open probability increases with increasing Ca^{2+} concentration. It is unknown whether there is a Ca^{2+}-binding site on the matrix or cytosolic side of the mitochondrial membrane (Siemen *et al.*, 1999). Like most BK-type channels, the mitochondrial channel is blocked by charybdotoxin in a dose dependent manner (EC_{50} = 1.4 nM) (Siemen *et al.*, 1999).

The function of the mitochondrial BK-type $K_{(Ca)}$ channel is unknown. In the plasma membrane it is thought to link the membrane potential to cellular metabolism (Petersen, 1992). It plays an important role in secretion and in repolarizing phase of action potential in some neurons and myotubules (Latorre *et al.*, 1989). It has been speculated that the BK-channel in the human glioma cell LN229 can cause the complete and irreversible uncoupling of mitochondria, thereby promoting apoptosis (Siemen *et al.*, 1999).

MITOCHONDRIAL CHLORIDE CHANNEL

Chloride channels are involved in several crucial cell processes regulating cell volume, membrane potential, transepithelial transport, signal transduction and acidification of organelles (Jentsch & Gunther, 1997). Recently, a novel intracellular chloride channel (mtCLIC) has been identified and shown to be localised in mitochondria (Fernández-Salas *et al.*, 1999). The mtCLIC cDNA is very similar to several reported sequences in the GeneBank™/EMBL/Protein Data Bank database. It is similar to several plasma membrane chloride channels and intracellular chloride channels (93% identity with rat intracellular chloride channel *p64H1*, 88% homology with human *H1* chloride channel and 87% homology with human intracellular chloride channel *p64H1*) (Fernández-Salas *et al.*, 1999). The mtCLIC cDNA codes for a 253 amino-acid protein with a predicted molecular mass of 27.8 kDa (Fernández-Salas *et al.*, 1999). mtCLIC protein also shows extensive homology with a family of chloride channels, especially the intracellular p64H1 chloride channel present in the endoplasmic reticulum of rat brain (98% homology) (Duncan *et al.*,1997) and human p64H1 (Chuang *et al.*, 1999). The analysis of mtCLIC protein revealed two putative transmembrane domains and possible cAMP-dependent protein kinase phosphorylation sites, several PKC, CK2 and tyrosine kinase phosphorylation sites and N-myristoylation sites (Fernández-Salas *et al.*, 1999).

mtCLIC mRNA is expressed to a greatest extent *in vivo* in heart, lung, liver, brain and skin. However, it has been detected in all tested tissues (Fernández-Salas *et al.*, 1999). This protein is the first intracellular ion channel shown to be differentially regulated. The expression in heart, lung, liver, kidney and skin is higher in $p53^{+/+}$ mice than in $p53^{-/-}$ mice ranging from a 2.5- to 5-fold difference but these differences were not detected in intestine, spleen, testis and kidney (Fernández-Salas *et al.*, 1999). Also, in cultured keratinocytes the levels of mtCLIC mRNA and protein are higher in $p53^{+/+}$ keratinocytes than in $p53^{-/-}$ cells and further increased after induction of differentiation of keratinocytes (Fernández-Salas *et al.*, 1999). Moreover, overexpression of p53 in primary mouse keratinocytes increases mtCLIC mRNA and the protein level. Exogenous human TNFα also increase the levels of mtCLIC mRNA and protein in both $p53^{+/+}$ and $p53^{-/-}$ keratinocytes (Fernández-Salas *et al.*, 1999).

mtCLIC is the first chloride channel shown to be localised in mitochondria. It has been shown that other proteins belonging to the CLIC family of intracellular chloride channels are involved in the control of transmembrane potential, ionic concentration and pH in intracellular organelles such as early endosomes (Edwards *et al.*, 1998). The mito-

chondrial chloride channel may be very important for establishing the pH gradient across the inner mitochondrial membrane, as chloride currents could compensate for charges during H^+ transport through the respiratory chain.

As mentioned above, mtCLIC expression is regulated by p53 and TNFα, proteins that are both important in apoptotic cell death (Fernández-Salas *et al.*, 1999). It has been suggested that mtCLIC can provide a potential common downstream effector for the p53 and TNFα pathways (Fernández-Salas *et al.*, 1999). This is an interesting hypothesis as both pathways can mediate UV-induced apoptosis in keratinocytes (Schwarz *et al.*, 1995; Ziegler *et al.*, 1994). It is possible that changes in inner mitochondrial membrane permeability to chloride ions and changes in mitochondrial membrane potential lead to the activation of mitochondrial permeability transition and apoptosis.

FINAL REMARKS AND FUTURE PERSPECTIVES

Due to experimental difficulties, mitochondrial K^+ and Cl^- channels are challenging targets for basic research. Recent observations on the role of mitoK_{ATP} channels in ischemic preconditioning open new perspectives for this field. For example, ischemic preconditioning has recently emerged as a new strategy for improving the preservation of heart transplants. Hence, there is a continuing effort to identify endogenous mediators of the preconditioning-induced signalling pathway in an attempt to use some of them therapeutically. Recently, using a model of prolonged cold heart storage, it has been shown that the mitoK_{ATP} channel opener diazoxide can reproduce the protection conferred by ischemic preconditioning (Kevelaitis *et al.*, 1999). Both ischemic and diazoxide preconditioning provided a similar degree of cardioprotection. These beneficial effects were abolished by 5-HD pretreatment. These data support the concept that the cardioprotective effects of ischemic preconditioning can be simulated by a mitoK_{ATP} channel opener and suggest that the activation of these channels could be an effective means of improving the preservation of globally ischemic cold-stored hearts, as occurs during cardiac transplantation (Ahmet *et al.*, 2000; Kevelaitis *et al.*, 1999). This example illustrates that the understanding of mitochondrial ion channel function could not only significantly deepen our knowledge of cellular physiology but also could lead to the better treatment of cardiovascular diseases.

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